Respiratory vasculatures of the intertidal air-breathing eel goby, *Odontamblyopus lacepedii* (Gobiidae: Amblyopinae)

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Synopsis

Lacking a propensity to emerge over the mud surface, *Odontamblyopus lacepedii* survives low tide periods by continuously breathing air in burrows filled with hypoxic water. As with most marine air-breathing fishes, *O. lacepedii* does not possess an accessory air-breathing organ, but holds air in the buccal-opercular cavity. The present study aimed to clarify how the respiratory vasculature has been modified in this facultative air-breathing fish. Results showed that the gills apparently lacked structural modifications for air breathing, whereas the inner epithelia of the opercula were richly vascularized. Comparison with two sympatric gobies revealed that the density of blood capillaries within 10 μm from the inner opercular epithelial surface in *O. lacepedii* (14.5±3.0 capillaries mm^{-1}; mean±s.d., n=3) was significantly higher than in the aquatic non-air-breathing *Acanthogobius hasta* (0.0±0.0) but significantly lower than in the amphibious air-breathing mudskipper *Periophthalmus modestus* (59.1±8.5). The opercular capillary bed was supplied predominantly by the 1^{st} efferent branchial arteries (EBA1) and drained by the opercular veins, which open into the anterior cardinal vein. Deep invaginations at the distal end of the EBA1 and the junction with EBA2 are suggestive of blood flow regulatory sites during breath-holding and apnoeic periods. It remains to be investigated how blood flow through the gills is maintained during breath holding when the buccal-opercular cavity is filled with air.

Introduction

While many freshwater air-breathing fishes possess a wide array of specialized accessory air-breathing organs (e.g. lungs, gas bladder, stomach, intestine, and suprabranchial chambers), most brackish and marine air-breathing fishes lack such specializations but use the buccal-opercular epithelia, skin and possibly the gills for aerial gas exchange (for review see Graham 1997; Martin & Bridges 1999; Sayer 2005). Typical fish gills are highly efficient gas exchangers in water but not in air since the lamellae are prone to gravitational collapse, resulting in a significant reduction of their functional respiratory surface area and an elevation of vascular resistance (Maina 2000). This is partly the reason Randall et al. (1981) attributed the non-existence of a single terrestrial vertebrate species that exclusively relies on gills for aerial respiration.

It is a general perception that the gills of air-breathing fishes reduce their surface area with increasing reliance on aerial gas exchange (Munshi 1976; Graham 1997). This holds true especially for amphibious mudskippers residing in intertidal mudflats whose adaptation to terrestrial life, albeit varies widely among species, is apparently reflected in different degrees of morphological alterations of the gills to prevent them from collapsing in air (Schöttle 1931; Tamura & Moriyama 1976; Low et al. 1988). The eel goby, *Odontamblyopus lacepedii* (family Gobiidae, subfamily Amblyopinae), is one of the recently reported brackish-marine species that breathe air using the buccal-opercular cavity when it stays in its hypoxic mudflat

burrow during low tide (Gonzales et al. 2006). However, our laboratory experiment demonstrated that the fish is a facultative air breather, and therefore probably relies exclusively on aquatic gas exchange while migrating along the coastal water (see Discussion). Thus, this provides a unique opportunity to inspect how the gills, which are important during migration, can withstand gravitational collapse in air during aerial respiration in the burrow. In addition, the role of mouth epithelia as potential sites of aerial gas exchange needs scrutiny.

In the present study, we described the gross and vascular morphology of the gills and buccal-opercular epithelia of *O. lacepedii*. Our main objective was to clarify how the respiratory vasculature has been modified in this facultative air-breathing fish. The use of two other sympatric gobiid species allowed us to draw comparisons of the branchial morphology and vascular anatomy of the aquatic (i.e. does not emerge from water) air-breathing *O. lacepedii* to the non-air-breathing *Acanthogobius hasta* (subfamily Gobionellinae) and the amphibious air-breathing mudskipper *Periophthalmus modestus* (subfamily Oxudercinae). The lack of air-breathing capability of *A. hasta* has been confirmed by subjecting the fish to stepwise aquatic hypoxia, a similar method described by Gonzales et al. (2006).

Materials and Methods

Fish Collection and Maintenance

Adult individuals of *O. lacepedii* were netted at 3-5 m depths in the coastal waters of Ariake Bay, Japan (33°10’ N, 130°15’ E). *P. modestus* were caught by a handheld net over the mudflat surface in the same area during low tide, and *A. hasta* were purchased from a commercial trader. Fishes were kept in either glass aquaria or a fiberglass tank equipped with a recirculating system, and the water temperature and salinity were adjusted to 25±1ºC and 17‰, respectively. The tank of *P. modestus* was provided with a diagonal platform of which the upper half plane was out of the water for fish emergence. Fishes were given chopped fish or squid during captivity of more than a week, but starved for 24 h prior to use.

Tissue Fixation

The heads of *O. lacepedii* [21.5-46.0 g body mass (BM), 22.5-32.6 cm total length (TL); n=3], *A. hasta* (70.0-95.0 g BM, 26.9-35.6 cm TL; n=3), and *P. modestus* (3.9-4.6 g BM, 7.6-8.2 cm TL; n=3) were severed from the body under 2-phenoxyethanol anesthesia (1 ml l^{-1}) and fixed in 2% paraformaldehyde-2% glutaraldehyde (PFA-GA) in 0.1 M phosphate buffer (PB; pH 7.4) for 48 h at 4°C. The gills were excised and immersed overnight in 0.1 M PB-10% sucrose solution at 4°C. Gill samples were dehydrated in a 30-min graded series of ethanol solutions (EtOH: 70%, 80%, 90%, 95%, 100%) at room temperature (20-25ºC) followed by two mixtures (1:1, 1:2) of EtOH and 2-methyl-2-propanol (*t*-BuOH) at 50ºC. After two final changes of absolute *t*-BuOH, samples were put in a freezer for at least 5 min, freeze-dried

(JFD-310, JEOL, Japan), mounted on specimen stubs using a double-sided adhesive tape, coated with platinum in an ion sputter (JFC-1600, JEOL, Japan), and photographed with a scanning electron microscope (SEM, JSM-6380LAKII, JEOL, Japan).

To examine the possible gill modification of the burrow-dwelling *O. lacepedii*, three individuals (19.9-25.7 g BM, 23.1-24.8 cm TL) were fished directly from burrows using a hand-operated steel shaft with barbless hook at the tip. Immediately after capture, the fish was anesthetized, head was severed from the body and fixed in the same mixture of PFA-GA as described above. Subsequent processing for SEM observation was done in the laboratory.

Vascular Casting of O. lacepedii

Anesthetized *O. lacepedii* (56.0-98.0 g BM, 31.8-39.1 cm TL; n=12) was supinely mounted on an operating table and the gills were continuously irrigated with air-saturated water (salinity: 17‰) containing 0.5 ml l^{-1} 2-phenoxyethanol. To gain access to the heart, the fused pelvic fins were carefully removed and a small amount of heparin (10,000 IU ml^{-1}) was injected through the ventricle to inhibit blood coagulation. A small incision in the ventricle permitted insertion of a flared polyethylene tubing (Hibiki #7, Kunii Ltd., Tokyo, Japan) up to the bulbus arteriosus. The tubing was secured by a ligature at the junction of the bulbus and the ventricle. After cutting the sinus venosus, the vasculature was flushed with a 0.9% NaCl solution. When the outflowing saline became almost colorless, a 40:1 mix ratio of

thoroughly stirred Mercox and its accompanying solidifying agent (Dainippon Ink, Inc., Tokyo, Japan) was injected into the vasculature by moderate hand pressure. The fish was kept wrapped in a wet towel for 1-2 h and then was immersed in 50-60°C water bath for another 3-4 h to ensure complete resin polymerization. Tissues were macerated in concentrated hydrochloric acid leaving only the vascular cast. The cast was rinsed in gently flowing tap water and then several times in distilled water. Casts destined for SEM observations were air dried, mounted on a specimen stub, and coated with platinum. Digital photography (Camedia C-7070, Olympus Corp., Tokyo, Japan) and vascular tracing using a stereomicroscope (Olympus SZH10, Olympus Corp., Tokyo, Japan) fitted with camera lucida were made on casts in water.

Histology

Three individuals each of *O. lacepedii* (35.8-49.0 g BM, 29.2-32.8 cm TL), *A. hasta* (66.0-102.0 g BM, 30.0-36.6 cm TL), and *P. modestus* (2.6-3.9 g BM, 7.6-8.2 cm TL) were used to compare the capillary density and diffusion distance in the epithelial margins of the palate, opercula, and tongue. These three regions in the buccal-opercular cavity are apparently in contact with air during breath holding in *O. lacepedii* and *P. modestus*, and therefore the potential sites for gas exchange. To avoid loss of blood during dissection, the whole fish were fixed in Bouin's solution for 48 h and subsequently transferred to 70% EtOH

for another 48 h. Portions of the palate, left operculum, and tongue were excised approximately from the same site of the respective surfaces, dehydrated in a graded alcohol series, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Histological sections were photographed at 40x magnification and digitized with Fujifilm Camera Shooting Software v.1.0.0 running under an Apple Macintosh computer (OS 8.5). Quantification of the capillary density and diffusion distance was performed using a public domain image processing software (ImageJ 1.36b, National Institute of Health, Bethesda, USA). The diffusion distance was ascertained from the shortest line made between the luminal surface of the capillary endothelial cell and epithelium (Park 2002). Three replicate (2-3 mm per replicate) measurements were made on each fish.

Statistical Analysis

Capillary density was compared between species using either one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple-range test if variances were equal or by Kruskal-Wallis ANOVA on ranks if variances were unequal. Values are expressed as means ± standard deviation (s.d.). The significant level was set at $P<0.05$.

Results

Gill morphology

Odontamblyopus lacepedii possessed four pairs of gill arches which were lined with pairs of filaments (holobranch) bearing well-developed lamellae on both sides (Figures 1b, 1b', 3a). The gills of three individuals captured from the burrows exhibited gross morphological features similar with those netted from the coastal water. The thin and wide lamellae of *O. lacepedii* resembled those of the non-air-breathing *A. hasta* (Figure 1a, a'), whereas the lamellae of the amphibious *P. modestus* were distinctly different with thicker epithelia and occasional fusions (Figure 1c, c').

Vascular architecture and blood circulation in O. lacepedii

The vascular architecture of *O. lacepedii* basically retains typical circulatory pattern of teleosts. A single ventral aorta (VA) emanates from the heart, which gives rise to four pairs of afferent branchial arteries (ABA). The 1st and 2nd ABAs (ABA1 and ABA2, respectively) arise independently from VA whereas ABA3 and ABA4 are the result of bifurcation of a vessel arising from each side of the posterior aspect of VA (Figure 2b). After traversing the gills, the oxygenated blood is collected into the four pairs of efferent branchial arteries (EBA). Two pairs of the lateral aortae (LA), formed by anastomoses of EBA1 and EBA2 as well as of EBA3 and EBA4, serve as conduits of the effluent blood into the dorsal aorta (DA). At its distal end, EBA1 divides into three vessels (Figure 2c); the orbitonasal artery (ONA) supplying the head and brain, the opercular artery (OpA_2) supplying the operculum, and the

connecting artery (CnA) of the EBA1 and EBA2. Deep invaginations of the arterial lumen occur at the distal end of EBA1 and the caudal end of CnA (Figure 2b, c), suggesting sites for blood flow regulation. The coeliacomesenteric artery (CMA) and a pair of subclavian (=pectoral) arteries (ScA) branch off from the anterior aspect of DA.

Microvasculature of the gills

The microvasculature of *O. lacepedii* gills consists of respiratory (arterio-arterial) and intrafilamental circuits as in many other fishes (Laurent 1984). The respiratory circuit directs blood from the heart through the afferent filamental artery (AFA), lacunae in the secondary lamellae (L), and the efferent filamental artery (EFA). The regularly spaced lamellae run throughout the length of the filament (Figure 3b). The lamellar vasculature consists of inner (IMC) and outer (OMC) marginal channels (Figure 3c, d) and central lacunar area where the pillar cells (PC) are arranged more or less in parallel with the OMC (Figure 3d). This type of arrangement is thought to provide directional control of blood flow in the lamellar sinus (Olson 2002). Unlike the OMC, the IMC is discontinuous (Figure 3c) as in most other fishes. An extensive network of vessels occurs in both branchial arches and afferent but not efferent filaments (Figure 3c). The vessels in the afferent filaments show no direct connection with AFA, suggesting vascular pathway either within or outside the branchial artery. These elaborate vessels and the vessels running in the core of the filaments (Figure 3c) are

presumably associated with the arterio-venous circulation (Olson 2002; Evans et al. 2005).

Microvasculature of the buccal-opercular region

The opercula are perfused with blood from the two arteries (OpA_1 and OpA_2) emanating from EBA1 (Figures 2b, 4a). The dense capillary network near the opercular epithelial surface (Figure 4b, c) indicates its importance in gaseous exchange. Measurements of vascular casts from 5 individuals showed that the capillaries have a mean diameter of 7.86±1.64 (s.d.) µm. The opercular capillary bed is drained by a large opercular vein (OpV), which eventually joins the anterior cardinal vein (ACV, Figures 2b, 4a; see also Figure 7).

The representative histological sections of the opercular epithelium from three sympatric gobies are shown in Figure 5. *P. modestus* had the highest capillary density (59.1±8.5 capillaries mm^{-1}; mean±s.d., n=3) bordering the epithelial surface (0-10 µm), followed in decreasing order by, *O. lacepedii* (14.5±3.0) and *A. hasta* (0.0±0.0). Comparison among the three regions in the buccal-opercular cavity revealed that the operculum was the most heavily vascularized in *P. modestus* and *O. lacepedii* (Figure 6).

Discussion

The gills of *O. lacepedii* appear to be functionally well-suited for aquatic respiration, possessing the thin and wide lamellae, and lacking any morphological specializations for

aerial gas exchange. Instead, a novel aerial gas exchange surface has developed in the inner opercular epithelia that are supplied by the efferent blood from the gills. The gills therefore must maintain blood flow during air breathing, even though the present results failed to satisfactorily resolve how the gills prevent from collapsing to permit sufficient blood flow.

Examinations of the gills of three individuals recovered inside the mudflat burrows showed morphological characteristics apparently similar to the gills of the migrating eel goby, thus discounting the possibility of alteration of the gill morphology by burrow confinement (Nilsson 2007). Although the life history of *O. lacepedii* has not been sufficiently documented, the high catch of this species by stow nets in Ariake Bay, Japan (Takita et al. 2003) attests to the abundant populations of migrating individuals. The migrating fish do not probably experience hypoxic conditions since oxygen is usually not a limiting factor in their habitats in coastal waters. On the mudflat, however, *O. lacepedii* periodically experiences severe hypoxia of the burrow water during low tide (Gonzales et al. 2006). Air breathing, therefore, has been strongly selected to enable the fish to sustain its aerobic metabolic activity during burrow confinement (Gonzales et al. 2006). The use of the buccal-opercular cavity for air-breath storage with no accessory air-breathing organ implies that aerial gas exchange takes place in either the gills or the buccal-opercular linings, or both. We previously hypothesized the gills as the main aerial gas exchange organ on the basis of the apparent lack of rich vascularization on the mouth lining (Gonzales et al. 2006). However, close inspection

of the inner opercular lining by light and scanning electron microscopy undoubtedly established the occurrence of a dense capillary network on the surface, and its significant respiratory role for the fish.

It is generally supposed that the gills of ordinary water-breathing fishes work efficiently in water but not in air because the filaments and lamellae cohere and collapse in air, thereby restricting blood circulation across the lamellae (Maina 2000). However, to our knowledge, this general supposition has not been experimentally verified. Morphological alterations seen in some air-breathing fishes may help sustain blood circulation in air. For example, the mudskipper, *Periophthalmodon schlosseri*, has thick, fused lamellae (Low et al. 1988), as was observed in *P. modestus* (Figure 1c'). The gill filaments of the swamp eel, *Synbranchus marmoratus*, are supported by hypertrophied, spirally configurated gill rays (Liem 1987), and the gills of the electric eel, *Electrophorus electricus*, were reported to be degenerative (Carter 1935). These three species use the buccal-opercular cavity for air breathing. Using X-ray cineradiography, Liem (1980) demonstrated that the entire buccal-opercular cavity of *S. marmoratus* was filled with air during breath holding, indicating that the gills were probably held in air. The similarity of air-breathing behavior of *O. lacepedii* (Gonzales et al. 2006) to *S. marmoratus* (Graham et al. 1995) signifies aerial exposure of gills during breath holding in the former. During air breathing, both species have the anterior body held nearly vertical to the water surface, the mouth fully filled with air and

the opercula distended, and the head floating at the surface with positive buoyancy afforded by the buccal air volume. Even though the gills of *O. lacepedii* lack apparent morphological specialization, such as bypass vessels between the afferent and efferent sides of the gills, they must be patent during breath holding so that blood could flow across the lamellae in order to maintain perfusion into the opercular epithelium. Maintenance of branchial blood flow is important even if the gills do not play a significant role for aerial gas exchange. Understanding how this is made possible requires further physiological investigation.

Observation of the corrosion casts revealed that the vasculature of the opercular epithelia receives blood from two arteries (OpA_1 and OpA_2) derived from the first gill arch. This agrees with the early observations of Schöttle (1931) that the operculum in the three species of mudskippers (*Boleophthalmus boddarti*, *Periophthalmodon schlosseri* and *Periophthalmus vulgaris*) and one species of aquatic goby [*Gobius* (=*Knipowitschia*) *panizzae*] was supplied by an efferent artery originating between the first and second gill arches. However, the serial arrangement of the gills and aerial gas exchange epithelia necessitates that during aquatic ventilation, the capillary bed of the operculum, which is in contact with the same respiratory water as irrigating the gills, is perfused by the blood already oxygenated at the gills. The gills are usually highly efficient in aquatic gas exchange due to countercurrent disposition of the water and the blood across the lamellae (Evans et al. 2005; Graham 2006), and therefore the serial vascular arrangement of the gill-operculum complex

seems to be functionally redundant. Thus, from a teleological standpoint, it would be reasonable to expect some kind of perfusion control mechanism to modulate blood flow distribution between the gill arches. The deep invaginations at the distal end of the EBA1 and at the junction of the CnA with the EBA2 might serve for such a role, including blood partitioning in the head, opercular epithelia, and the general trunk vasculature. In general, perfusion rate of air-breathing organs increases immediately after an air breath when the oxygen partial pressure (PO_2) of the gas in the air-breathing organ is highest (Boutilier 1990). The increasing role of the accessory respiratory surfaces for aerial gas exchange is generally expected to be accompanied by an increase of blood supply to the air-breathing organ, and thus probably a modification in the circulatory vasculature. Lacking detailed comparative data from other species, future studies are necessary to address this crucial issue.

Similar with other gobies reported by Schöttle (1931), blood drains from the opercula via the opercular veins. During air breathing, oxygenated blood leaving the opercula mixes with deoxygenated blood from the head, and the resulting mixed blood enters the sinus venosus through the ductus Cuvier (Figure 7). This represents functional inefficiency from the viewpoint of oxygen transport, but is common among all air-breathing fishes except lungfish and *Channa*, for which partial separation of oxygen-rich blood from the air-breathing organ and oxygen-poor systemic venous blood has been documented (Ishimatsu & Itazawa 1983; Graham 1997). Farmer (1999) argued that the admixture of oxygen-rich

blood to systemic venous blood is vital for oxygenation of the spongy myocardium in the lower vertebrates that often lack coronary circulation, and that this circulatory pattern likely provided the impetus for the evolution of lungs in the early fishes. She also interpreted the reduction of the gill surface area, which is increasingly obvious with greater importance of aerial gas exchange among air-breathing fishes, as an adaptation that reduces oxygen loss into hypoxic water. A similar view was advanced earlier by Randall et al. (1981), citing examples from the lungfish, *Lepidosiren* and *Protopterus*. However, the universality of this thesis might be put into question considering that the gills diminish also among marine air-breathing fishes, such as mudskippers (Figure 1; see also Low et al. 1988), in which hypoxic exposure may be periodical and depend upon the duration of burrow confinement during high tide. In fact, hypoxic exposure is least likely for the more terrestrially adapted species such as *Periophthalmus*, which usually stays above water's edge during high tide (Colombini et al. 1995; Ikebe & Oishi 1996). Nevertheless, the gills of *Periophthalmus* species are the most vestigial among mudskippers (Figure 1; see also Graham 1997). In comparison, burrow-dwelling *O. lacepedii* experiences aquatic hypoxia for longer durations, but retains well-developed gills as shown in this study. Likewise, less terrestrial mudskippers of the genus *Boleophthalmus* also possess well-developed gills with no apparent modifications as evident for *Periophthalmus* and *Periophthalmodon* (Low et al. 1988). These observations apparently favor the more traditional view that gill reduction is the result of increasing

reliance on a novel air-breathing organ for oxygen supply (Munshi 1976). This optimization in structural design of the respiratory organ is consistent with the principle of symmorphosis (Weibel 2000).

The present study has demonstrated that the opercular epithelia of *O. lacepedii* are richly vascularized, and therefore of importance in aerial gas exchange, yet the relative contribution of the opercular and lamellar epithelia to aerial respiration remains to be elucidated. Apparently, facultative air-breathing fishes benefit from retaining typical gill morphology during much of their aquatic existence. To understand the roles of the gills during transition from water to air breathing, direct determinations must be made for branchial blood flow and pre- and post-branchial blood gas levels during water and air breathing.

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List of abbreviations

ABA1-4	1st to 4th afferent branchial arteries
ACV	anterior cardinal vein
AFA	afferent filamental artery
ALA	afferent lamellar arteriole
BV	blood vessel
CA	cerebral artery
CMA	coeliacomesenteric artery
CnA	connecting artery between EBA1 and EBA2
DA	dorsal aorta
DC	ductus Cuvier
EBA1-4	1st to 4th efferent branchial arteries
EFA	efferent filamental artery
ELA	efferent lamellar arteriole
H	heart
HV	hepatic vein
IMC	inner marginal channel of the lamella
L	lamella
LA	lateral aorta

MnA	mandibular artery
MxA	maxillary artery
OMC	outer marginal channel of the lamella
ONA	orbitonasal artery
ONV	orbitonasal vein
Op	operculum
OpA_1	opercular artery from the anterior end of EBA1
OpA_2	opercular artery from the distal end of EBA1
OpV	opercular vein
PC	pillar cell
PCV	posterior cardinal vein
ScA	subclavian (=pectoral) artery
VA	ventral aorta

Figure legends

Figure 1. Comparison of gross morphological features of the gills in the three sympatric gobies. Gill samples are from the 3rd branchial arch. Top rows (a, b, c) are the branchial arches of individual species, and the representative filaments are shown below (a', b', c'). Scale bars: (a, b, c) 1 mm; (a', b', c') 200 μm.

Figure 2. A corrosion cast of the head region of *Odontamblyopus lacepedii* as viewed from the dorsal side (a) with matching direct tracing of the major blood vessels (b). The boxed area in (b) highlights the connecting artery (CnA) between EBA1 and EBA2 as detailed in SEM photograph in (c). An invagination of the arterial lumen (arrowheads) was noticeable between EBA2 and CnA and at the junction immediately before EBA1 branches off the CnA, ONA and OpA_2 (c). See text and the list of abbreviations for details. Scale bars: (a) 1 cm; (c) 1 mm.

Figure 3. Corrosion casts showing the four intact branchial arches (I-IV) of the left gills (a) and vascular details of the filaments and lamellae (b-d) in *Odontamblyopus lacepedii*. A pair of filaments (holobranch) bears regularly spaced, well-developed lamellae (b). A network of vessels is apparent in the afferent but not efferent filament (c). Several lamellae have been removed from a single filament (hemibranch) to expose the intrafilamental circuit

(arrowheads in c). The pillar cells (holes in the lamellae denoted by arrowheads in d) are arranged more or less in parallel with the outer marginal channel (OMC, d). Asterisks in (c) denote the discontinuous inner marginal channels (IMCs) of the lamellae. Arrows in (c and d) indicate the direction of blood flow. See the list of abbreviations for vessel names. Scale bars: (a) 1 cm; (b) 500 μm; (c, d) 200 μm.

Figure 4. Lateral view of the corrosion cast of the right operculum of *Odontamblyopus lacepedii*, anterior to the right (a). The inner epithelial surface of the operculum is overlaid by a dense network of capillary vessels (b). The boxed area in (b) is shown in a higher magnification SEM photograph in (c). See the list of abbreviations for vessel names. Scale bars: (a) 5 mm; (b) 200 μm; (c) 50 μm.

Figure 5. Representative photomicrographs of the hematoxylin/eosin-stained inner opercular epithelia of the three sympatric gobies, *Acanthogobius hasta* (a), *Odontamblyopus lacepedii* (b), and *Periophthalmus modestus* (c). Asterisks in (a) are goblet cells. BV: blood vessel. Arrowheads denote blood capillaries. The scale bar is 100 μm and applies to all panels.

Figure 6. Blood capillary density and diffusion distance in the buccal-opercular epithelia of three sympatric gobies. Bars are means + s.d. (n=3). For each size class of the diffusion distance, significant differences (P<0.05) are denoted by bars with different letters.

Figure 7. Schematic representation of the circulatory system of the head region in *Odontamblyopus lacepedii*. The vascularized operculum (Op) is supplied by two arteries (OpA_1 and OpA_2) derived from the first branchial arch (see also Figures 2 and 4a) and drained by a systemic opercular vein (OpV). Solid arrows denote the direction of blood flow. A broken arrow indicates potential blood flow from EBA2 to the head, including opercular vasculature. See text and the list of abbreviations for details.

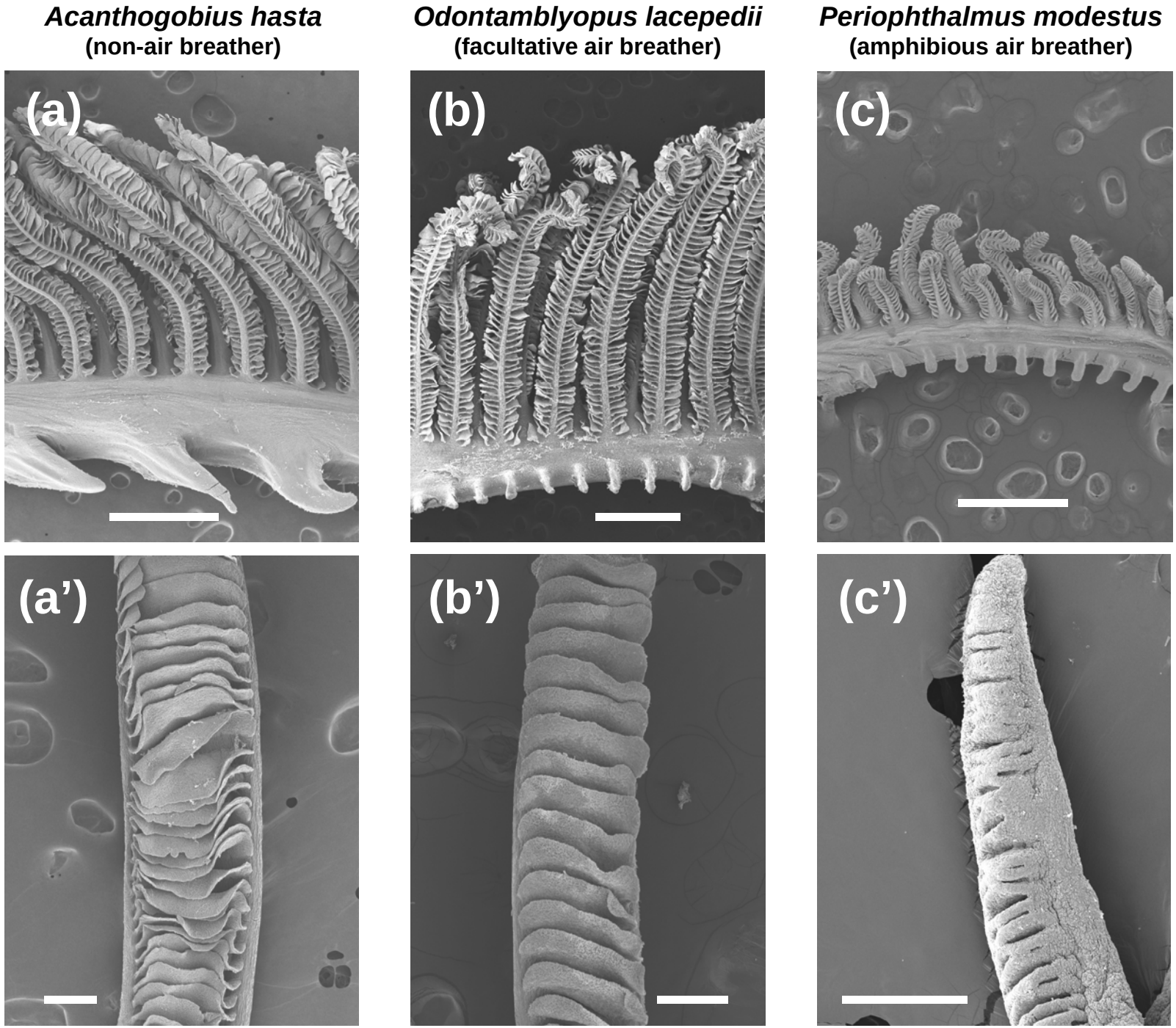
Acanthogobius hasta
(non-air breather)
Odontamblyopus lacepedii
(facultative air breather)
Periophthalmus modestus
(amphibious air breather)
(a)
(b)
(c)
(a')
(b')
(c')

Figure 1

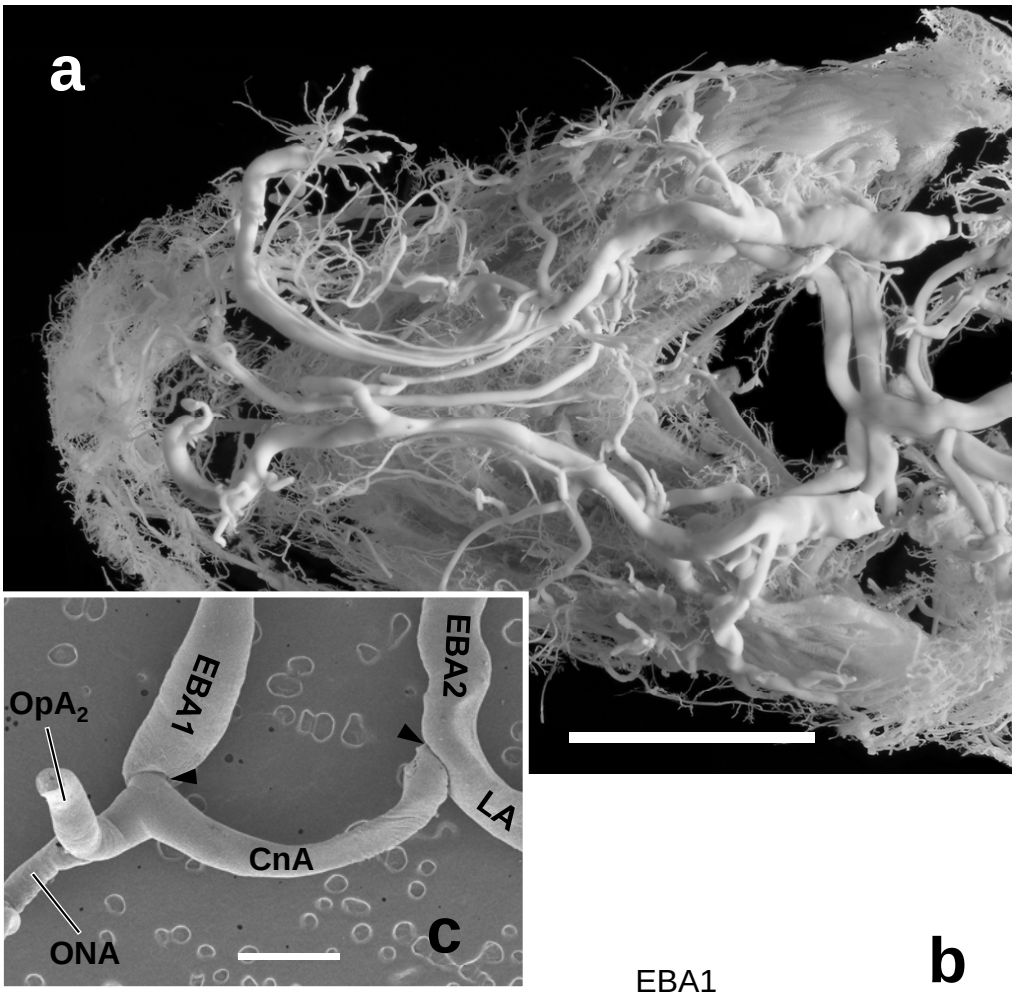
a
EBA1
EBA2
OpA2
LA
CnA
ONA
c
b
EBA1
ABA3
ABA4
CnA
ABA2
OpA2
EBA2
OpA1
EBA3
ABA1
MnA
EBA4
LiA
ScA
LA
CA
ONA
VA
DA
MxA
CMA
ONV
ACV
OpV
OpA2

Figure 2

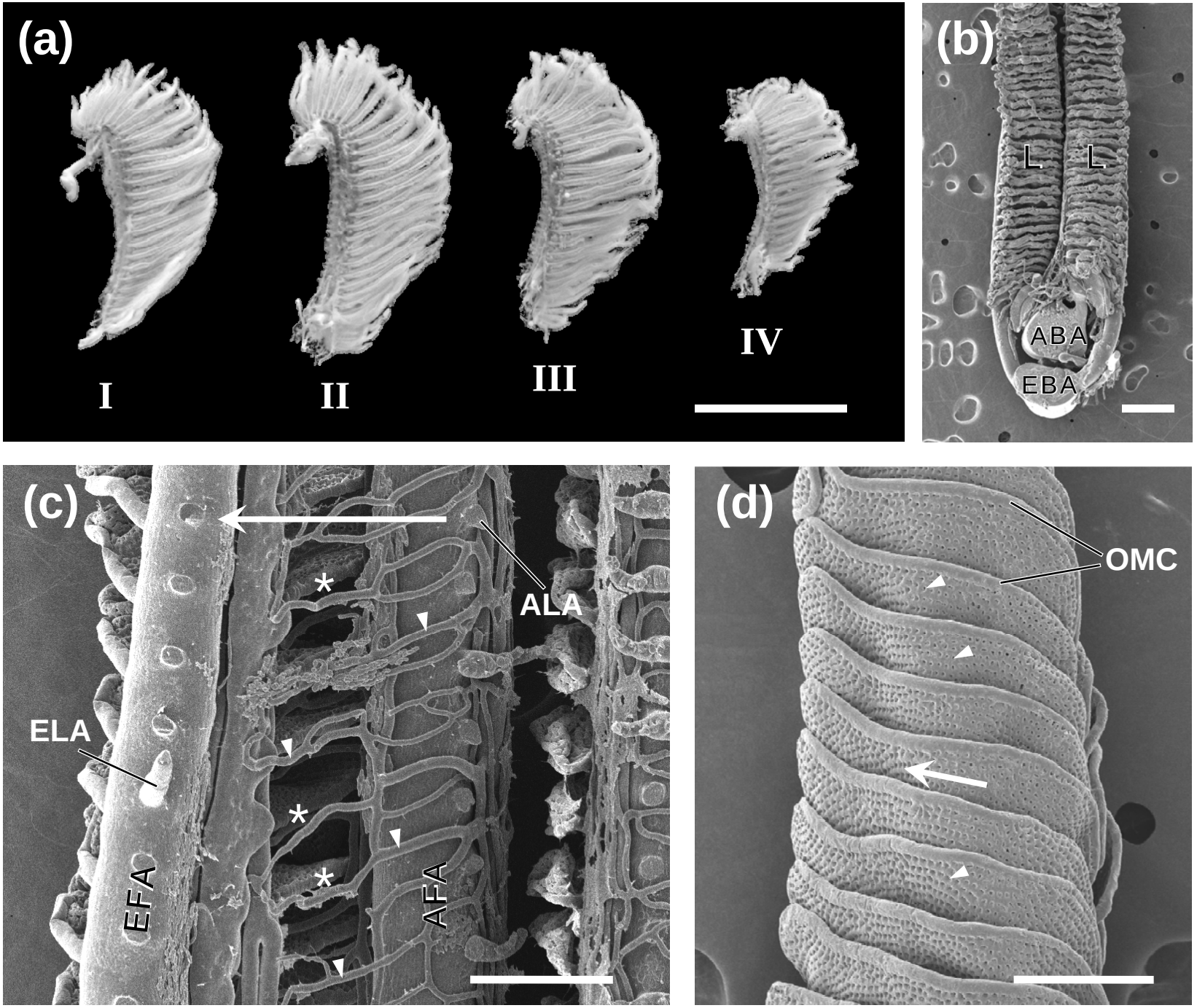

Figure 3

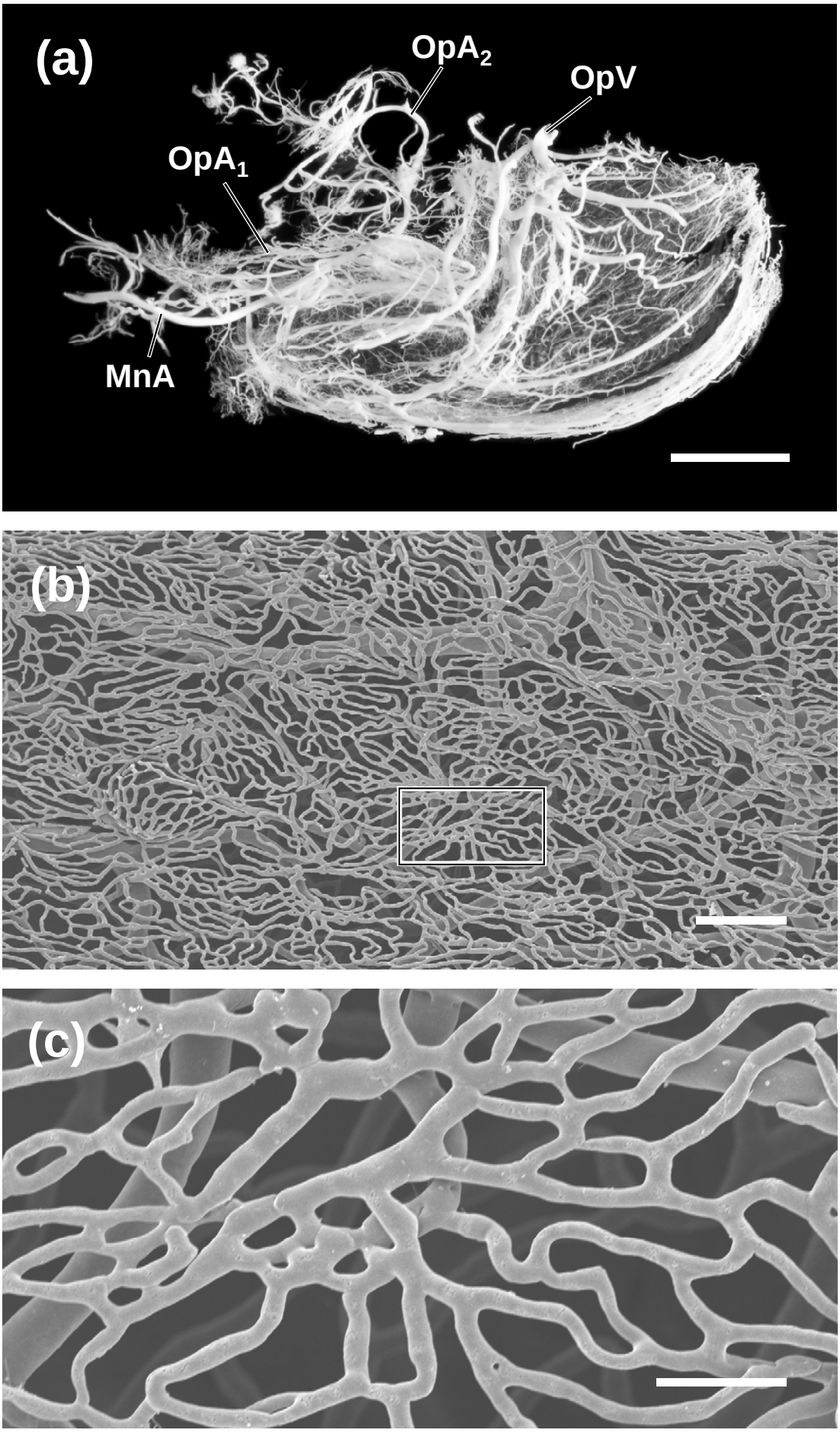

Figure 4

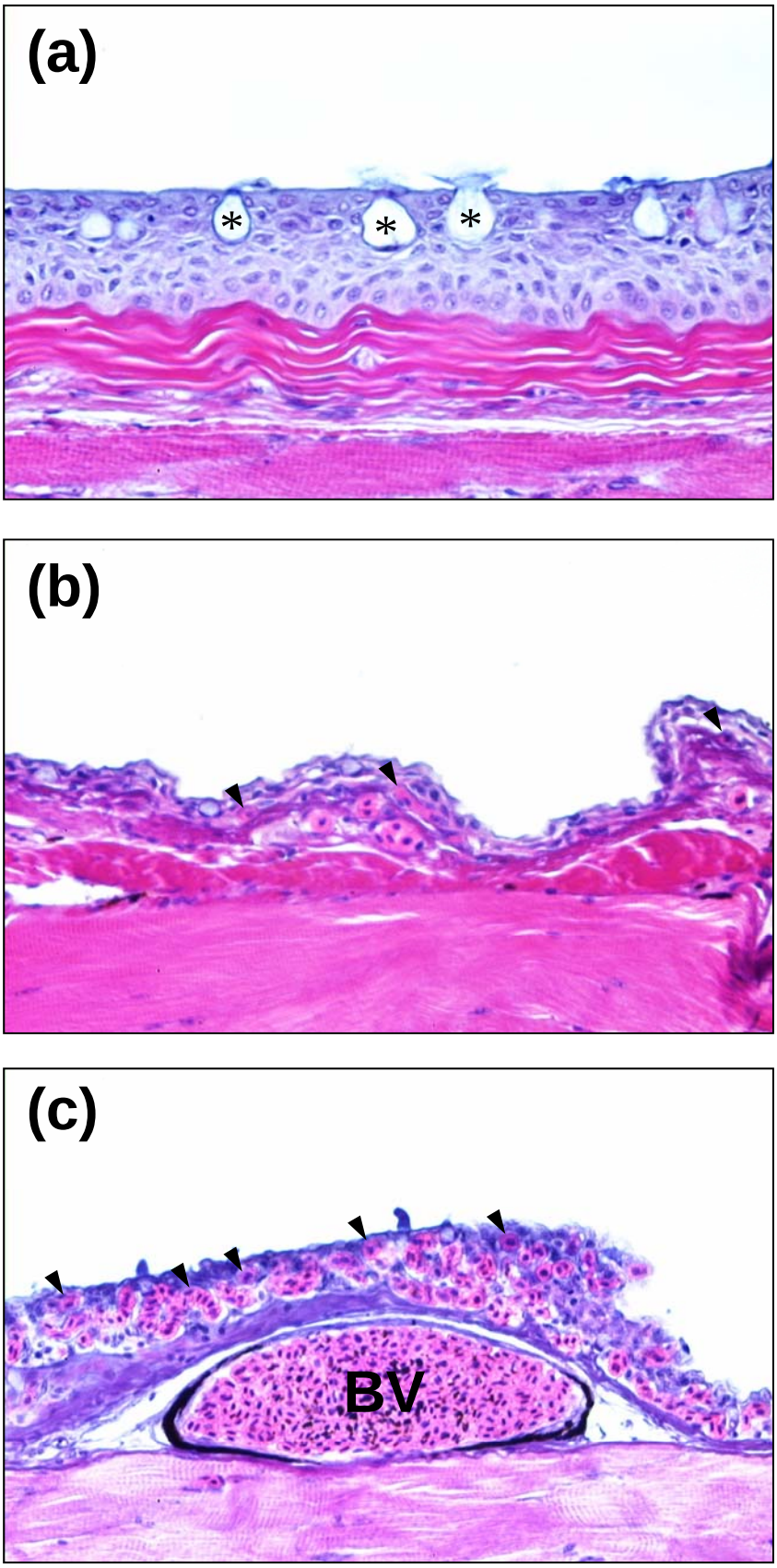

Figure 5

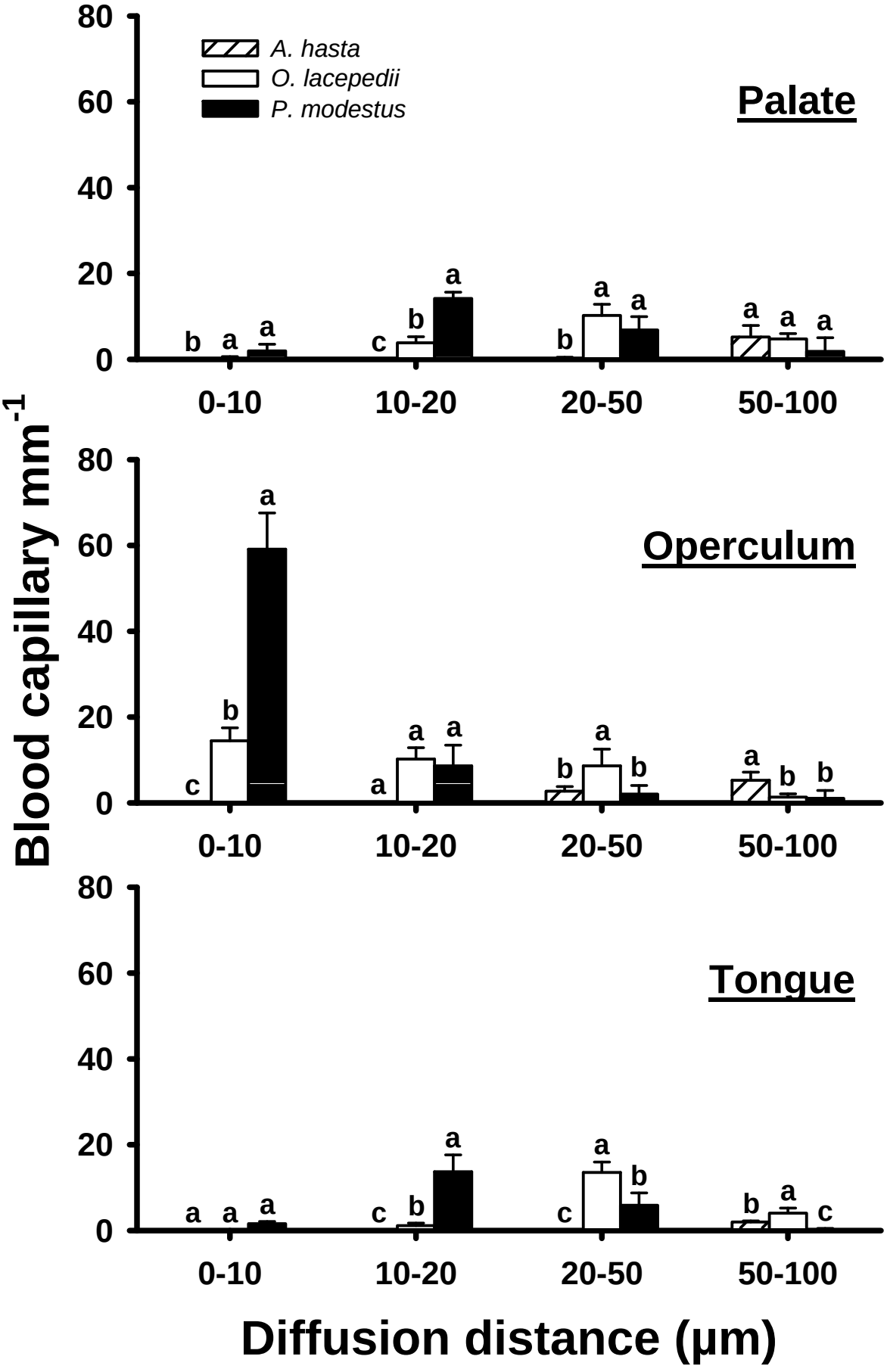
A. hasta
O. lacepedii
P. modestus
Palate
Operculum
Tongue
Blood capillary mm-1
Diffusion distance (μm)
0-10
10-20
20-50
50-100
0
20
40
60
80

Figure 6

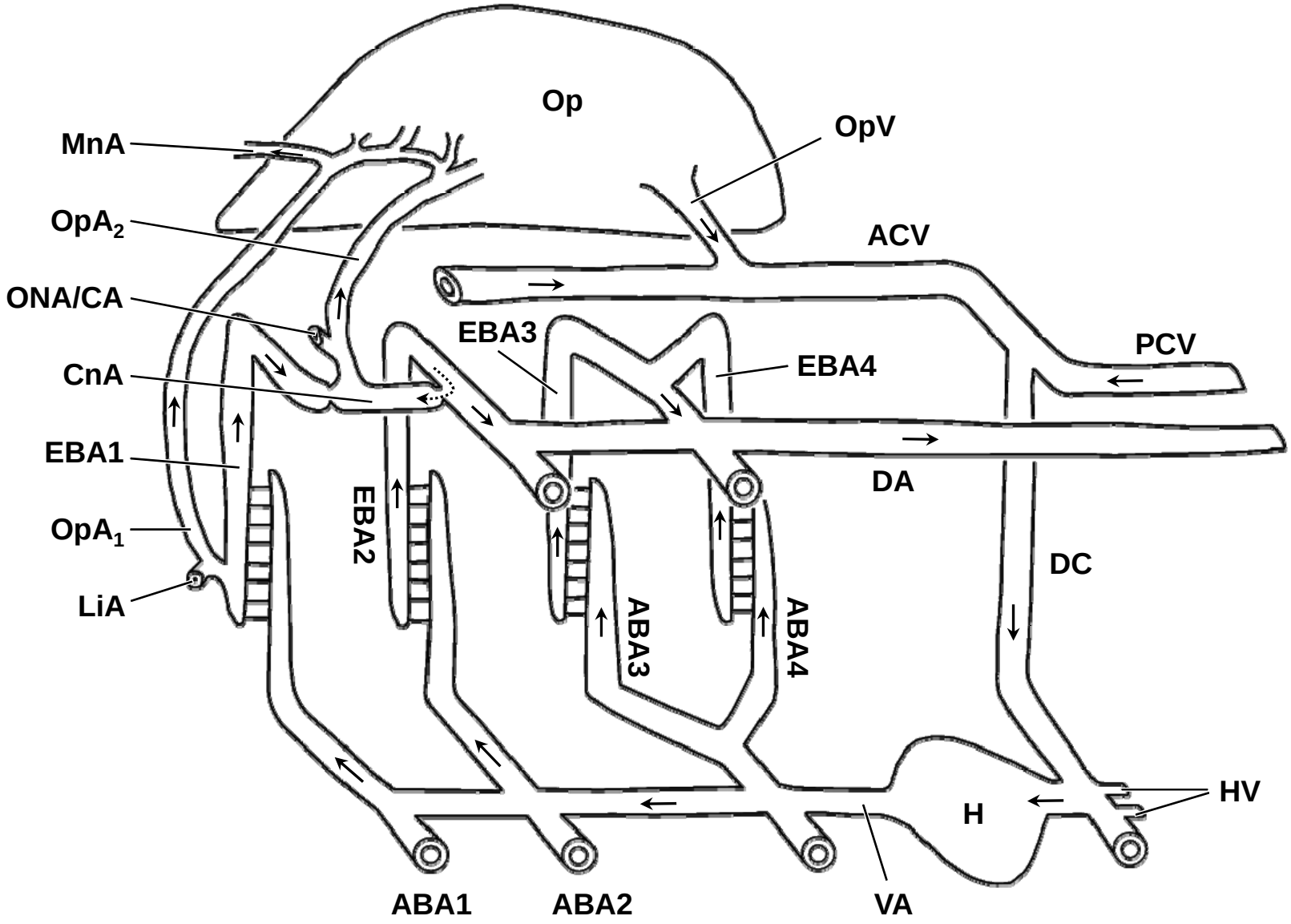
Op
OpV
MnA
OpA2
ACV
ONA/CA
EBA3
PCV
CnA
EBA4
EBA1
DA
EBA2
OpA1
DC
LiA
ABA3
ABA4
HV
H
ABA1
ABA2
VA

Figure 7