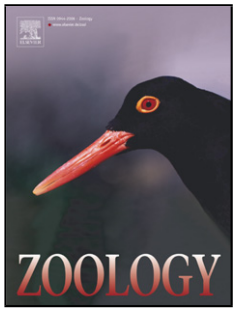

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Identification and distribution of neuronal nitric oxide synthase and neurochemical markers in the neuroepithelial cells of the gill and the skin in the giant mudskipper, *Periophthalmodon schlosseri*

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Highlights

- We document the presence of neuroepithelial cells (NECs) in the gill and skin tissues of *Periophthalmodon schlosseri*.
- NECs and their associated innervation may represent a functional system of O_2 chemoreceptors.
- NECs in the gill and skin are innervated by catecholaminergic nerves, suggesting their involvement in the control of respiration.

Abstract

Mudskippers are amphibious fishes living in mudflats and mangroves. These fishes hold air in their large buccopharyngeal-opercular cavities where respiratory gas exchange takes place via the gills and higher vascularized epithelium lining the cavities and also the skin epidermis. Although aerial ventilation response to changes in ambient gas concentration has been studied in mudskippers, the localization and distribution

of respiratory chemoreceptors, their neurochemical coding and function as well as physiological evidence for the gill or skin as site for O_2 and CO_2 sensing are currently not known. In the present study we assessed the distribution of serotonin, acetylcholine, catecholamines and nitric oxide in the neuroepithelial cells (NECs) of the mudskipper gill and skin epithelium using immunohistochemistry and confocal microscopy. Colocalization studies showed that 5-HT is coexpressed with nNOS, Na^+/K^+-ATPase, TH and VAChT; nNOS is coexpressed with Na^+/K^+-ATPase and TH in the skin. In the gill 5-HT is coexpressed with nNOS and VAhHT and nNOS is coexpressed with Na^+/K^+-ATPase and TH. Acetylcholine is also expressed in chain and proximal neurons projecting to the efferent filament artery and branchial smooth muscle. The serotonergic cells c labeled with VAChT, nNOS and TH, thus indicating the presence of NEC populations and the possibility that these neurotransmitters (other than serotonin) may act as primary transmitters in the hypoxic reflex in fish gills. Immunolabeling with TH antibodies revealed that NECs in the gill and the skin are innervated by catecholaminergic nerves, thus suggesting that these cells are involved in a central control of branchial functions through their relationships with the sympathetic branchial nervous system. The Na^+/K^+-ATPase in mitochondria-rich cells (MRCs), which are most concentrated in the gill lamellar epithelium, is colabeled with nNOS and associated with TH nerve terminals. TH-immunopositive fine varicosities were also associated with the numerous capillaries in the skin surface and the layers of the swollen cells. Based on the often hypercapnic and hypoxic habitat of the mudskippers, these fishes may represent an attractive model for pursuing studies on O_2 and CO_2 sensing due to the air-breathing that increases the importance of acid/base regulation and the O_2-related drive including the function of gasotransmitters such as nitric oxide that has an inhibitory (regulatory) function in ionoregulation.

Keywords: Gill; Skin; Neuroepithelial cells; Neurotransmitters; *Periophthalmodon schlosseri*

1. Introduction

Specialized air-breathing respiratory epithelia on surfaces of the buccal, pharyngeal, branchial, and opercular chambers have been reported for no less than 16 air-breathing genera (Graham, 1997). Most fishes possessing this feature are either amphibious or hold air in their mouths while air breathing. Air-breathing species occur in all ostariophysan orders and, while air breathers do not constitute a high proportion of all species, their respiratory specializations are diverse. There is more documentation of the terrestrial behavior of a few species in two cyprinodont families, the rivulines (Aplocheilidae), and the killifish (Cyprinodontidae). The rivulines are capable of aerial respiration, but not necessarily emerge from water (Graham, 1997). In contrast, mudskippers and their allies (subfamily Oxudercinae) are most notable among the air-breathing gobies. They are a diverse group and their taxonomy is not fully known; their air-breathing and terrestrial behavior have mainly been studied so far.

Amphibious mudskippers breathe air by opening their mouths or by filling the buccal, pharyngeal, branchial, and opercular cavities with air. All of the surfaces, together with the gills and the skin, are considered to be functional for aerial respiration in these fishes (Graham and Kramer, 1976; Graham, 1997). Structural modifications favoring air breathing have been identified in *P. schlosseri* (Yadav et al., 1990) and are likely present in all mudskippers. These include the formation of an inhalant aperture into the branchial and opercular chambers, a well vascularized branchiostegal membrane surrounding the gills and an increased volume in both pharynx and opercular chambers that is 16% of the body volume of this fish (Graham, 1997; Aguilar et al., 2000). Since mudskippers are advanced teleosts adapted for amphibious life in mudflats, their terrestrial activity is associated with a capacity for aerial respiration. But it is also possible that the behavioral preference of mudskippers and their air-breathing physiology may change with

the conditions in their microhabitat (Graham, 1997). So these fishes are not obligatory, but facultative air breathers. More recent studies by Ishimatsu et al. (2007) have emphasized the environmental challenges on the reproduction of mudskippers. This comprises egg development in air and a complex behavioral repertoire including egg guarding, O_2 sensing and air deposition by burrow-guarding mudskippers in the field (Ishimatsu et al., 2000).

The ability of fishes to exchange respiratory gases through their skins was reported in a variety of species (see Graham, 1997 for review). Compared to other gas-exchanging surfaces, fish skin is relatively thick and neither particularly well ventilated nor perfused; its role in gas exchange is thus always auxiliary including the potential for vascular transport of cutaneously obtained O_2 to other body regions. Transcutaneous diffusion distances in most air-breathing fishes vastly exceed those of gills and other vascular surfaces. Although high vessel density is found in various epidermal regions of mudskippers (Park et al., 2003), no studies are available regarding cutaneous air breathing that is only determined on the basis of skin structure. The skin of mudskippers is composed of epidermal cell layers, mucous cells and swollen cells (Park et al., 2003). The respiratory mode has not been quantified in these fishes, and the presence of cutaneous neuroepithelial cells first reported in this study might account for a possible function of these cells as multimodal sensors during their amphibious air breathing. In fact, there is no evidence of NEC plasticity in response to hypercapnia in fully aquatic fishes, but in amphibious fishes it may play a role in the adaptation to hypercapnic environments (Robertson et al., 2015).

Peripheral chemoreceptors associated with oxygen sensing are present in all the vertebrates studied to date. Oxygen-sensing cells have been comprehensively studied in fish where these cells are called neuroepithelial cells (NECs) that are found dispersed throughout the gill arches of fish and the lungs of air-breathing fish (Zaccone et al., 2003, 2006, 2015a; Porteus et al., 2012, 2015; Jonz et al., 2016). They are innervated by trigeminal and cranial nerves (Dunel-Erb et al., 1982; Zaccone et al., 1992, 1994, 1997; Bailly, 2009; Milsom, 2012). A broader spectrum of neurotransmitters (serotonin, ACh, NO and neuropeptides such as enkephalins and neuropeptide Y) is noticed in these cells and the localization of these substances is species-specific. O_2-sensing cells are conserved among vertebrates with their location, stimulus modalities and plasticity in their reflex responses, and have evolved to more effectively match ventilation and perfusion, particularly in animals with central cardiac shunts. In fish some NECs are presumed to be involved in paracrine regulation of gill blood flow, while others are implicated in cardiorespiratory control. So the chemosensing cells sit in locations where they monitor O_2 levels in water, blood and both, but the specific tissue distribution has not been determined yet (Porteus et al., 2012; Wright and Turko, 2016).

Virtually nothing is known about the distribution of O_2-chemosensing cells in the amphibious mudskippers. In *Periophthalmodon schlosseri* hypoxia elicits an increase in ventilation (Aguilar et al., 2000). Hypoxia in most fish elicits a reduction in heart rate and an increase in ventilation. The increase in breathing frequency appears to be regulated primarily by chemoreceptors distributed in all the gill arches in fish (Sundin et al., 2000; Milsom, 2012).

Control of breathing, whether gill ventilation or air breathing, is influenced in fishes by the feedback from peripheral and/or central nervous system receptors that respond to changes in PO_2, PCO_2, and/or pH (Hedrick and Katz, 2015). Given the diversity of respiratory surfaces reported for mudskippers' aerial respiration (Graham, 1997; Ishimatsu, 2012), it is not clear to what extent the breathing patterns of these fishes which exhibit terrestriality are determined by the presence of two anatomically separated groups of skin and gill chemoreceptors. These were reported for the first time in the species under study by Ishimatsu

(2012), including the modifications of the circulatory system regarding the vascularization of the skin and the bucco-opercular cavity.

Although the roles of specific chemoreceptors and their innervation in the control of breathing in mudskippers are not known, the aim of the present study was to investigate the anatomical distribution of the neuroepithelial cells (NECs) implicated in water-breathing in teleosts (Porteus et al., 2012, 2013, 2015) in the gills and the skin of the amphibious mudskipper *P. schlosseri*.

2. Materials and methods

2.1. Study animals and tissue preparation

Specimens of *P. schlosseri* (35–90 g body mass) were purchased from fishermen at Benut, Malaysia, imported to Singapore and transferred to the National University of Singapore. The lowest salinity during low tides and the highest salinity during high tides recorded in the natural habitat of *P. schlosseri* at Benut were salinity 3 (pH = 6.8–7.4) and salinity 30 (pH = 7.8–8.3), respectively. Procedures adopted in the present study were approved by the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore (501/06).

Since specimens of *P. schlosseri* were obtained from the estuaries where salinity fluctuates twice daily during high and low tides, they were immersed in brackish water of salinity 15 in individual plastic aquaria (L 29 cm x W 19 cm x H 17.5 cm) at 25–27 °C under a 12 h light : 12 h dark regime. The tanks were filled up to at least 6 cm with brackish water. Salinity was monitored using a YSI Model 30/10 FT salinometer (YSI Inc., Yellow Springs, OH, USA). No aeration was provided because *P. schlosseri* is a facultative air breather. Fish were fed fish meat and water was changed daily. No attempt was made to separate the sexes. After one week of acclimatization to laboratory conditions, fish were killed with an overdose of neutralized MS-222 (0.2%) (Sigma-Aldrich, St. Louis, MO, USA). The whole left and right gill arches and skins from the branchiostegal membrane, head, operculum and dorsal and lateral body walls were excised and immersed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH = 7.4 and further processed for paraffin embedding. After hydration, some sections were routinely stained with hematoxylin-eosin.

2.2. Immunohistochemistry, confocal immunofluorescence microscopy and antibody characterization

Techniques for immunolabeling were similar to those previously described for a wide variety of fish tissues (Zaccone et al., 2014, 2015 b, c). Double immunostainings were carried out at room temperature. 5–10 µm sections were rinsed in PBS between each incubation; all primary and secondary antibodies used in this study as well as their dilutions are listed in Table 1. The antigens in the double-labeling experiments, with primary antisera raised in different species, were detected by means of indirect immunofluorescence. The sections were rinsed three times with double-distilled water and transferred for 1 h to a blocking solution with PBS containing 0.5% Triton-100 (Sigma-Aldrich), 0.2% bovine serum albumin (BSA; Jackson Immunoresearch, West Grove, PA, USA), 1% dimethyl-sulphoxide, 0.02% sodium azide and 5% normal horse serum (NHS; Jackson Immunoresearch). Next, the permeabilized tissue sections were incubated in primary antibodies for 24 h at 4 °C, and in secondary antibodies at room temperature for 1 h in darkness. Preparations were then placed on glass microscope slides in Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) to reduce photobleaching during confocal scanning.

5-HT, VaChT, nNOS and TH are widely occurring neurotransmitters in fish (Table 2). All the primary antibodies against 5-HT, VaChT, nNOS and TH (Table 1) were chosen on the basis of previous morphological studies performed in both zebrafish and other fish species. NEC cells in the gills and skins of *P. schlosseri* were identified using three different antibodies against serotonin (5-HT) (Table 1). Monoclonal 5-HT antibody has been used to characterize the serotonergic NECs in the gills of several teleost fish species (Saltys et al., 2006; Zaccone et al., 2006; Jonz et al., 2016) including those in the lungs of the bichir (Zaccone et al., 2007). Monoclonal 5-HT antibody (Code MO758; Dako/Agilent Pathology Solutions, Santa Clara, CA, USA) was raised in mouse against 5-hydroxytryptamine hydrochloride and used at a dilution range 1:50 to 1:100, and localized with goat anti mouse secondary antibodies conjugated with Alexa 594 (red) (1:100; Invitrogen, Burlington, ON, Canada). Polyclonal 5-HT antibody (Cat. N° s 5545; Sigma-Aldrich) was also used. It was raised in rabbit against a 5-HT creatinine sulfate complex conjugate with bovine serum albumin. This antibody was used at a dilution of 1:250 and localized with goat anti-rabbit secondary antibodies conjugated with fluorescein isothiocyanate (FITC, 1:50; Cedarlane Laboratories, Burlington, ON, Canada). The specificity of this 5-HT antibody is assumed to be similar in all vertebrate species (Olsson et al., 2008). Moreover, this antibody has already been used to detect serotonergic neurons in the enteric nervous system of mutant and wild-type zebrafish embryos and other fish species (Olsson et al., 2008; Zaccone et al., 2015 b). We pre-incubated the 5-HT antibody with 20 µl/ml 5-HT/BSA conjugate (Immunostar, Hudson, WI, USA) which completely abolished immunoreactivity. The polyclonal VAChT antibody V 5387 (Sigma-Aldrich, dilution 1:250, raised in rabbit) has been used to characterize the distribution of acetylcholine in the chain and proximal neurons and the extrinsic innervation in the filaments of both trout and goldfish (Porteus et al., 2013). We also used VAChT antibody AB 1588 (Merck Millipore, Billerica, MA, USA), dilution 1:100, as employed by Shakarchi et al. (2013) and Stoyek et al. (2015). They reported that the pre-absorption with a synthetic peptide that corresponded to the C-terminus sequence eliminated immunoreactivity in fish gills. Stoyek et al. (2015) used this antibody to label intrinsic and extrinsic innervation of the gills in zebrafish. The specificity and reliability of the antibodies against nNOS and their application in morphological studies of NECs in the gills and swimbladder have already been described by Zaccone et al. (2003, 2006, 2015a), Porteus et al. (2015), and Jonz et al. (2016). Preabsorption of the nNOS antibody sc-648 (Santa Cruz Biotechnology, Dallas, TX, USA) with the homologous blocking peptide containing the sequences to which the antibody was raised (sc-648p; Santa Cruz Biotechnology) – with 100, 10 and 1 ng blocking peptide/ml – led to complete or partial elimination of the immunostaining in all cases. The antibody was diluted to 1:500 and 1:300, respectively, according to the supplier's specifications. Nearly complete and partial blocking of nNOS immunoreactivity was found in MRCs, NECs and neuronal structures of the species studied with 10 and 1 ng/ml of the blocking peptide, respectively. The mouse monoclonal TH antibody recognizes an epitope on the outside of the regulatory N-terminus of TH. It shows homology with the zebrafish TH. This antibody has been used to detect catecholaminergic neurons in the central and peripheral nervous system of the zebrafish (Chen et al., 2009). Evidence of TH labeling in the nerve terminals in the pseudobranch of the trout is reported by Porteus et al. (2013). The application of the antibody to Na^+/K^+-ATPase in morphological studies of the larval zebrafish ionocytes is described by Perry et al. (2016). In all immunohistochemical procedures, omission of the primary antibody resulted in no discernible labeling of the cell distribution and nerves.

2.3. Morphometric analysis

Confocal images of *P. schlosseri* gills and skin were used to perform a morphometric analysis of NEC length and to count them. Numbers and specific regions of measurements are presented in Table 3. Measurements were performed in analogous regions of the particular organs using the Fiji image processing package (https://imagej.net/fiji/downloads), analyzed in accordance with the NECs' size

differences between organs (Student's *t*-test) and between three particular regions of each organ (Kruskal-Wallis ANOVA test).

2.4. Image processing and analysis

Quantitative colocalization analysis was performed using the Fiji image processing package. To reduce noise signals, backgrounds for red and green split channel images were corrected via background removal. Pearson's correlation coefficient (PC) and Mander's (M) overlap coefficient were recorded. Values for the PC coefficient ranged between –1.0 and 1.0, with 0 indicating no significant correlation and 1.0 indicating total colocalization. M coefficient values ranged from 0 to 1.0, with values of 0 and 1 indicating no and total overlap of channel-positive pixels in the image, respectively. We considered combined PC values above 0.5 and M values above 0.9 as significant indicators of colocalization.

Specimen tissues were examined using a confocal microscope (Zeiss LSM 700; Zeiss, Jena, Germany) equipped with argon (Ar) and helium-neon (He-Ne) lasers with peak outputs of 488 nm and 543 nm, respectively.

3. Results

3.1. Structural organization of the gill

As previously reported by Wilson et al. (2000), *P. schlosseri* has four pairs of gill arches divided into two parallel rows, or hemibranchs, as occurs in teleost fishes. The gill filaments bear an alternating series of lamellae on both surfaces. These filaments, like those from most air-breathing fish, interdigitate and the interbranchial septa are much reduced. Blood flow through the gill filaments and lamellae occurs by way of the filament arteries (afferent and efferent) and vascular sinus; it proceeds opposite to the flow of water over the gill to increase gas exchange during ventilation (Jonz and Nurse, 2009). The gill filaments and lamellae are covered by squamous and columnar pavement cells, large mucous cells and mitochondria-rich cells (MRCs). The lamellar epithelium is composed almost entirely of MRCs with ovoid or cuboidal shape. This epithelium consists of a thick layer of MRCs and it is a characteristic feature of the species studied (Wilson et al., 2000).

NECs were localized by immunolabeling methods in the distal half of the gill filament near the efferent arterial vasculature and vascular sinus and lamellar epithelia.

3.2. Structural organization of the skin

The epidermis of the gill membrane covering the ventral portion of the branchial cavity (branchiostegal membrane), operculum, and other skin regions, could be divided into three layers, the outermost layer, middle layer and stratum germinativum. The outermost layer consists of polygonal cells or rather flattened cells arranged in one layer. Mucous cells and capillaries are also present in this layer. The middle layer consists of several layers of the so-called small or voluminous swollen cells forming a web-like structure (Park et al., 2003). Dermal bulges (dermal tissues) are found in this layer; they push up the middle layer. The stratum germinativum consists of a single layer of cuboidal cells (basal cells).

NECs were present throughout the skin epidermis and labeled with specific antibodies.

3.3. Immunohistochemistry of the NECs in the gills

3.3.1. Double immunolabeling of 5-HT and VAChT

As reported in the trout and goldfish gills (Porteus et al., 2013), NECs containing 5-HT were distributed in the tips of the gill lamellae and mainly concentrated in the distal half of the filaments where these cells are often seen in clusters (Fig. 1 A–C). The NECs could be classified into three populations: (1) those containing 5-HT; (2) those containing VAChT; and (3) those containing both 5-HT and VAChT. VAChT immunoreactivity was seen in nerve fibers associated with 5-HT and 5-HT-VAChT-immunopositive NECs. Bipolar neurons along the length of the filament were consistently labeled by VAChT antibodies and colabeled by 5-HT antibodies; their projections extended exclusively along the filament. Additionally, neurons projecting specifically to the base of the efferent filament artery, previously described as superficial and deep proximal neurons (Jonz and Nurse, 2009), also labeled for 5-HT and VAChT. VAChT nerve bundles ran parallel to the efferent filament artery. A collection of neurons colabeled for 5-HT and VAChT was also seen very close to the branchial muscles at the base of the filament (Fig. 1 D–F). 5-HT-immunoreactive nerve bundles were seen running in the muscles, suggesting they are part of the intrinsic innervation of the gills. These neurons were not of similar size to the serotonergic and VAChT-positive NECs.

3.3.2. Double immunolabeling of 5-HT and TH

TH immunoreactivity was detected in perivascular subepithelial nerve fibers around the arterial vasculature of the filament. A large number of 5-HT-immunoreactive NECs colabeled for TH; they were seen isolated and in clusters in the distal half of the filament both in its deeper layer and reaching the external surface, whereas single and few clustered NECS were seen in the tips of the gill lamellae (Fig. 2 A–C). A small proportion of the NECs contained 5-HT and was not colabeled for TH. TH-immunopositive nerve endings were close to the basal pole of some NECs. The apposition of more than the nerve profile to the same NEC indicated that either several nerves converged on a single cell or the same nerve fiber had several contacts. TH nerve fibers were also seen close to the apical pole of some NECs and are catecholaminergic nerve fibers of the sympathetic system (Bailly, 2009). Analysis of the nerve fibers revealed that MRCs are innervated by TH-immunopositive nerve varicosities (Fig. 2 D).

3.3.3. Double immunolabeling of 5-HT and nNOS

Simultaneous detection of 5-HT and nNOS was observed in the NECs of the gill lamellae and the distal half of the filament (Fig. 3 A–C). As previously reported (Jonz and Nurse, 2009; Porteus et al., 2015), it is important to note that all the gill NECs have been identified by the expression of 5-HT and also contain nNOS. This suggests that the neurochemical analysis involves one population of NECs (i.e. 5-HT- and nNOS-positive NECs of the filament and lamellae).

3.3.4. Double immunolabeling of TH and nNOS

Immunostaining with antibodies to TH and nNOS revealed a diffuse organization of the NECs throughout the lamellae and the distal regions of the filaments (Fig. 3 D, E). In all NECs, TH colabeled with nNOS, thus showing that one population of these cells is present. All the NECs are confined to the efferent aspects of the filament and lamellar epithelium indicating both the location of the efferent filament artery and the flow of water over the gills during ventilation. In numerous NECs located in the distal halves on the efferent side of the filament, nerve varicosities came into contact with them (Fig. 3 E). These smaller terminal nerve profiles were suggested by Bailly (2009) to indicate that the nerve fibers are in close association with the NECs. In the gill lamellar epithelium the NECs were often seen exposed directly to the external environment. In the distal halves of the filament, the NECs were seen in clusters. Some cells were exposed directly to the external surface, others were internally oriented and located in the basal layer of the gill

epithelium. MRCs in the gill lamellae revealed a dense innervation by TH-immunopositive nerve fibers (Fig. 3F).

3.4. Immunohistochemistry of MRCs

One type of MRCs or ionocytes (corresponding in terms of its ion transporter to those in the skin and gill of fishes) could be located by immunohistochemistry in the gill lamellar epithelium. MRCs colabeled for Na^+/K^+-ATPase and nNOS (Fig. 4A). In many MRCs a punctate distribution of Na^+/K^+-ATPase was found throughout the cells and the apical and basolateral cell membranes. The search for new transporters is still in progress since MRCs in fishes express distinct sets of ion transporters and thus perform transepithelial H^+ secretion/Na^+ uptake/NH_4^+ excretion/Ca^+ uptake/, Na^+/Cl^- uptake and K^+ secretion (Hwang and Chou, 2013; Perry et al., 2016). No immunoreactivity was noticed in the MRCs when using the nNOS antiserum preabsorbed with the synthetic peptide (Fig. 4 B, C).

Confocal imaging of the gill filaments immunolabeled with antibodies to TH with nNOS and TH with 5-HT revealed a close association of MRCs with TH-immunopositive nerve fibers (Fig. 3 F).

3.5. Immunohistochemistry of the NECs in the skin

Confocal immunofluorescent studies with antibodies to 5-HT, nNOS, TH and VAChT showed that NECs were scattered throughout the skin epithelium and could clearly be classified into both the closed and the open type (Fig. 5 A–J). NECs containing 5-HT colabeled for TH or nNOS, but never for VAChT. In addition to double immunostaining with antibodies to 5-HT and nNOS, colocalization (Fig. 6 A, B) showed at least three NEC subpopulations: a serotonergic and a nitrergic (nNOS) subpopulation, with one subpopulation expressing both 5-HT and nNOS (Fig. 5 E, J). These cells were intimately associated with nerve fibers immunolabeled with antibodies to 5-HT and TH (Fig. 5 D). The present investigation confirmed the source of this innervation both from nerve fibers with corresponding cell bodies that are extrinsic to the gill (Bailly, 2009) and from nerve fibers with cell bodies that are intrinsic and located within the gill filament (Jonz and Nurse, 2009). After 5-HT/TH and TH/nNOS double immunolabeling procedures a basket of TH-immunopositive nerve fibers showed up that surrounded the capillaries in the outermost surface of the epidermis and the swollen cells in the deeper epidermal layers (Fig. 5 D).

3.6. Morphometric analysis

Neuroepithelial cells are more numerous in gills than in the skin (Table 3) and their length does not differ significantly between these two organs (Fig. 7) ($p < 0.05$, Student *t*-test, $n = 668$). Kruskal-Wallis test of NEC lengths in different regions (gill: leading edge, gill filament, gill lamellae; skin: basal surface, middle surface, outermost surface) indicated significant length differences in gills ($H = 17.56$, $p < 0.05$, $n = 442$) and no significant differences in the skin ($H = 4.14$, $p < 0.05$, $n = 226$).

4. Discussion

4.1. Localization and innervation of the neuroepithelial cells (NECs) as putative O_2 chemoreceptors in the gills and skin of P. schlosseri

This is the first study on the distribution and innervation of the NECs in the gills and the skin of amphibious mudskippers. NECs were found in the epithelial layer covering the efferent aspect (i.e. facing the incident flow of water) of the filaments and lamellae, and have been observed in all fish species studied (Zaccone et al., 2006; Perry et al., 2009; Jonz and Nurse, 2009; Portus et al., 2015). NECs typically contain neurotransmitters such as 5-HT as primary transmitters (Zaccone et al., 2006; Jonz and Nurse, 2009). Studies in teleosts such as trout, zebrafish, goldfish and catfish have shown that NECs are closely apposed to nerve fibers originating from multiple sources (Zaccone et al., 2003; Bailly et al., 2009). Innervation patterns generally indicate that NECs form synapses with catecholaminergic nerve fibers with ultrastructural characteristics of afferent and efferent nerve terminals (Bailly et al., 2009). These nerves are extrinsic, having their cell bodies localized within the cranial nerve ganglia. An additional source of NEC innervation includes neurons intrinsic to gill filaments synapsing with a contractile segment of a filament artery, thus indicating a mechanism of vascular control (Jonz and Nurse, 2003; Zachar and Jonz, 2012).

The NECs of *P. schlosseri* show a diffuse organization along the lamellae, the apical end of the filament, and all the cell epidermal layers of the skin epithelium. This may constitute a potential network of O_2-, CO_2-and ammonia-sensitive NECs distributed for sensing changes in either environment, arterial PO_2 or other chemostimuli. In the species studied, like zebrafish and goldfish (Zachar and Jonz, 2012), as many as five populations of NECs in both skin and gills were identified with diverse morphological and neurochemical markers (Jonz and Nurse, 2009).

In the present study there is a possibility that the size (but not the density) of skin NECs was overestimated as may be surmised from the known effect of low water oxygen levels on the site of NECs in *Kryptolebias marmoratus* (Regan et al., 2011) and *Amia calva* (Porteus et al., 2014), which needs further investigation.

4.2. Expression of serotonin and neurochemical markers in the gills and the skin, and in the gill neurons, of P. schlosseri

Immunohistochemistry and confocal microscopy were used to study the presence of serotonin (5-HT)-containing NECs in the gill filaments and the skin of *P. schlosseri*. NECs are thought to be the primary O_2 chemosensors in fish. 5-HT is the primary neurotransmitter in fish NECs (Zaccone et al., 2003; Jonz and Nurse, 2009; Porteus et al., 2015) and one of the many neurotransmitter substances involved in the transduction of hypoxic stimuli, with acetylcholine (ACh) being the primary fast-acting excitatory neurotransmitter (Van Lommel, 2009; Zachar and Jonz, 2012) in the mammalian carotid body. The use of antibodies to VAChT, TH and nNOS to localize neurochemical markers in the NECs of the species studied has suggested that these cells contain acetylcholine (ACh), catecholamines and nitric oxide (Von Bohlen und Halbach and Dermietzel, 2006). Other studies have indicated that, in addition to 5-HT, other candidates may include nitric oxide, catecholamine and ACh (Zaccone et al., 2006; Burleson et al., 2006; Zachar and Jonz, 2012). The expression of AChE and nNOS has been described in the lungs of bichirs (Zaccone et al., 2007, 2015a) and may play important modulatory roles in O_2 sensing. Catecholaminergic nerves of the sympathetic system displaying degenerated varicosities were demonstrated in contact with NECs after surgical and chemical (5-,6-OHDA) sympathectomy (Bailly, 2009). The extrinsic nerves showing TH immunoreactivity and associated with NECs in the distal halves of the gill filaments of *P. schlosseri* could correspond to the first type of catecholaminergic nerve endings in the trout gill as suggested by Bailly (2009). These data show that many NECs, particularly in the apical filament and in the skin of the species studied, establish synapses with catecholaminergic sympathetic nerve fibers. TH-immunopositive nerve fibers are seen coursing in the extensive capillary network at the epidermal surface of *P. schlosseri*. These

findings are in agreement with the study by Cooper et al. (2012) demonstrating alpha adrenoreceptor-mediated vasoregulation in the fin of the rivulus *K. marmoratus*.

Cells expressing VAChT as well as serotonergic NECs were observed in the amphibious fish *Kryptolebias marmoratus* (Regan et al., 2011) and in zebrafish larvae (Porteus et al., 2015). The skin NECs of *K. marmoratus* are morphologically quite different from those of the species studied herein where the serotonergic cells colabeled with TH and nNOS, but not with VAChT, and show an innervation associated with TH-positive nerve fibers. The NEC population in the skin of *K. marmoratus* could play an adaptive role during emersion on land (Regan et al., 2011), but *P. schlosseri*, in contrast, is one of the mudskipper species routinely coping with hypoxia and hypercapnia in its natural habitat, i.e. in the burrows where it resides (Aguilar et al., 2000). The NECs in the skin of this species could be sensitive to changes in O_2, CO_2 and/or H^+. CO_2-oriented ventilatory responses are absent in air-breathing organs of fishes (Sundin et al., 2006). But acid/base regulation is accomplished by ion exchanges. The gill lamellae of *P. schlosseri* are endowed with MRCs that have ion transporters and antiporters involved in the active transport of ions, but we have not identified MRCs by immunohistochemistry. The role of skin in gas exchange is also auxiliary in that gas exchanging surfaces are relatively thick and neither ventilated nor perfused (Graham, 1997). So the role of skin NECs in the mudskipper is not clarified and needs further physiological and genetic studies. NECs in the gill of *P. schlosseri* resemble those in the skin, but we have no evidence of their homology. NECs in fish gills were strongly believed to have a phylogenetic relationship with mammalian O_2 chemoreceptors, but recent experiments have proven that mammalian O_2 chemoreceptors and NECs of fish gills have different origins (Hockman et al., 2017). In fact, studies using genetic lineage tracing and neural crest-deficient mutants in zebrafish have excluded the origin of NECs in amniote gills and orobranchial epithelia from the neural crest, but confirmed their homology to hypoxia-sensitive pulmonary neuroendocrine cells in lung airway epithelia. Also, it may be possible that NECs in both gill and skin epithelium are simply “epithelial“, i.e. can be differentiated in situ into both endoderm-derived and ectoderm-derived epithelia (Hockman et al., 2017).

ACh was expressed in chain and proximal neurons and extrinsic nerve bundles in the filament. In the filament neurons, VAChT colabeled with 5-HT. The second type of nerve fibers close to NECs as suggested by Bailly (2009) is seen in the subepithelial parenchyma beneath these cells, indicating that in addition to synaptic contacts there may be an association between NECs and filament neurons in the subepithelial tissues. The serotonergic NECs found by double labeling to also contain TH and nNOS appear innervated by 5-HT-positive nerve fibers probably originating from neurons of the filament and by TH- and nNOS-positive nerve fibers originating from extrabranchial cell bodies as also emphasized by Jonz and Nurse (2003).

Chain neurons in *P. schlosseri* are seen in close association with subepithelial smooth musculature attached to the basal lamina of the filament epithelium, which is innervated by TH- and 5-HT-positive nerve fibers. Some of the filament neurons are likely candidates for relaying hypoxic signals from the NECs (Bailly, 2009) and so have afferent sensory functions, in addition to a local motor control of the efferent respiratory vasculature in response to hypoxia. Smooth branchial muscle may modulate the surface area and orientation of the filament epithelium, a mechanism that could also involve serotonin from the neurons that could also project to it.

Although several neurochemical markers such as ACh, TH and nNOS found both in the NECs and associated innervation in the amphibious mudskipper species studied here have not yet been supported by physiological data, the immunohistochemical analysis suggests that the neurochemical basis of O_2 chemoreception in the gill includes multiple populations of NECs and a broad spectrum of

neurotransmitters. These data are in agreement with the fact that a variety of neurochemical substances such as ACh, 5-HT and dopamine applied exogenously to the gill have been shown to have stimulatory effects on sensory nerve fibers and cardiorespiratory reflexes (Burleson and Milsom, 1995).

4.3. Expression of Na^+/K^+-ATPase *and nNOS in the MRCs of the gills and associated innervation*

P. schlosseri lives in mud burrows at the top of the intertidal zone of mangrove mudflats, where burrow water is hypoxic and hypercapnic and has relatively high ammonia levels (Randall et al., 2004). This fish can maintain tissue ammonia levels in the face of higher ammonia concentration of the water. This is probably achieved by active ammonium ion transport across the MRCs via an apical Na^+/H^+ exchanger and a basolateral Na^+/K^+-ATPase (Randall et al., 2004; Chew et al., 2014). In the present study we tested MRCs for Na^+/K^+-ATPase in combination with nNOS and found colabeling of both enzymes. Randall et al. (2004) also reported a low NHE3 permeability in the skin that is responsible for reducing ammonia influx. But we did not test this and did not find any presence of MRCs in the skin epithelium.

Recently, Perry et al. (2016) have reviewed the role of gasotransmitters in the ion uptake in zebrafish, and in particular the reduced activities of branchial Na^+/K^+-ATPase and H-ATPase in the presence of nitric oxide (NO) donors, suggesting an inhibitory regulatory role of NO in fish osmoregulation. Effects of exogenous NO application or inhibition of endogenous NO synthesis on the Na^+ balance were not investigated in the mudskippers. But colocalization of nNOS and Na^+/K^+-ATPase was noticed by Ebbeson et al. (2005) in the gill ionocytes of SW-acclimated Atlantic salmon.

MCR innervation has not been investigated in other fish species except for zebrafish (Jonz and Nurse, 2008). Nerve fibers in fish gills are associated with basolateral regions of MRCs where Na^+/K^+-ATPase activity is localized (Evans et al., 2005), and with apical regions of MRCs positioned toward the external environment. Neurotransmitters and neuropeptides, such as catecholamines, nitric oxide, vasoactive intestinal polypeptides and prostaglandins have been shown to mediate the movement of ions across gill opercular epithelia, and stimulation of chloride extrusion is mediated by adrenergic receptors (Evans et al., 2003; Jonz and Nurse, 2008). MCRs of the filament epithelium make contact with extrinsic nerves derived from the nerve plexus of the filament that also innervates the NECs (Jonz and Nurse, 2008). Our immunohistochemical findings show that MRCs in the gill of *P. schlosseri* appear to make contact with TH-immunopositive nerve fibers, thus suggesting catecholamine innervation of these cells. They also demonstrate that gill MRCs must carry membrane receptors tuned to the neurohormones (catecholamines) released by the nerve endings of the species studied. In fact, catecholamines contribute to Na^+ uptake in zebrafish (Guh et al., 2015) and play an important role through their effects on alpha- and beta-adrenergic receptors (Evans et al., 2003, 2005). Neuropeptides such as endothelin 1 were also localized by immunohistochemistry in large round cells adjacent to MRCs in the gill of killifish (*Fundulus heteroclitus*) (Hyndman and Evans, 2007) and in the NECs of fish gills (Zaccone et al., 1996).

5. Conclusions

The NECs and oxygen-sensitive glomus cells of the carotid body of fish share in common some chemoreceptive mechanisms and the same embryologic origin (Jonz and Nurse, 2009), while we have no available information on the skin NECs in this respect. Previous studies suggested that, like the glomus cells of the carotid body, some of the NECs in the skin are multimodal sensors. Many air-breathing fish increase air-breathing frequency in response to aquatic hypercapnia (Graham, 1997). Aquatic hypercapnia has been studied most extensively because it represents a potentially natural environmental condition, but *P. schlosseri*, one of the mudskippers which store air in their burrows, may be the only air-breathing fish

which routinely experiences hypercapnia (Aguilar et al., 2000). Therefore the multiple NEC cell populations sitting in its gills and skin may represent multimodal sensors. In fact, in vivo serotonergic NECs respond to both PO_2 and PCO_2 (Qin et al., 2010) while others respond to changes in ammonia (Zhang et al., 2011). Although more physiological studies were conducted in some mudskipper species, nothing is yet known on the cellular basis of their O_2- and CO_2-sensing as well as the receptor responses.

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Figure Captions

Fig. 1. (A) Longitudinal section of the gill of *P. schlosseri* stained with haematoxylin-eosin showing the presence of filaments (FE) and lamellar (L) epithelia. (B) A population of mitochondria-rich (MR) cells is present in the gill lamellar epithelium. (C). Longitudinal section of the branchiostegal membrane with skin showing the presence of a large number of swollen cells (SW) in the middle layer (ML), except for the outermost layer (OL). BL = basal layer. (D-I) Confocal images from double immunolabeling of the gill of *P. schlosseri* using antibodies against 5-HT (green) and VAChT (red). (D) Note the localization of the NECs (arrows) in the distal end of the filament epithelium with nearby VAChT-positive nerve bundles along the wall of the efferent filament artery (eFA). (E) Higher magnification of the gill filament in (D) showing serotonergic NECs (green), VAChT NECs (red) and 5-HT-VAChT-positive NECs (orange) as marked by arrows. (F) NECs in the gill lamellae and the distal filament. The serotonergic NECs on the tips of the lamellae (arrows) directly contact the external environment (EE). In the distal filament (DF) both a serotonergic NEC and a 5-HT-VAChT positive NEC (arrows) are visible. (G) Chain neurons (ChN) labeled with 5-HT and VAChT antibodies at the base of the efferent filament artery (eFA). Note the presence of serotonergic NECs (arrows) dispersed throughout the gill lamellae (G) and gill arch (Ga). (H and I) Chain multipolar neurons (ChN) labeled with 5-HT and VAChT antibodies at the base of the filament artery vasculature and in close vicinity to branchial smooth muscle (SM). Note the presence of 5-HT-positive bundled nerves (arrows) in the muscle, probably originating from 5-HT-VAChT-positive chain neurons. Numerous serotonergic NECs (arrows) are seen in the gill lamellar tissue. Scale bars = 100 μm in A and C, 50 μm in B, 20 μm in D, F, G, H and I, and 10 μm in E.

Fig. 2. Localization of the NECs in the distal end of the filament and the gill lamellae using antibodies against 5-HT (red) and TH (green). (A) In the distal end of the gill filament the 5-HT-TH-immunopositive NECs are seen in clusters. One NEC shows a point of contact with a TH-positive bundled nerve. (B) A region of the image in (A) shown at higher magnification reveals the close association of NECs and nerve fibers by colocalization of 5HT and TH labeling (arrowheads). (C) NECs of gill lamellae dispersed in the interfilament epithelium (arrows) and others at the tips of the lamellae in contact with the external environment. (D) Confocal image revealing the close association of TH-immunopositive innervation with mitochondria-rich cells (MRCs) marked by arrows. Scale bars = 20 μm in A, C, D, 10 μm in B and insert.

Fig. 3. Confocal images of the gill filament and lamellae. (A–C) Distribution of NECs in the gill lamellae and distal filament regions. (A) Double labeling with antibodies against 5-HT (green) and nNOS (red) showing NECs in the tips of lamellae (arrows). (B) NECs are located within the efferent aspect of the filament epithelium. Some of these cells reach the external environment by a long process (arrow). eFA = efferent filament artery. (C) High magnification image showing NECs in the distal filament region. (D–F) Distribution of NECs in the distal filament regions, and of nerves in the gill lamellae. Double immunolabeling with antibodies against TH (green) and nNOS (red). (D) Clustered NECs near the efferent filament artery (eFA). Some NECs are facing the external environment (arrow). (E) Note the large nuclei in NECs and points of contact with nerve fibers (arrows). (F) Confocal image showing a close association between MRCs (arrows) and TH-immunoreactive dense nerve fibers. Scale bars = 20 μm in A, B, 10 μm in C, 20 μm in E and F.

Fig. 4. Expression of Na^+/K^+-ATPase and nNOS in the MRCs of the gill lamellae. (A) General view showing Na^+/K^+-ATPase and nNOS colocalization in MRCs of the lamellae. In some MRCs the punctate Na^+/K^+-ATPase immunostaining (arrows) is visible on the cell membranes. (B) nNOS-positive cells are abundant in the gill lamellar epithelium. (C) The same cells showing a nearly complete or partial blockade of nNOS immunoreactivity with 10 ng/ml blocking peptide. Scale bars = 20 μm.

Fig. 5. Merged confocal images of NECs and nerves in skin from the branchiostegal membrane of *P. schlosseri*. (A) Double immunostaining for VAChT and 5-HT revealing a 5-HT-positive NEC in the outer epidermis (arrow). (B and C) Double immunostaining for TH and nNOS revealing the coexpression of the two neuronal markers in the NECs that are located deep in the epidermis (arrows in C), and the TH-immunopositive dense innervation in the outer epidermis (OE) and among the swollen cells (SC). (D) Double immunolabeling for TH and 5-HT revealing the presence of a NEC (arrow) with associated TH-positive nerve bundles (arrows). Fine varicose TH-immunopositive nerves are also found at the skin surface and among the layers of the swollen cells (SC). (E–I) Double immunostaining for 5-HT and nNOS revealing the presence of NECs in the intermediate, outer and outermost skin layers. An nNOS-positive NEC (H) is confined to the outermost skin layer, whereas a NEC with a long process is seen reaching the epidermal surface. (J) Double immunostaining for Na^+/K^+-ATPase and nNOS revealing the absence of MRCs and the presence of three nNOS-positive NECs in the mid epidermis. BL = basal layer; M = muscle; MC = mucous goblet cell; SC = swollen cells. Scale bars = 10 μm.

Fig. 6. An average proportional distribution of 5-HT, nNOS, Na^+/K^+-ATPase, TH and VAChT (A) in the skin and (B) in the gill of *P. schlosseri*. Proportional distribution of most markers remained constant. Bar heights and whiskers represent sample means (for $n > 1$) and standard deviation, respectively.

Fig. 7. Mean dimensions (length and width) and standard deviation of NECs in three regions of the gills (leading edge, gill filament and gill lamellae) and skin (basal surface, mid surface, outermost surface) of *P. schlosseri* based on immunostained images. Measurements as shown in Table 3.

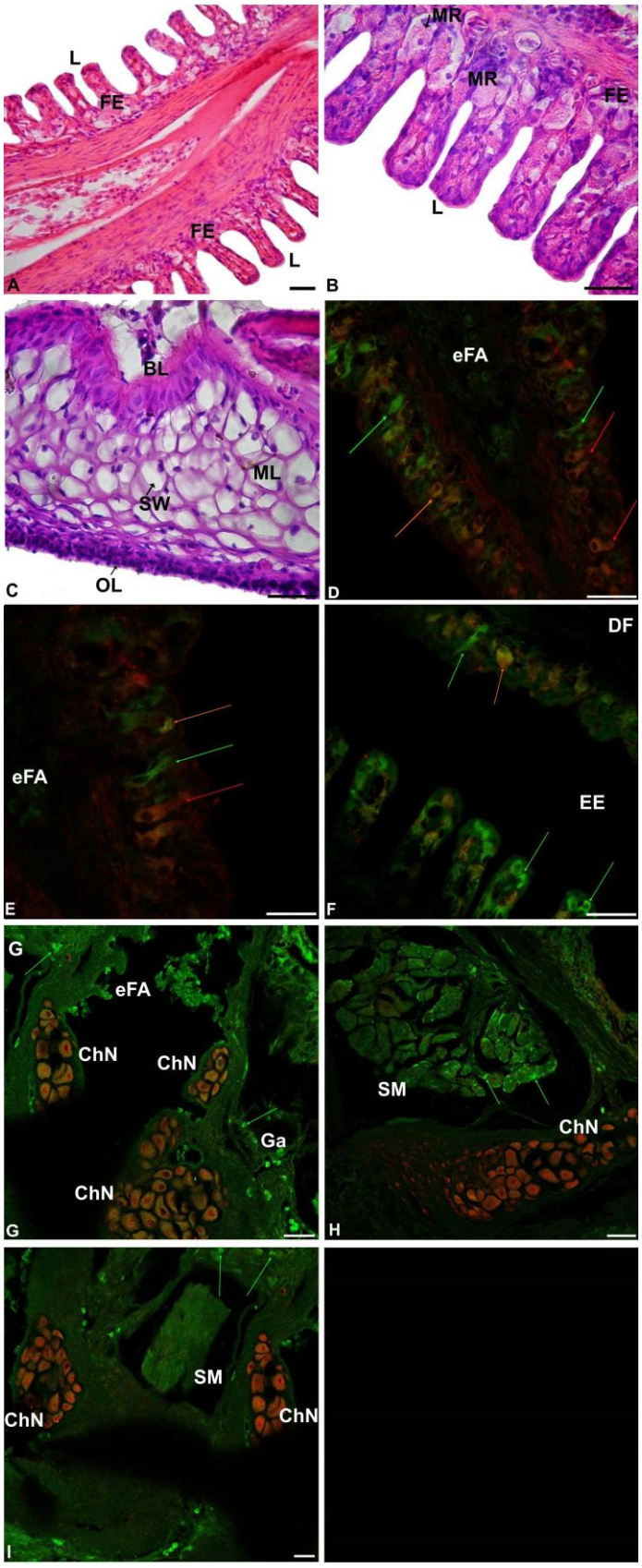

Figure 1

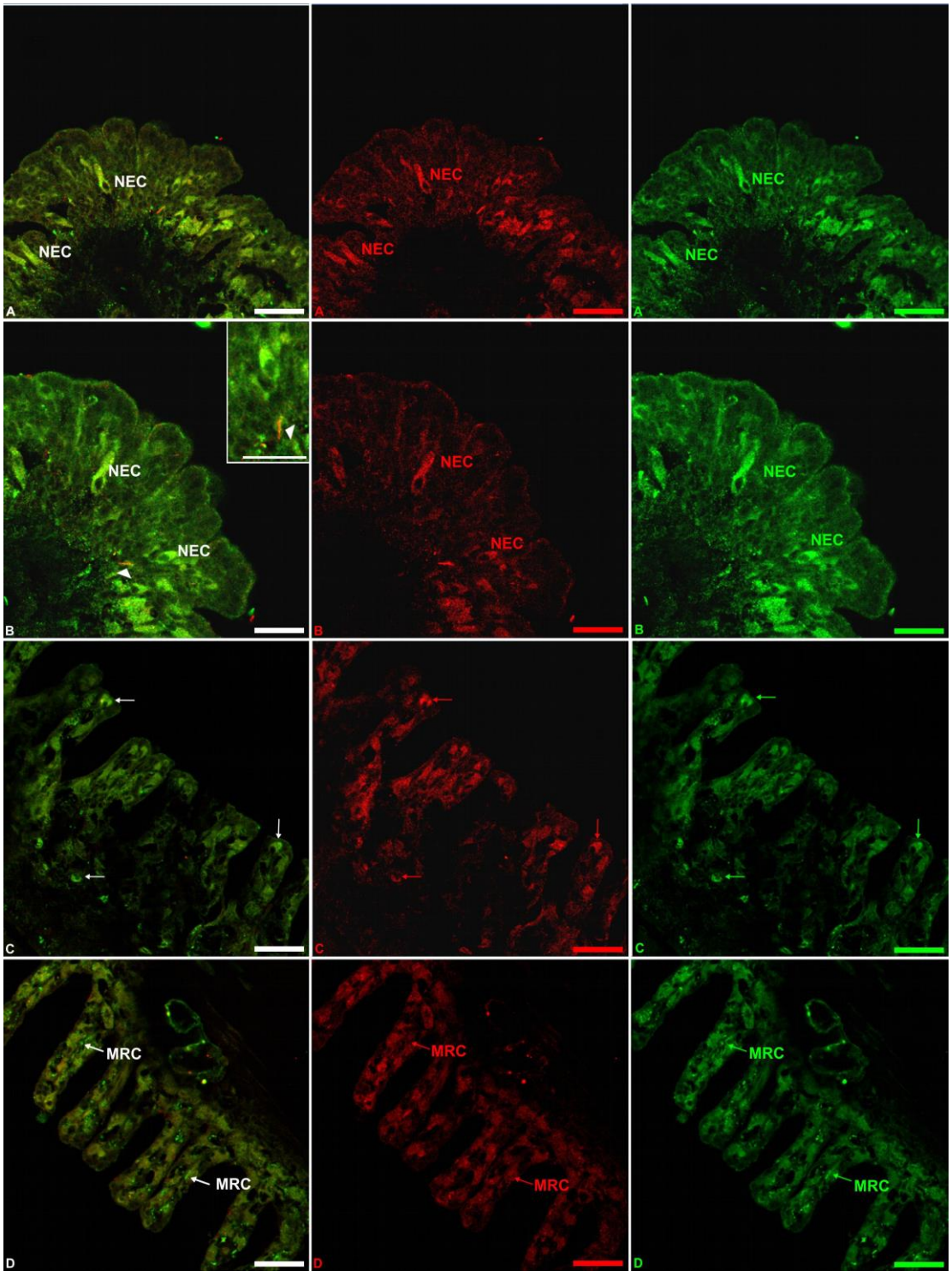

Figure 2

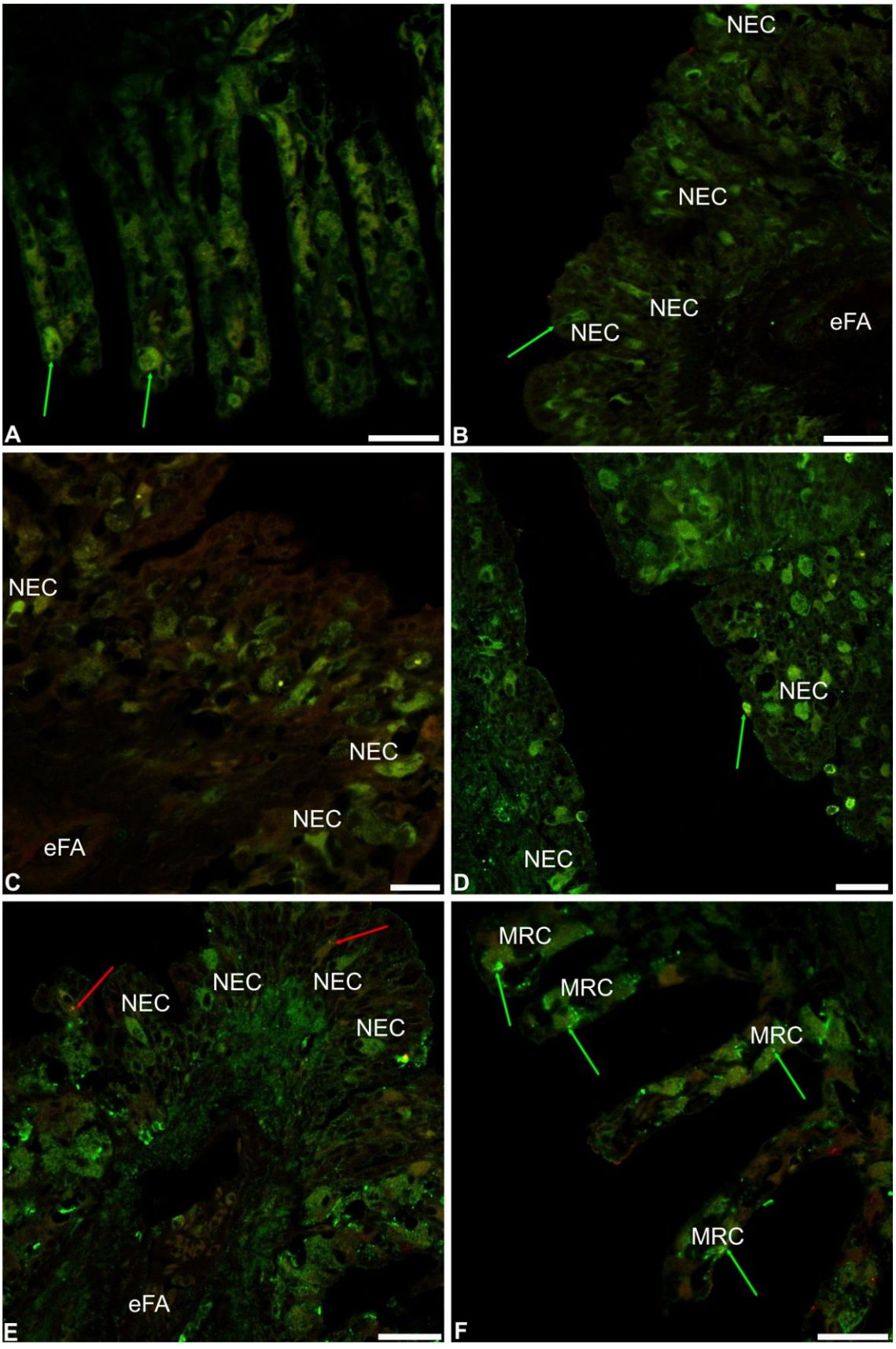

Figure 3

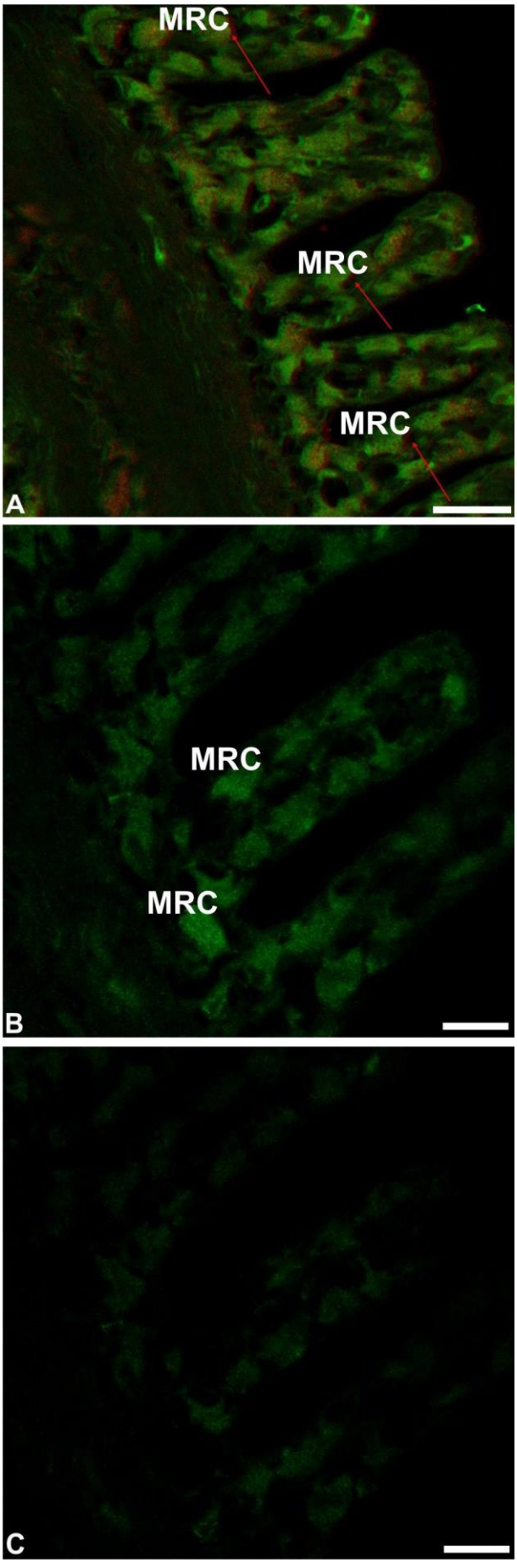

Figure 4

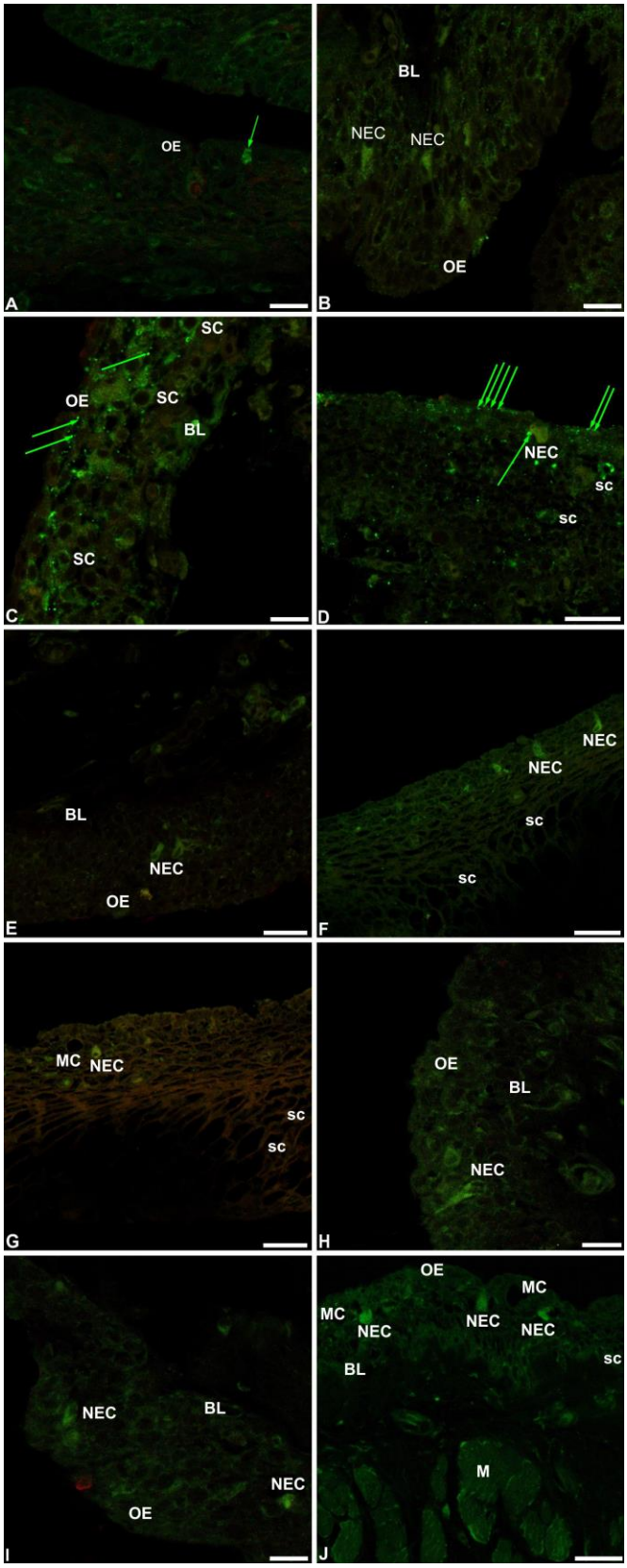

Figure 5

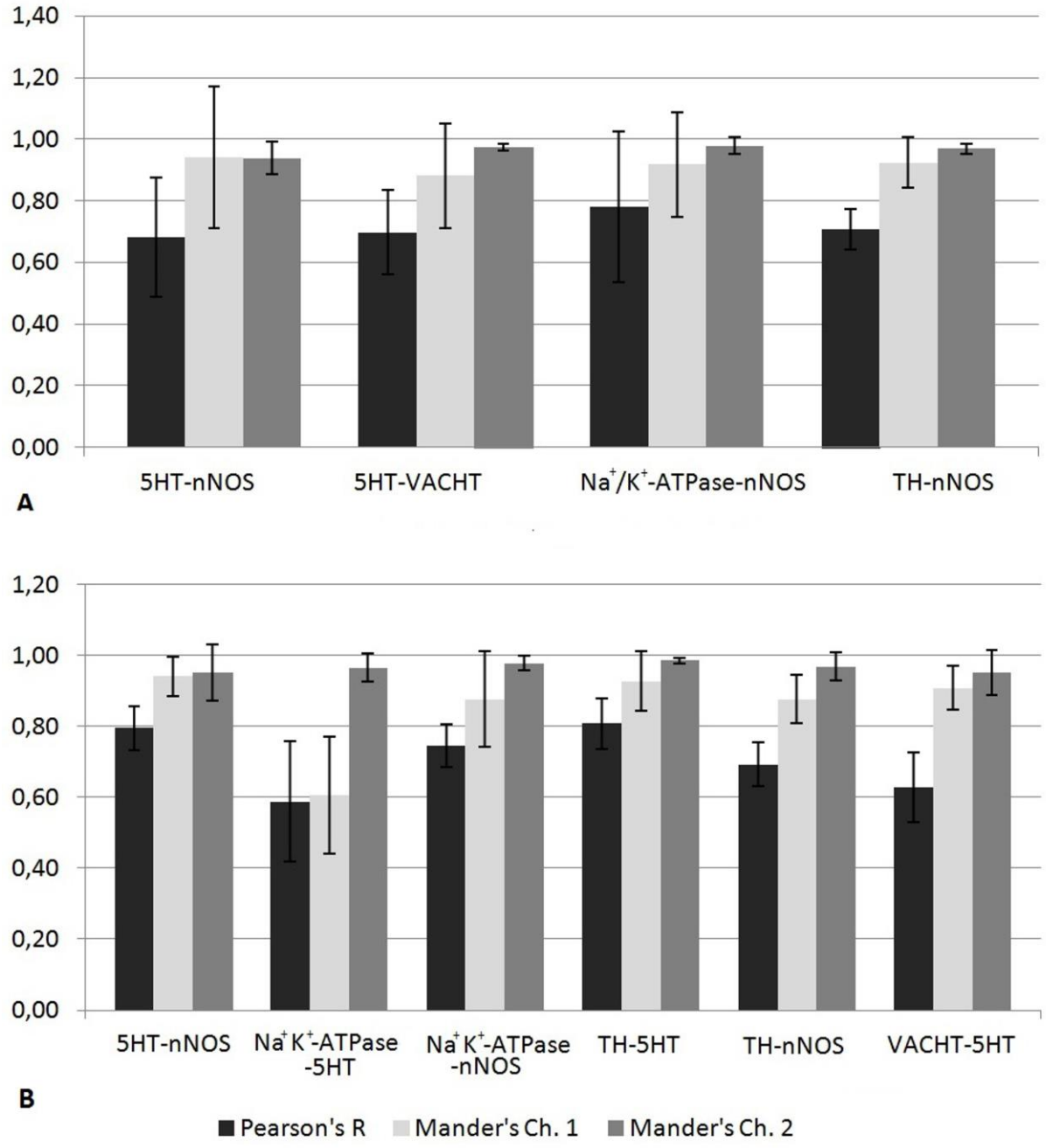

Figure 6

Table 1. Primary and secondary antibodies used in the present study of *P. schlosseri*.

Primary antibodies	**Manufacturer**	**Dilution**	**Catalog No**
5-HT	Sigma-Aldrich	1:4000	S5545
5-HT	Dako	1:50	MO758
5-HT	Immunostar	1:10000	20080
VAChT	Sigma-Aldrich	1:500	
VAChT	Millipore	1:100	AB 1588
nNOS	Transduction Labs	1:250	SAB4200559
nNOS	Abcam	1:25	ab67002
nNOS	Santa Cruz Biotechnology	1:500	Sc-648
TH	Sigma-Aldrich	1:100	T2928
TH	Chemicon	1:200	MAB318
Na+/K+-ATPase	Alomone Labs	1:200	

Secondary antibodies	**Antigen**	**Manufacturer**	**Dilution**
*Alexa Fluor 488	Mouse IgG	Invitrogen	1:100
*Alexa Fluor 594	Rabbit IgG	Invitrogen	1:100

*Secondary antisera antigen corresponds with the primary antibody host which is donkey.

Table 2. Summary of neural markers and the transport enzyme Na^+/K^+-ATPase found in fish species studied to date.

5-HT	Saltys et al., 2006
	Zaccone et al., 2006, 2007
	Olsson et al., 2008
	Jonz et al., 2016
VAChT	Regan et al., 2011
	Porteus et al., 2013
	Shakarchi et al., 2013
	Stoyek et al., 2015
nNOS	Zaccone et al., 2003, 2006, 2015a
	Porteus et al., 2015
TH	Zaccone et al., 2006, 2007, 2015a
	Chen et al., 2009
	Porteus et al., 2013
Na^+/K^+-ATPase	Uchiyama et al.,2012

Table 3. Numbers and specific regions of measurements in the gills and skin of *P. schlosseri*.

	Measurements							
	No of images	Gill			No of images	Skin		
		leading edge	gill filament	gill lamellae		basal surface	middle surface	outermost surface
5HT-nNOS	17	34	14	45	17	18	32	27
NaKatpase-5HT	7	13	9	27	N/A	x	x	N/A
NaKatpase-nNOS	7	7	12	54	N/A	x	x	N/A
TH-5HT	10	15	11	26	7	9	7	9
TH-nNOS	9	12	16	73	10	16	15	16
VACHT-5HT	11	26	15	33	N/A	x	x	N/A
Act-VAChT	N/A	x	x	N/A	5	6	12	59
subtotal	61	107	77	258	39	49	66	111
		total: 442				total: 226		