Morphological correlates of signal variation in weakly electric mormyrid fish

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Overview

Weakly electric fish occupy a special place in the field of neuroethology as a model system for the study of the neurobiological basis of natural behavior. Comprising two orders of freshwater teleosts, the Gymnotiformes and the Mormyriformes, weakly electric fish have evolved diverse electric organ discharges (EODs) that are used for electrolocation of objects and for sex- and species recognition, courtship, aggression, and appeasement, among other behaviors (Bullock 1982; Heiligenberg & Bastian 1984). Aside from the variety of EODs, the elements of electric communication that make it a model system include the presence of a highly specialized electrosensory system with a subpopulation of receptors and neurons dedicated to communication signal sensing, as well as the structure of the electrical signals themselves, which is relatively simple and amenable to study by experimental manipulation (Hopkins 1988).

This paper presents the results of two studies on mormyrid fish and the electric organs (EOs) responsible for weak electrogenesis. In mormyrids, the electric organ is composed of four columns of serially stacked, disk-shaped electrocytes, two on each side of the spinal cord (Bennett 1970; Bass 1986). The central theme of this thesis is the relationship between electric organ morphology and EOD waveform. The first chapter presents a study of *Paramormyrops kingsleyae* that shows geographic variation in signals correlated with variation in electric organ anatomy. The second chapter is a descriptive study of larval and juvenile *Brienomyrus brachyistius* that identifies morphological correlates of EOD change in the course of development.

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INTRODUCTION

The focal point of this study is the process of signal evolution: variation in communication signals, the mechanism of their evolution, their adaptive function, and their relationship to an organism's underlying physiology and morphology. In weakly electric fish, EOD waveforms evolve faster than feeding morphology, size, or trophic ecology, suggesting that sexual selection is a potential driving force in mormyrid evolution (Arnegard et al., 2010). This idea is supported by behavioral and neurophysiological evidence from playback studies of species recognition (Hopkins and Bass 1981). With this explanation in mind, the goal of this project was to examine other evolutionary processes that could lead to the observed evolution of divergent signals.

For a study of communication signals and rapid species divergence, the *Paramormyrops* genus was selected, as the species flock consists of an estimated 22 species (Lavoué et al., 2008). While the genetic divergence between *Paramormyrops* species is low, suggesting recent speciation, their EODs are nonetheless highly divergent and distinguishable, allowing for analysis of phenotypic differences at relatively high resolution.

Early in the course of the project, EOD variation was analyzed by principal components analysis (PCA), and it was discovered that variation in EODs is best explained by EOD duration and magnitude of P0, a weak head-negative pre-potential (Gallant, 2010). Each electrocyte is characterized by three elements that determine the appearance of the EOD waveform: the anterior face, the posterior face, and their relationship to a stalk structure that is innervated by electromotor neurons (Fig. 1) (Bass, 1986). Stalk systems in *P. kingsleyae* can originate on the posterior face and pass through the electrocyte to the anterior face, where they are innervated

(*P*enetrating with *a*nterior innervations, or *Pa*). The other type of stalk system remains on the posterior face, where it is innervated (*N*on-*P*enetrating with *p*osterior innervation, or *NPp*). *Pa* type electric organs produce P0-present EODs, while *NPp* type electric organs P0-absent EODs.

Gallant et al. (2010) found that as EODs varied geographically, fish with intermediate phenotypes could be found at the boundary between regions with distinct populations of *Pa* or *NPp* type fish. My contribution to this project began at this point to confirm these initial observations of phenotypic divergence at the anatomical level: to analyze the structure and fine-scale variation in electric organ morphology of *P. kingsleaye* that contribute to subtle differences in the EOD signals that they produce.

METHODS

Specimens: A total of 19 *P. kingsleyae* fish were chosen from the Louétsi drainage basin of Gabon: Apassa (n=4), Le Soungou (n=4), Bambomo (n=7), Mouvanga (n=4) (Fig. 2). This drainage basin is of special interest because it is one of two sources of P0 signal variation. Data from 1998 and 1999 collecting seasons, high-resolution topographic maps, GIS coordinates, and detailed signal analysis revealed that only P0-absent individuals were collected in rivers draining into the Louétsi river above Bongolo Falls and that P0-present individuals were predominantly captured in rivers draining into the Louétsi and Ngounié rivers below Bongolo Falls.

Electric Organ Light Microscopy: After their EODs were recorded, specimens were killed with an overdose of MS-222 and fixed in 10% formalin. Electric organs were removed from fixed specimens and decalcified overnight using 100% CalEx-II solution (Fisher Chemicals, Fair Lawn, NJ, USA). Next, tissue samples were dehydrated in a graded alcohol series up to 95%,

then infiltrated and embedded in JB-4 glycol methacrylate resin (Polysciences, Inc., Warrington, PA, USA). Once the samples were embedded, we prepared 6 µm thick, serial sagittal sections, which were mounted on glass slides and stained with a 0.5% Toluidine blue solution for 30 s. Sections were then imaged using a Leica Leitz DMRB microscope equipped with at SPOT Flex 15.2 64MP Shifting Pixel digital camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Serial Reconstruction: For each specimen, we reconstructed one of four columns of electrocytes from serial, sagittal 6 µm sections. We began our reconstructions at the lateral edge of the electric organs, proceeding medially before stopping at clear sight of the spinal cord. For each section, the number of penetrations in each electrocyte was counted (27–72 electrocytes per section) from anterior to posterior. Penetrations were counted as the number of times a stalk was observed to pass through either or both faces of the electrocyte. Each section surveyed is represented as a square shaded based on the number of penetrations observed. Each column represents a single electrocyte; each row represents a single 6 µm section made from lateral to medial. For each specimen, the number of electrocytes analyzed for each individual is reported (n = 27–72 electrocytes).

RESULTS

Three types of overall electric organ morphology were observed: *NPp* type morphology, featuring organs with few (3 or fewer) electrocytes with penetrating stalks; *Pa* type morphology, with penetrations in almost every electrocyte; finally, an intermediate or mixed-morphology, featuring organs with 7-10 clusters of electrocytes with penetrations. The mixed morphology fish have P0-present EOD despite having mostly *NPp* type electrocytes.

Populations with Homogenous EOD Type Have Uniform Electric Organ Morphology

Songou Creek fish have electric organs consisting primarily of *NPp* electrocytes, without penetrating stalks (Fig. 1). For all plots (n=4), horizontal axis represents distance from the anterior edge of the electric organ, and the vertical axis represents distance from the lateral edge of the electric organ (top lateral). The primarily *NPp* electrocyte morphology corresponds to the P0-absent EODs recorded in all fish from this locality (n=11). All fish from this locality were previously designated P0-absent from EOD recording.

With a similar degree of homogeneity, every fish chosen for reconstruction (n=4) from Mouvanga exhibited *Pa* type morphology in each electrocyte, characterized by a colored box in each column from anterior to posterior in the digital reconstruction (Fig. 2). This overall *Pa* type electric organ morphology corresponds to the P0-present EOD that was recorded from each fish in this locality (n=118).

Populations with Heterogeneous EODs Have Mixed Morphology at Population and Individual Level

Two localities within the Louétsi drainage basin included individuals with P0-present EODs and fish with P0-absent EODs in the same population: Bambomo Creek and Apassa Creek. The population of Bambomo Creek was composed predominantly of P0-absent fish (n=145) although P0-present fish were captured and recorded as well (n=14). From reconstructions of the electric organ (Fig. 3) both types of EO morphology are present in Bambomo, mostly *NPp* type electric organs (n=6, two not shown) and mostly *Pa* type electric organs (n=1), corresponding to the mixed EOD types.

The Apassa Creek population is composed of mostly P0-present fish (n=30) and one P0-absent fish. Four specimens with P0-present waveforms from were examined via reconstruction, of which three had mostly *Pa* type electric organs (Fig. 4, first three electric organs). The fourth specimen with P0-present EOD had an intermediate organ, with 7 clusters of 2-3 *Pa* type electrocytes separated by 6 *NPp* electrocytes.

DISCUSSION

The results from this study confirm the patterns of variation observed in the EODs of distinct populations, providing the morphological evidence for the first time of an intermediate electric organ type, in regions of sympatry.

Geographical Isolation as a Mechanism for Genetic Drift and EOD Diversity

We discovered two geographically distinct populations isolated by physical barriers to migration. A waterfall isolates the Upper Louétsi from the Lower Louétsi and acts as a barrier between populations. Only fish with P0-absent EODs were collected above the Bongolo Falls; only fish with P0-present EODs were collected below. At two collection sites, Apassa and Bambomo Creek, both along the boundary between P0-absent and P0-present populations, P0-absent and P0-present individuals were found to co-occur. In each with a distinct EOD character, the morphology of the electric organ changes accordingly. P0-absent EODs were the product of electric organs primarily composed of *NPp* electrocytes. P0-present EODs were correlated with electric organs that had predominantly *Pa* electrocytes.

Because the variation in P0 is clinal and occurs in the absence of systematic variation between populations in terms of selective or environmental factors, the phenotypic differences may be the result of populations isolated by geographic distance.

Intermediate Electric Organ Morphology

Earlier in the course of this project, two intermediate phenotypes had been described: one at Cocobeach and the other at Bambomo Creek. Both were correlated with P0-present EOD. There does not appear to be an exact intermediate phenotype: while the intermediate type of electric organ is characterized by clusters of *Pa* type electrocytes separated by clusters of *NPp* electrocytes, there is no fixed ratio of one type of electrocyte to the other.

The repeated, patterned appearance of the electrocyte clusters suggests early developmental determination of the stalk's penetrating character, an *NPp* to *Pa* transition that could reflect mormyrid phylogeny, as suggested by Sullivan et al. (2000).

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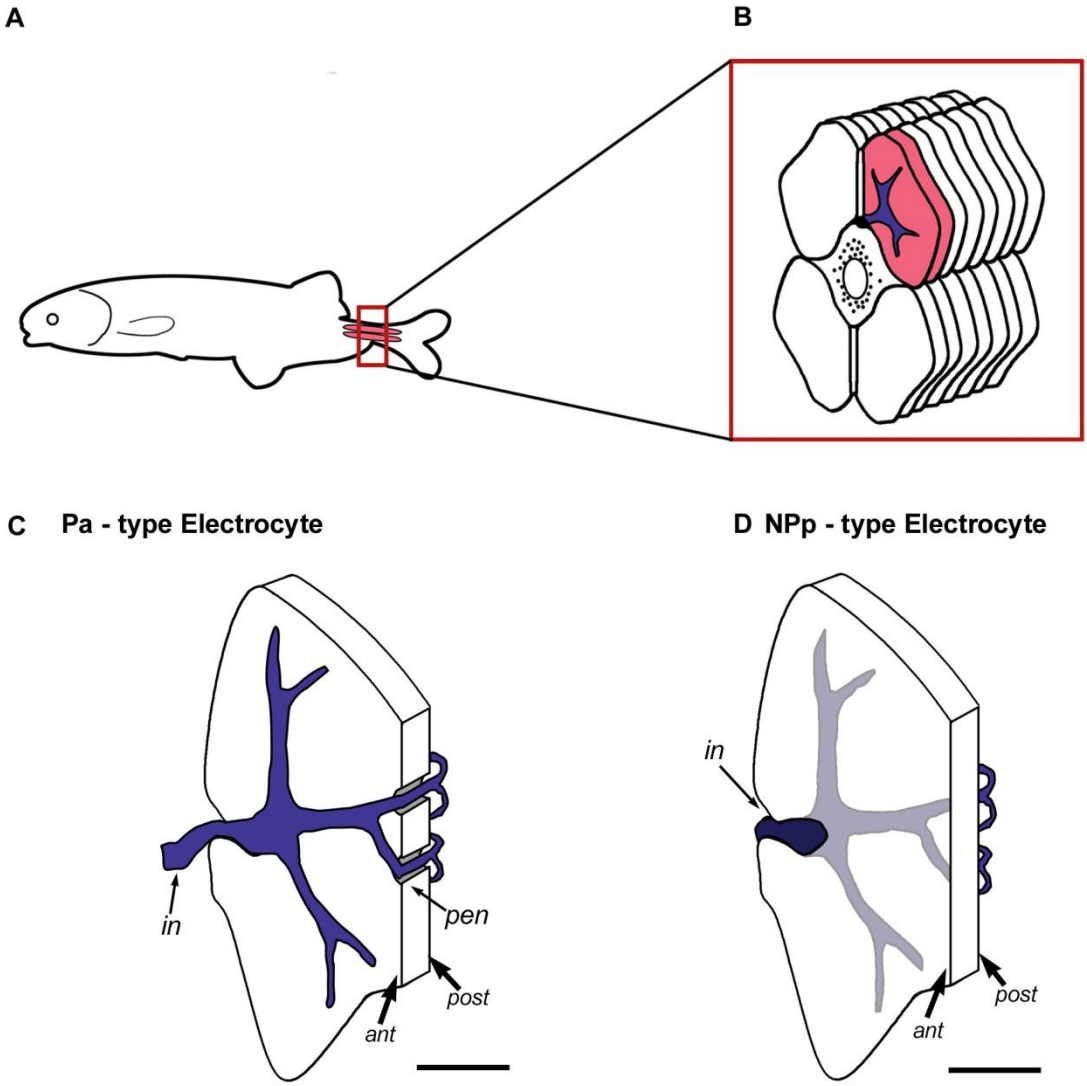

Figure 1. The electric organ (EO) of weakly electric fish.
A The electric organ (pink) is found in the caudal peduncle. **B** The EO is composed of four columns of serially stacked electrocytes (pink) activated by the stalk system (purple). **C** Electrocytes can be penetrating with anterior innervation (Pa) type, with stalks (purple) found on the anterior face before penetrating the electrocyte (*p*) and fusing with the posterior face (*post*) and the site of innervation (*in*) is found on the anterior face (*ant*). **D** Another type of stalk morphology is seen in electrocytes that are non-penetrating with posterior innervation (NPp) type, where the entire stalk is found on the posterior face (*post*), the stalk receives innervations on the posterior face (*in*), and no penetrations are observed. Adapted from Bass et al. 1986.

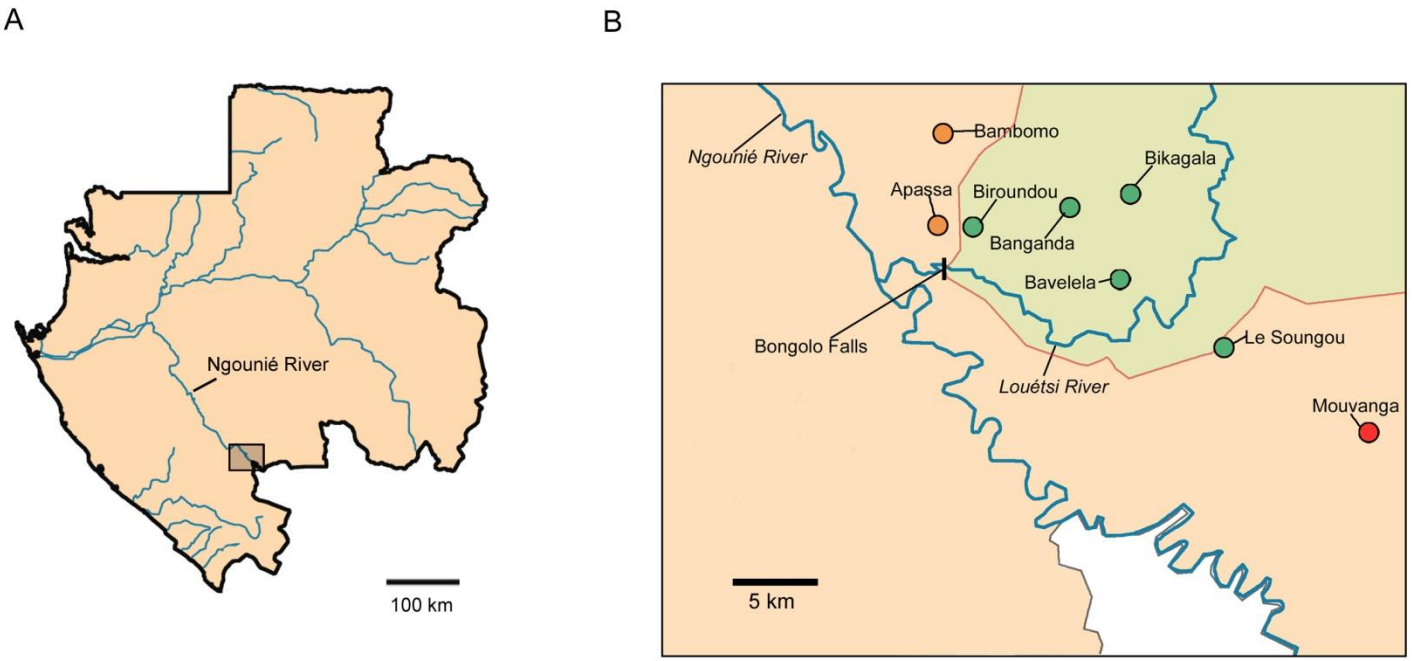

Figure 2. Geographic distribution of P0 present and P0 absent *P. kinglseyae.*
Adapted from Gallant et al. 2011. **A** Map of Gabon with major rivers indicated. The specimens examined in this study were collected from the Louétsi drainage basin of Gabon (gray shaded region). In the majority of localities, only P0-present individuals were captured (not shown). P0-absent individuals occurred in two geographically distinct regions. One such region was the Upper Louétsi watershed. **B** A detailed view of the region near the confluence of the Louétsi and Ngounié rivers. The green area, corresponds to the Upper Louétsi watershed, which is isolated from the Lower Louétsi watershed by the presence of a 15 m waterfall, Bongolo Falls. In the Upper Louétsi, several sample populations of P0-absent fish were observed (green marker). In the Lower Louétsi, P0-present populations were observed (red marker; majority not shown). Mixed populations with both signal types (orange marker) were observed at two collection sites: Apassa Creek and Bambomo Creek.

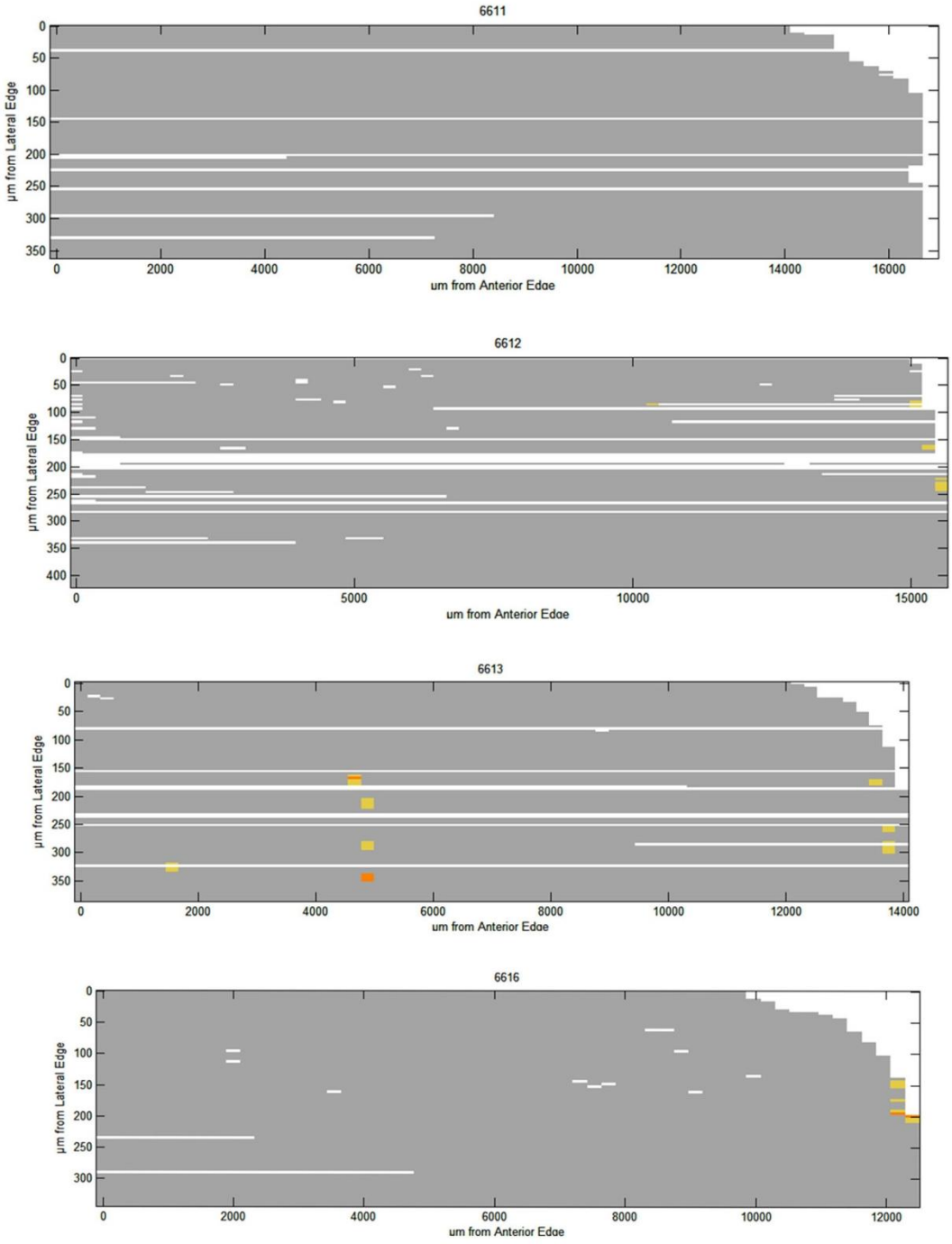

Figure 3. Specimens from Songou Creek are characterized by *NPp* electrocyte morphlogy.

Songou Creek fish have electric organs consisting primarily of electrocytes without penetrations (gray boxes). For all plots, horizontal axis represents distance from the anterior edge of the electric organ, and the vertical axis represents distance from the lateral edge of the electric organ. The *NPp* morphology corresponds to the P0-absent EODs recorded in all fish from this locality. (Number of penetrations: gray = 0; orange = 1; yellow = 2; red = 3; white = section missing/no data)

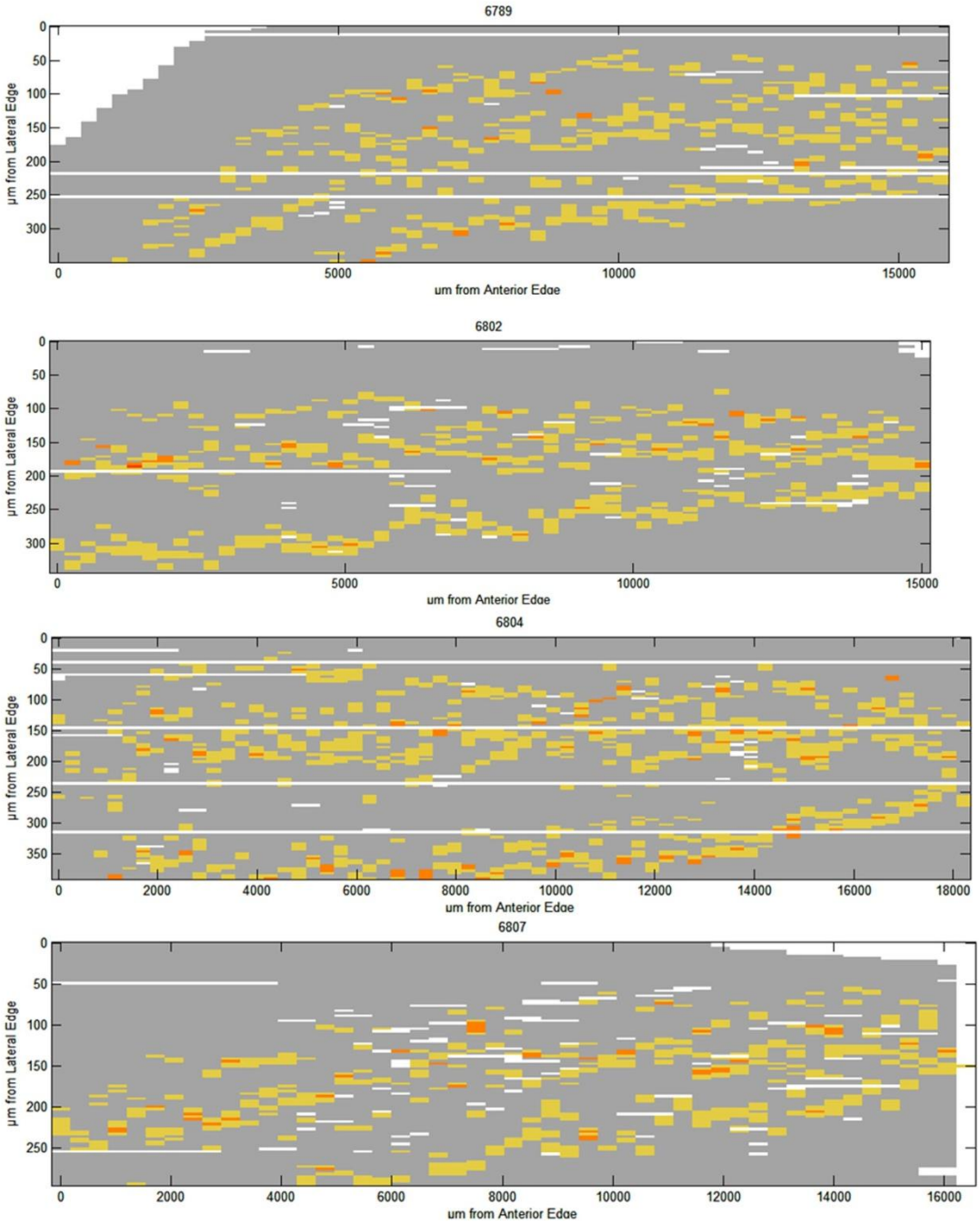

Figure 4. Fish from Mouvanga Creek are characterized by *Pa* electrocyte morphology

Mouvanga Creek fish have electric organs composed exclusively of electrocytes with penetrating stalks: each column represents an electrocyte, and each electrocyte from anterior to posterior contains a yellow or orange block representing one or more penetrations. This *Pa* type morphology is correlated with the P0-present EOD. (Number of penetrations: gray = 0; orange = 1; yellow = 2; red = 3; white = section missing/no data)

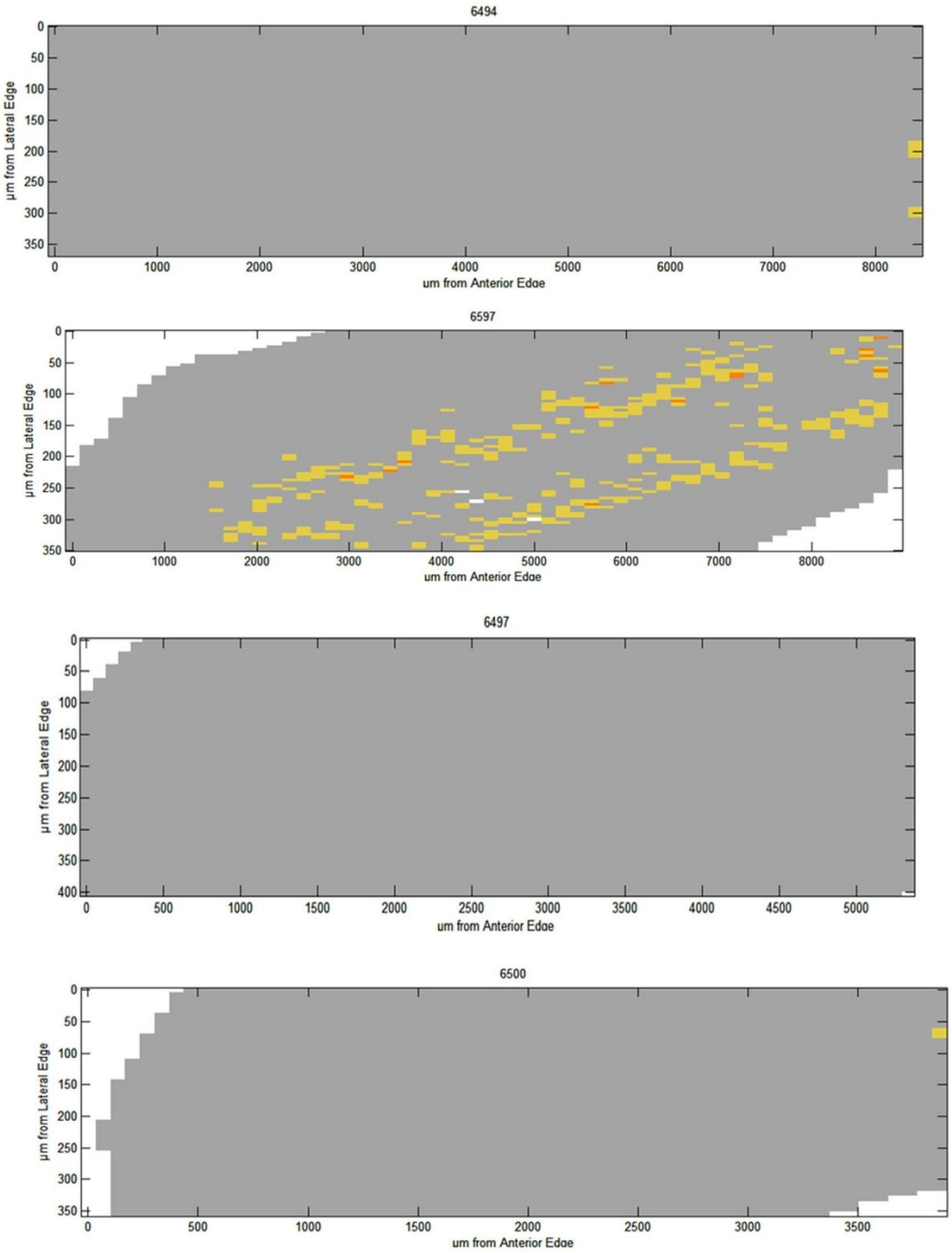

Figure 5. Both electric organ types are present in the Bambomo Creek population
Bambomo Creek fish exhibited both types of EODs corresponding to a mixed population with both *NPp* (first, third, and fourth electric organ reconstructions) and *Pa* individuals (second reconstruction from the top). (Number of penetrations: gray = 0; orange = 1; yellow = 2; red = 3; white = section missing/no data)

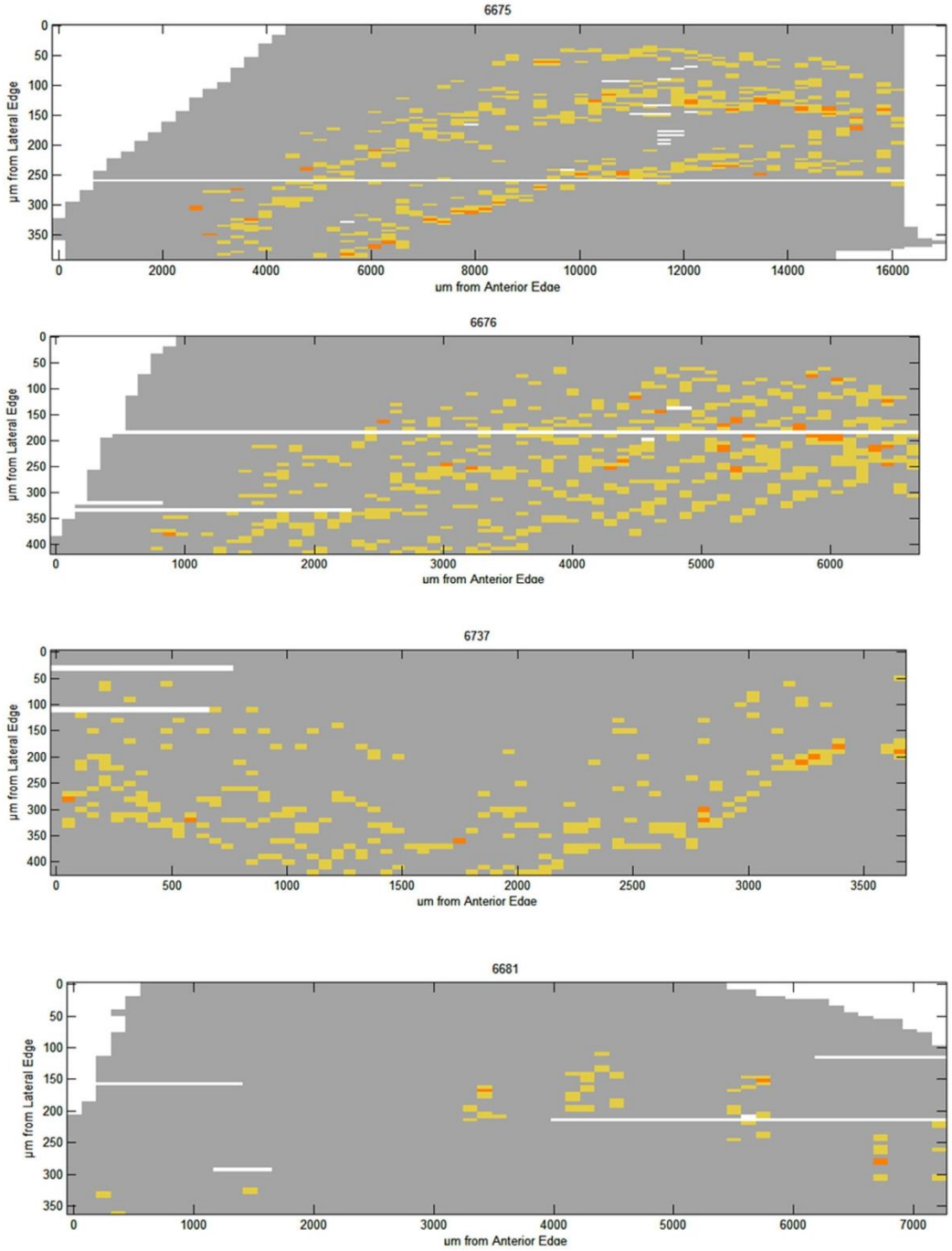

Figure 6. Apassa Creek specimens show evidence of both *Pa* and intermediate electric organ morphology

Apassa Creek fish show mixed electric organ morphology. Most Apassa fish have P0-present EODs and predominantly *Pa* type electrocytes in their electric organs (first, second, and third from the top). One specimen showed intermediate expression of *Pa* type electrocytes (bottom). (Number of penetrations: gray = 0; orange = 1; yellow = 2; red = 3; white = section missing/no data)

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INTRODUCTION

Mormyrids are a family of weakly electric African fish characterized by remarkable diversity. Many of the several hundred mormyrid species have a distinct electric organ discharge (EOD) waveform determined by the morphology of the electric organ. The electrocytes that constitute the electric organ are disk-shaped multi-nucleated cells, each with a complex, rootlike stalk system (Bennett, 1971; Bass, 1986). The stalk system provides not only a structure that propagates depolarization from a single site of innervation, but also a substrate for phenotypic variation between species: the smallest stalklets of the system can be fused to the anterior or posterior face; stalks can pass through the electrocyte to form penetrations; penetrations can be "single" if they simply pass from one face of the electrocyte to the other or "double" if they return to the original face before fusing again with the posterior face (Alves-Gomes & Hopkins, 1997).

In the course of development, two distinct electric organs are observed: the larval electric organ (LEO) and the adult electric organ (AEO), first described in *Pollimyrus isidori* larvae (Kirschbaum, 1977). Both larval and adult electric organs consist of four tubes of serially arranged electrocytes that originate from the medial part of deep lateral muscle (Szabo, 1960; Denizot et al., 1978; Denizot et al., 1982). The larval electric organ, so called because it only appears in larval fish, extends from the edge of the skull to the end of the dorsal fin, while the adult organ is found only in the caudal peduncle (Fig. 1). The larval electric organ becomes active in 8-day old fish, coexists with the adult organ between 40 and 80 days, and then degenerates, leaving only the adult electric organ as the source of EODs.

While the development of the larval electric organ and its corresponding EODs have been well-characterized by a series of studies on *Pollimyrus* (Denizot & Kirschbaum, 1978; Denizot &

Kirshbaum 1980), the early events in adult EO development are not understood, especially the anatomy of the complex penetrating stalk system. Thus, using light microscopic methods, we documented the development of adult electric organ with a focus on the stalk in larval and juvenile *Brienomyrus brachyistius*, an attractive organism for this study because it represents the earliest outgroup of the Mormyrinae sub-family and the possible evolutionary source of the penetrating stalk system (Sullivan and Hopkins, 2000).

METHODS

Specimens: The *Brienomyrus brachyistius* larvae and juvenile fish (n = 62) used in this study were bred in the laboratory by lowering water conductivity and simulating artificial rain conditions using the method described by Kirschbaum (1975). The specimens were collected, recorded, and fixed and embedded during an earlier study.

EOD recordings: Signals were recorded with bipolar silver chloride coated silver wire electrodes, and amplified with a differential bioamplifier (CWE, Inc, Ardmore, PA), and digitized at a 100 kHz–1 MHz sampling rate, with head-positivity upward using a Daqbook or WaveBook (IOTECH: Cleveland, OH, USA).

Ultrathin section: After EOD recording, specimens were first anesthetized with an overdose of MS222 (tricaine methane sulfonate) immersed in 0.1 M phosphate buffer followed by 2.5% glutaraldehyde in 0.1 M phosphate buffer. Fixed specimens were then either processed whole or in divided in three segments (A = head, B = trunk, C = caudal peduncle and tail). The specimens were rinsed in 0.1 M phosphate buffer before initial staining in 4% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA 19440). Tissue samples were dehydrated in a graded alcohol

series up to 70%, then infiltrated and embedded in Mollenhauer Mixture, a combination of Araldite and Poly/Bed resins (Polysciences, Inc., Warrington, PA, USA). We then made ribbons of serial sections (sagittal, n = 73; transverse, n = 29; horizontal, n = 11) using the method described by Campbell (1981). The sections, 0.99 – 7.0 µm thick were then mounted and stained with a 0.1% Toluidine blue solution for 30 seconds over heat. Sections were then imaged using a Leica Leitz DMRB microscope equipped with at SPOT Flex 15.2 64MP Shifting Pixel digital camera (Diagnostic Instruments, Sterling Heights, MI, USA).

3D Reconstruction: 3D reconstruction was done using Reconstruct® (Fiala, 2005). One electrocyte was chosen per specimen for reconstruction, cut in either transverse or sagittal section. A photograph was taken for each section in the electrocyte and aligned seriatim using 2 – 3 manually selected fiducial marks on each image. Profiles of element of the electrocyte, including the stalk, the stalklets, and the electrocyte itself were traced for each section until the spinal cord and surrounding connective tissue became visible (Fig. S1). Rendering was completed with the Boissonnat surface option.

RESULTS

The electric organ discharge was used to track the development of the electric organ. The following sequence of EODs was typically observed: monophasic (MP), monophasic-biphasic (MP-BP), biphasic (BP) and triphasic (TP) (Fig. 2). MP fish are defined by an EOD with a single head positive peak, representing the discharge of the larval electric organ. Fish with MP-BP signal actually have two EODs: a single head positive peak from the larval electric organ followed by a biphasic EOD from the adult electric organ. The initial formation of a functional

adult electric organ takes place in fish with the first two types of EODs. The next two types of EODs, BP and TP, are generated by fish as the stalks form penetrations within the electrocyte to form the mature form of the adult electric organ. The biphasic EOD is a head positive peak followed by head negative peak. In the BP stage, fish no longer have a functioning larval electric organ so the biphasic EOD from the adult electric organ is the only one that remains. The mature EOD is generated by fish in the TP stage and is characterized by a head negative P0 phase, followed by a head positive and head negative peak. Because length is a more reliable indication of the developmental state than the age of the fish is, we use this measure in our description of larvae in each stage (Denizot et al., 1982; Fuiman et al., 1998).

Fish with Monophasic EOD have Larval Electric Organs (n = 10)

The larval EOD first appears in fish with a standard length of 7 mm. At this stage, the larval electric organ can be identified as a region of light-staining cells (Fig. 3). These larval electrocytes are arranged in parallel and resemble muscle fibers, with clearly visible striations and similar orientation within the myotomes. Larval electrocytes can be distinguished from muscle fibers by the presence of a pedicle that extends from the posterior electrocyte into the space between myotomes.

The adult electric organ first begins to form in the caudal peduncle of 9.5 mm fish, between the 6^{th} and 8^{th} vertebrae counting anterior from the last vertebra. The first signs of differentiation appear near the base of the neural and hemal spines in the dorsal and ventral myotomes, respectively (Fig. 4). The myomere is divided into segments parallel to the muscle fibers which are separated by loose connective tissue (Fig. 5). While most of each segment is still striated, the edges appear light-staining and lack the striations characteristic of muscle tissue.

At 13 mm, adult electrocytes appear closely packed and in myomeric arrangement, arising from 7 myomeres and oriented approximately as they are in the mature electric organ, perpendicular to the spine (Fig. 6). Each myomere gives rise to 10-14 electrocytes. Nuclei are mainly found at the periphery of the electrocyte, surrounding an unstriated core. Aside from a single stalk that arises from the middle of the posterior face of each electrocyte, there are no visible differences between the anterior and posterior faces at this stage (Fig. 7).

As the electrocytes differentiate, the distance between each consecutive cell increases and the posterior face is reduced both in thickness and in number of nuclei with the formation of stalks that run the length of the electrocyte from lateral edge to the site of innervations (Fig. 8). These stalks are lighter-staining than the electrocyte and has a single line of nuclei in its center. At 16.5 mm, more than two-thirds of the muscle tissue in the caudal peduncle has been transformed to electrocytes, although a superficial layer of the remaining myotomes can be observed throughout the electrocyte column (Fig. 6). However, as shown by the single head positive peak from the larval electric organ in the EOD, the adult electrocytes remain nonfunctional at this stage.

Fish with Monophasic – Biphasic EOD have both Larval and Adult Electric Organs (n=5)

The adult electric organ is functional for the first time in fish 15 mm long. The biphasic discharge is a result of the depolarization of the posterior face from the point of innervation, leading to an initial head positive phase from the positive ion flow in the rostral direction. This is followed by depolarization of the anterior face, positive ion flow toward the tail, and a resulting head-negative phase in the second half of the EOD waveform. The remaining layer of myotomes observed in fish at the previous stage is no longer visible throughout the electrocyte column. The stalks have increased in diameter and resemble that of a mature fish: they contain ribosomes and

mitochondria, and their nuclei are found dispersed throughout the stalks. Stalklets have begun to form across the posterior face, resembling the early stalk with a single line of nuclei (Fig. 8). Four of the six fish with the MP-BP EOD had electrocytes with fully differentiated anterior faces: these edges are dark-staining from development of extensive invaginations that increase surface area (Fig. 9).

Fish with Biphasic EOD have an Adult Electric Organ without Penetrating Stalks (n=4)

The larval electric organ discharge is no longer detectable in fish as early as 17 mm, suggesting that the LEO is no longer functional. The adult electric organ has reached its full size, having replaced most or all of the axial muscle in the caudal peduncle. The core of each electrocyte shows faint striations, especially at the lateral edges where it is thicker.

Development of the penetrations takes place in fish between 18 and 20 mm of size. As the stalk begins to make contact with the electrocyte, material from the electrocyte is displaced, making it thinner at these points of contact (Fig. 10). The parts of the stalk that make contact differ from those that do not in several ways: color, size, and distribution of mitochondria within the stalk (Fig. 10). No penetrations are present at this stage, but several openings in the electrocyte at the points of contact between stalk and electrocyte were observed (Fig. 10). The edges of these holes lack invaginations and appear to be continuous with the posterior face . The largest points of contact are found approximately 150 µm dorsal and ventral from the site of innervation, near the first bifurcation along the stalk.

Triphasic EOD Stage have an Adult Electric Organ with Penetrated Stalks (n=13)

Penetrations first form at the points of contact defined in the previous stage. Stalks pass through the electrocyte as a loop, forming a double penetration. The part of the stalk that is found on the

anterior face is similar to the region that first comes in contact with the electrocyte: light in color, with mitochondria and other organelles clustered deep within the stalk, in contrast to the part of the stalk that remains on the posterior face (Fig. 11). The preceding head-negative phase of the triphasic EOD is first detected in 19 mm fish. At this stage, the penetrating stalks have not yet reached the anterior face proper of the electrocyte, although the electrocyte has already re-formed around the penetration. The electrocyte appears darker and uneven where it has reformed around each penetration.

The color and unevenness of the electrocyte disappears with increasing distance from the penetration site, and is not detectable in older fish, where the penetrations are bounded by a thickened collar (Fig. 12). The stalk continues to emerge from the electrocyte until the majority of the stalk is found on the anterior face, comparable to the adult fish.

DISCUSSION

Formation of the Adult Electric Organ

We have confirmed that the general order of events in the development of the adult electric organ in *Brienomyrus brachyistius* closely resemble that of *Pollimyrus isidori* as reported by Denizot et al. (1982), where the larval EOD is replaced by the adult EOD after a period during which both organs are active. We also confirm that the adult EO arises myomerically from 7 myomeres in the caudal peduncle.

We have found that the adult electric organ originates from deep within the lateral muscle in 9.5 mm fish near the base of the vertebral spine, between the 6th and 8th vertebrae from the last vertebra. Because the myomeres in this region are divided into segments of similar size to the

core of the first electrocytes and are found in the same region within the myotomes of the caudal peduncle. However, it is still unknown how these segments, which are parallel to the muscle fibers, are reoriented to lie perpendicular to the longitudinal axis of 13 mm fish.

In each of the early electrocytes still arranged within the first 7 differentiated myomeres, nuclei are mainly found at the periphery around an unstriated core. The single distinguishing feature between the anterior and posterior face of each electrocyte is a single stalk. The origin of this single stalk is unclear, although ultrastructural examination of this structure in *Pollimyrus* reveals that it is surrounded by exploratory nerve fibers that make synaptic contact in older fish (Denizot et al. 1982). This observation suggests a model for the development of the stalk system where the single stalk from each electrocyte within the first 7 myomeres becomes the site of innervations, whereas the branches arise from surrounding muscle tissue as it is converted to electrocyte.

As the electrocytes differentiate, the distance between each consecutive cell increases and the posterior face is reduced both in thickness and in number of nuclei with the formation of stalks. At 16.5 mm, the electrocyte resembles that of the adult, with clearly differentiated stalks, numerous stalklets and a densely invaginated anterior face. However, the adult electrocytes remain nonfunctional at this stage until most or all of the axial muscle in the caudal peduncle has been converted to electrocyte.

Development of Penetrating Stalks

Development of the penetrations took place in fish between 18 and 20 mm of size, beginning with migration of the stalk. The stalk first makes contact with electrocyte approximately 150 μm dorsal and ventral from the site of innervation, near the first bifurcations along the stalk. The stalk appears to modify the electrocyte at points of contact, thinning and eventually creating

openings in the electrocyte. The stalks then pass through the holes in the electrocyte as a loop, with the electrocyte re-forming around it to create a double penetration results in a corresponding head negative preceding phase first detected in the EODs of 19 mm fish. The stalk continues to emerge from the electrocyte until the majority of the stalk is found on the anterior face, generating an EOD comparable to the adult.

Two observations call for further investigation: the changing appearance of the stalk and the thickened electrocyte collar. The part of the stalk that is found on the anterior face in a penetration is similar to the region that first comes in contact with the electrocyte: light in color, with mitochondria and other organelles clustered deep within the stalk and not dispersed throughout the stalk or concentrated at the periphery. Similarly, the collar of thickened electrocyte that forms around sites of penetration appears to be a possible determinant in the extent of penetrations. It is still unclear what prevents the entire stalk from passing through the electrocyte, whether it is mechanical resistance or biochemical change.

Recent work in *Paramormyrops kingsleyae* suggests that the presence or absence of penetrating stalks could be the product of a relatively simple genetic mechanism (Gallant et al., 2010). This study has identified major steps and components of electrocyte and stalk development. Understanding the differences in protein expression in the stalk or electrocyte during these key stages could provide useful insights in identifying factors that influence the extent of penetrations in the adult electrocyte.

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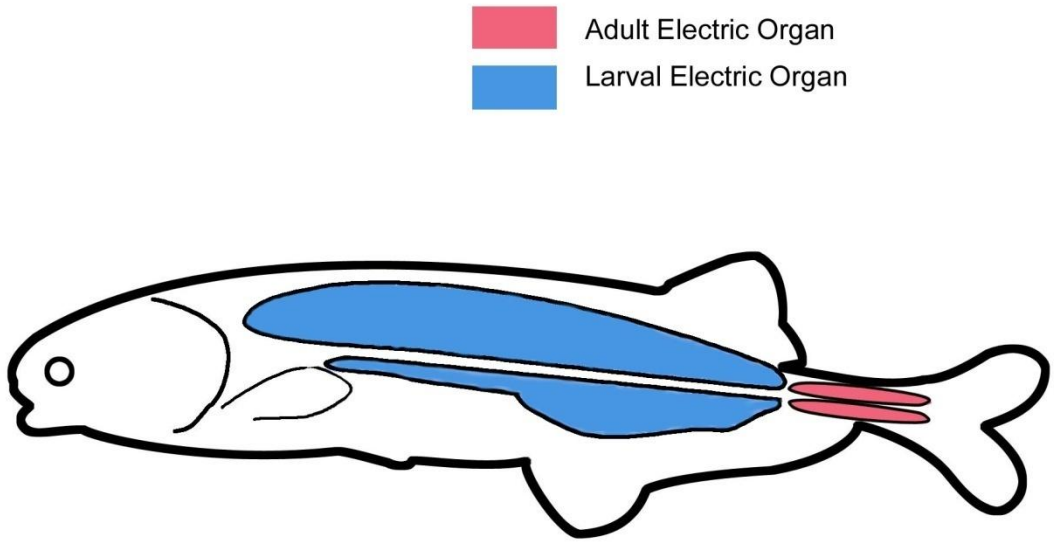

Figure 1. Location of the electric organ and the adult electric organ
The larval EO and the adult EO both arise from the medial part of deep lateral muscle in different regions along the body axis.The larval electric organ (blue) extends from the edge of the skull to the end of the dorsal fin. The adult electric organ (pink) is found only in the caudal peduncle.

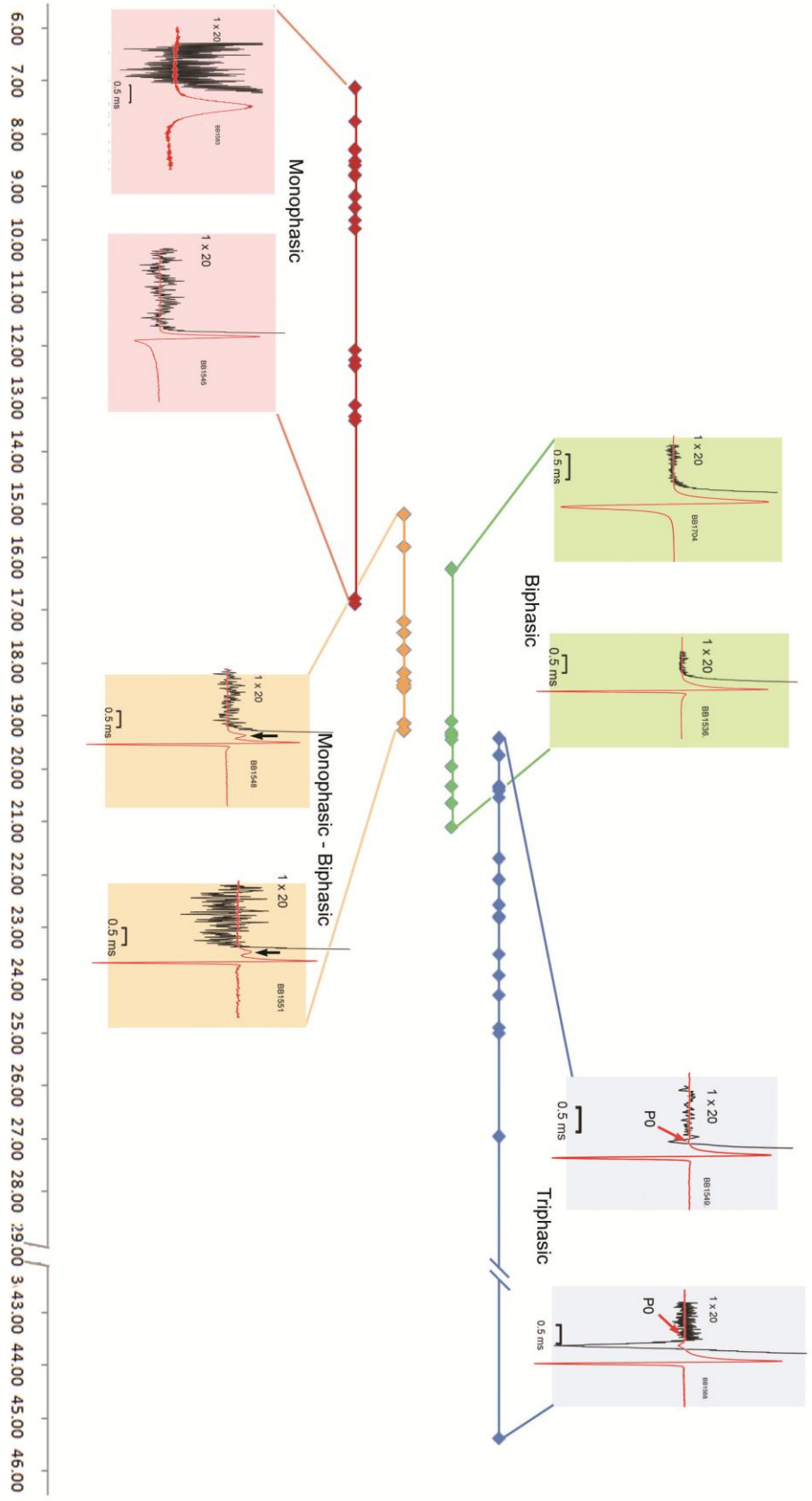

Figure 2. Changes in the electric organ discharge occur in sequence during the course of development
The electric organ discharge (EOD) undergoes successive changes according to age as estimated by standard length. Each point represents an individual fish, plotted according to standard length and colored according to EOD type. The EOD from the smallest and largest fish of each EOD type are shown. Red trace: EOD. Black trace: 20X magnification of EOD. The first type of EOD to appear is monophasic (red), a single head-positive peak generated by the larval electric organ (LEO). The monophasic-biphasic EOD (orange) is a result of two discharges, the monophasic EOD (black arrow) from the LEO and the biphasic EOD from the adult electric organ (AEO). The biphasic EOD (green) is generated when the LEO is no longer functional. Finally, the presence of penetrating stalks within the electric organ adds a head-negative P0 (red arrow) to the EOD, creating a triphasic signal.

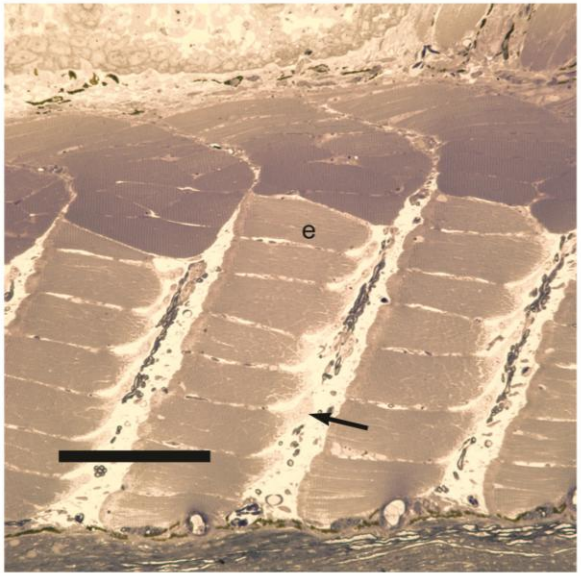

Figure 3. The larval electric organ
The larval EO is light-staining, with electrocytes (e) that resemble muscle fibers. These electrocytes can be differentiated from muscle by the presence of pedicles (arrow). Anterior, left; dorsal, up. Scale is 100 um.

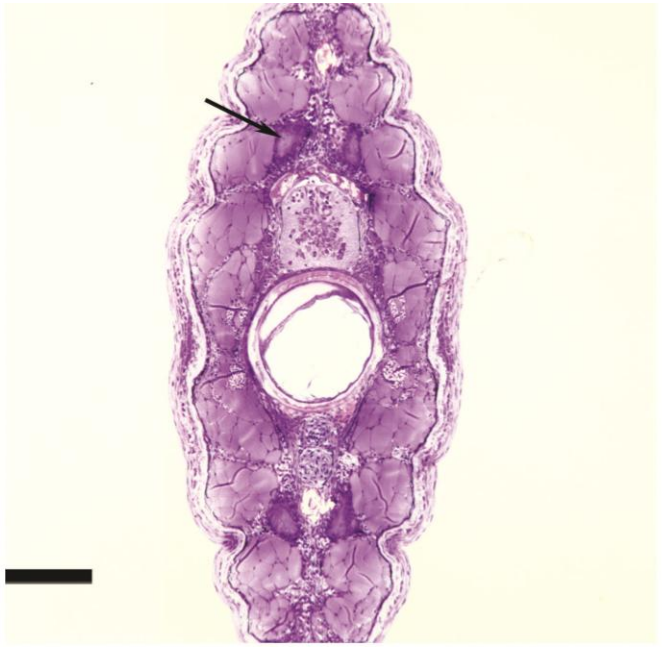

Figure 4. Location of the adult electrocyte
The first signs of differentiation appear near the bse of the neural and hemal spines. The electrocyte (arrow) begins forming within a single myomere. Transverse section; dorsal, up. Scale bar = 100 um

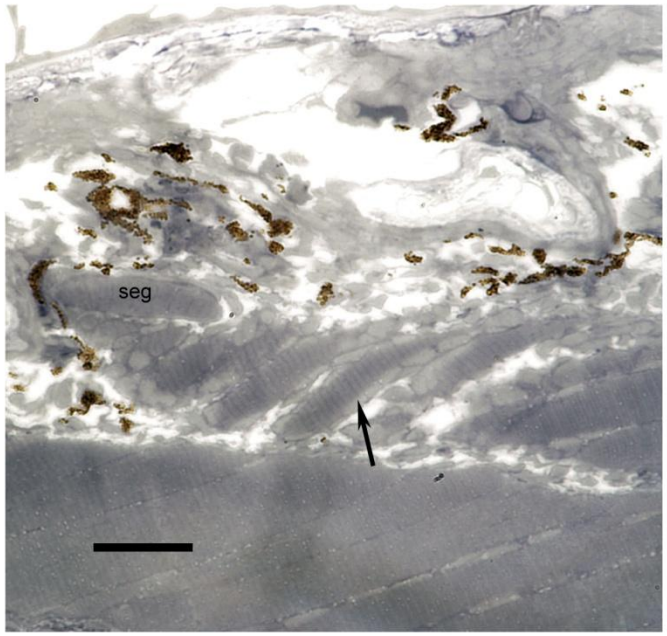

Figure 5 First signs of AEO differentiation.
The myomere is first divided into segments (seg) parallel to the muscle fibers. While most of the segment is still striated, the edges (arrow) appear light-staining and lack the striations characteristic of muscle tissue. Anterior, left; dorsal, down. Scale = 20 um.

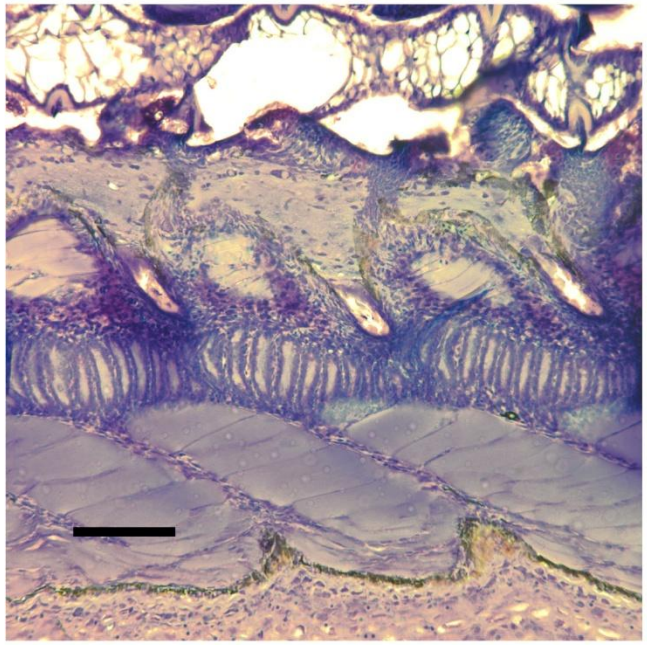

Figure 6 The adult electric organ emerges myomerically
Adults electrocytes (arrow) first appear closely packed in myomeric arrangement, oriented approximately as they are in the mature electric organ, perpendicular to the spine. Anterior, left; dorsal, up. Scale = 100 um.

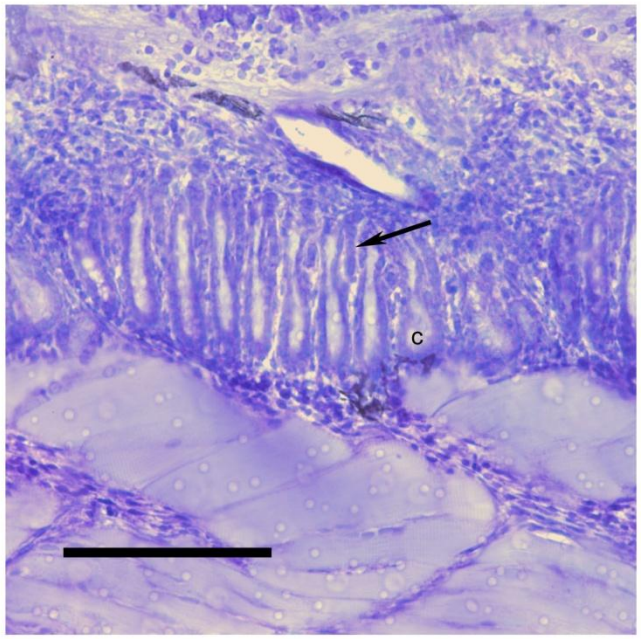

Figure 7 Morphology of the early electrocyte
In the early electrocyte, nuclei are found at the periphery of the electrocyte surrounding an unstriated core (c). A single stalk (arrow) arises from the middle of the posterior face of each electrocyte. Anterior, left; dorsal, up. Scale = 100 um.

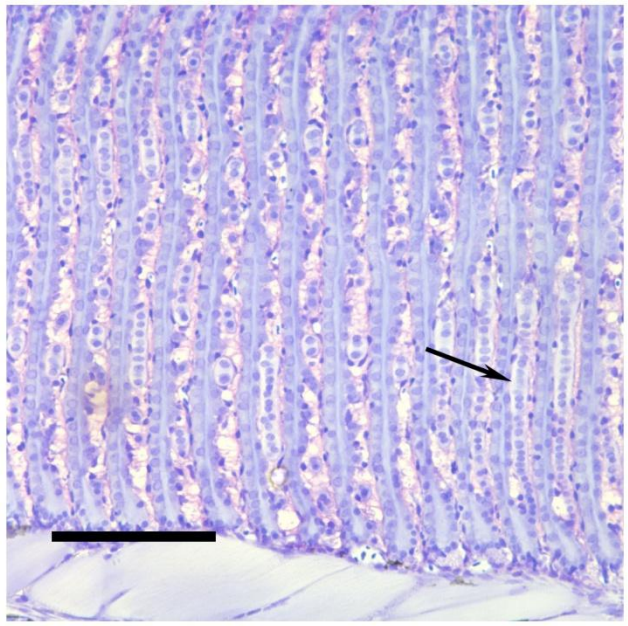

Figure 8 Formation of additional stalks and the differentiating electrocyte
The distance between each consecutive cell increases as the electric organ matures. The posterior face becomes thinner and less nucleated as stalks (arrow) that run across the entire face of the electrocyte are formed.
Very few stalklets have formed from the posterior face. Anterior, left; Dorsal, up. Scale = 100 um

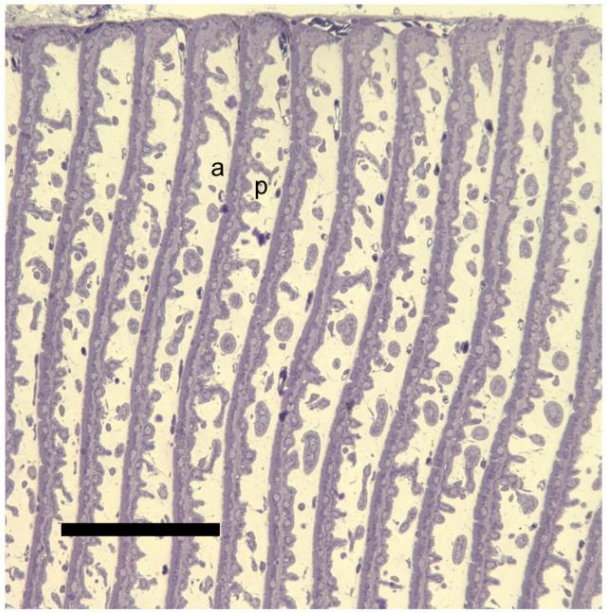

Figure 9 Formation of the functional electrocyte
The functional adult electric organ is composed of electrocytes with distinguishable anterior and posterior faces. The anterior face (a) has a dark band of invaginations. Many stalklets have now emerged from the posterior face (p). Anterior, left; dorsal, up. Scale = 100 um.

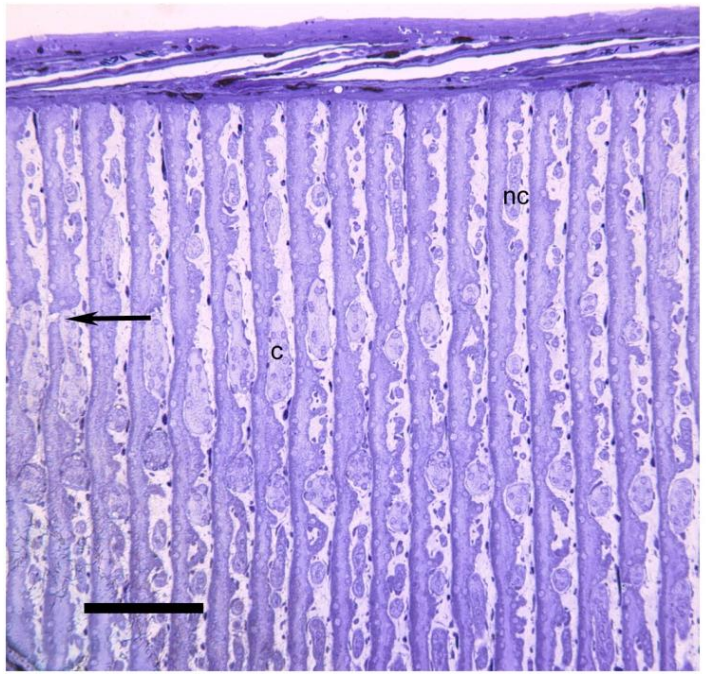

Figure 10 Formation of holes in the electrocyte at the points of contact
The electrocyte becomes thinner at the points where the stalk makes contact. The parts of the stalk that make contact (c) differs from the regions that do not (nc). Holes (arrow) begin to appear in the electrocyte. Anterior, left; dorsal, up. Scale = 100 um.

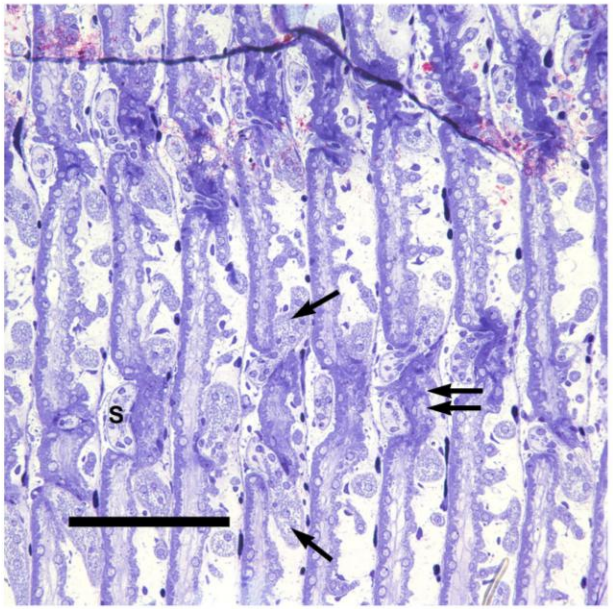

Figure 11 Formation of double penetrations
Stalks (s) pass through the holes in the electrocyte as a loop. The region of the stalk that remains on the posterior face appear darker (arrows) with the presence of more mitochondria and other organelles.The electrocyte that reforms around the future penetrations is darker in color (double arrow) and more uneven than the surrounding electrocyte. Anterior, left; dorsal up. Scale = 100 um

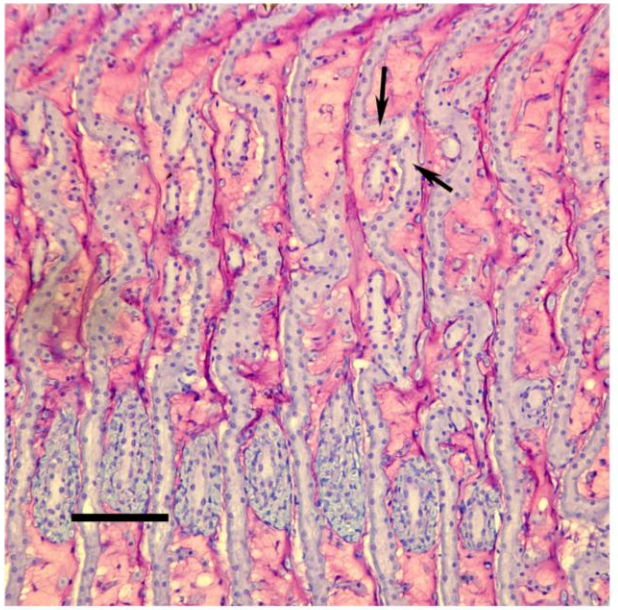

Figure 12 The fully mature electrocyte
The fully mature adult electric organ has electrocytes with double penetrations, invaginations along the anterior face, numerous stalklets on the posterior face, and a thickened collar (arrows) that bounds each penetration. Left, anterior; dorsal, up. Scale = 100 um.

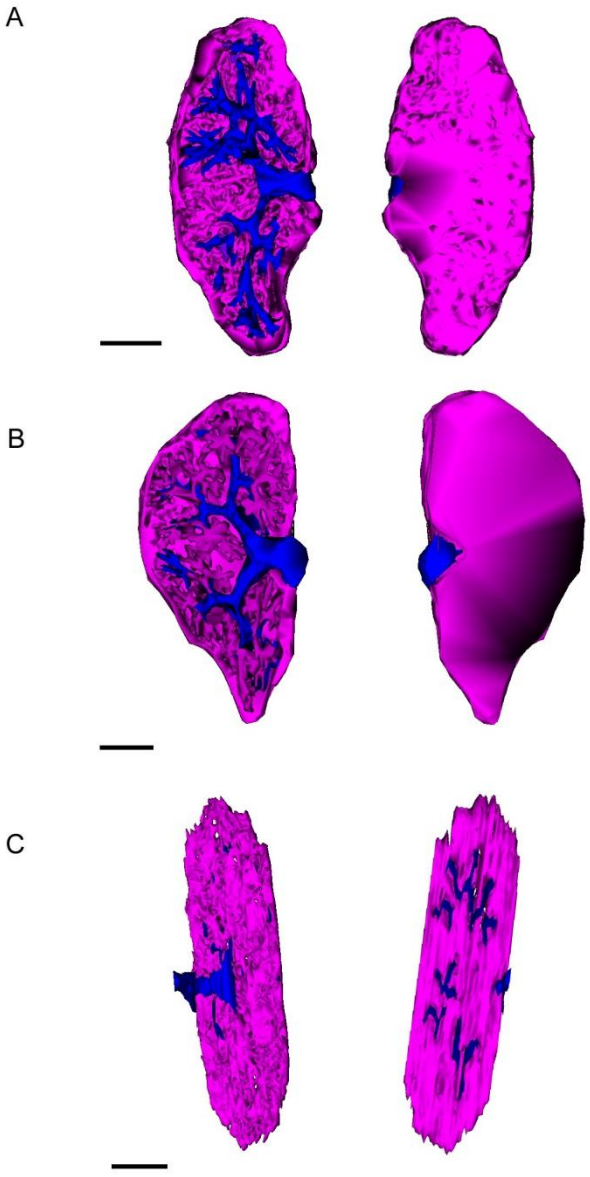

Figure 13 Movement of the stalk through the electrocyte of the AEO as the EOD changes from MP-BP to TP
Posterior face, left; anterior face, right. Scale = 100 um. When the adult electric organ first becomes functional in the MP-BP fish (A) the majority of the stalk is found on the posterior face. In BP fish, the stalk contacts with the electrocyte (C). The majority of the stalk is found on the anterior face of the electrocyte in the mature AEO after the formation of penetrations.

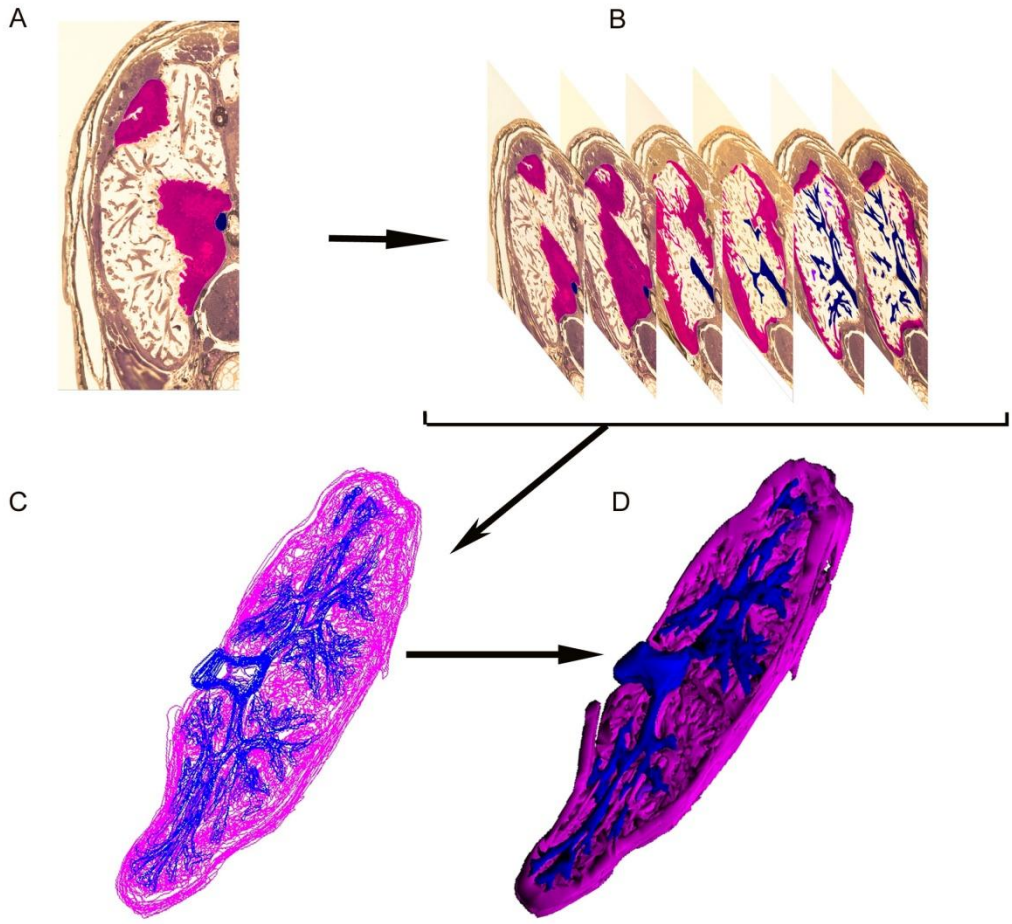

Figure S1: 3D Reconstruction of electrocytes
A-D: Overview of reconstruction process. First, each element of the electrocyte is traced; photograph of a single section showing one electrocyte in one column is shown (A). The photographs of consecutive sections are .aligned (B) . The traces are then compiled (C) and rendered using the Boissonat surface option in Reconstruct (D).

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