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Expression of acetylcholine, its contribution to regulation of immune function and O_2 sensing and phylogenetic interpretations of the African butterfly fish *Pantodon buchholzi* (Osteoglossiformes, Pantodontidae).

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Summary

Acetylcholine (Ach) is the main neurotransmitter in the neuronal cholinergic system and also works as a signaling molecule in non-neuronal cells and tissues. The diversity of signaling pathways mediated by Ach provides a basis for understanding the biology of the cholinergic epithelial cells and immune cells in the gill of the species studied.
NECs in the gill were not found surprisingly, but specialized cells showing the morphological, histochemical and ultrastructural characteristics of eosinophils were located in the gill filaments and respiratory lamellae. Much remains unknow about the interaction between the nerves and eosinophils that modulate both the release of acetylcholine and its nicotinic and muscarinic receptors including the role of acetylcholine in the mechanisms of O_2 chemosensing. In this study we report for the first time the expression of Ach in the pavement cells of the gill lamellae in fish, the mast cells associated with eosinophils and nerve interaction for both immune cell types, in the gill of the extant butterfly fish *Pantodon buchholzi*. Multiple roles have been hypothesized for Ach and alpha nAChR in the gills. Among these there are the possible involvement of the pavement cells of the gill lamellae as O_2 chemosensitive cells, the interaction of Ach positive mast cells with eosinophils and interaction of eosinophils with nerve terminals. This could be related to the use of the vesicular acetylcholine transporter (VAChT) and the alpha 2 subunit of the acetylcholine nicotinic receptor (alpha 2 nAChR). These data demonstrate the presence of Ach multiple sites of neuronal and non-neuronal release and reception within the gill and its ancestral signaling that arose during the evolutionary history of this conservative fish species.

Keywords: Immunomodulation; pavement cells; Eosinophils; Mast cells; *Pantodon buchholzi*; Respiration; Ach; nAChR; Phylogeny

1. Introduction

The gills in fish are organs with a multifunctional wide surface regulating balance and excretion of part of the vast nitrogen compounds and gas, sensing O_2 and regulating respiration, water and ion exchange (for review see [1]). Gills have mucous cells juxtaposed between respiratory and osmoregulatory cells; they provide a slimy polymer containing inhibitory activity against pathogens. Mucous cells produce mucus that cover most of fish external surface, mainly the skin, and is secreted by goblet, club and sacciform cells in the fish epithelium [2,3]. Also, antimicrobial peptides are well-known in fish mucus from three decades. Among the broad spectra of these peptides, as one of the principal group of antimicrobial molecules, piscidins have been found in various cell types of various visceral organs and immune cells in fish, [4]. Fish possess an active mucosal immune system comprising cellular components such as leukocytes, mast cells, dendritic cells, macrophages and granulocytes (see for review [5,6]).
As example, Zaccone et al. [7,8] used confocal microscopy to determine the coexpression of specific neurochemical markers, and in particular the co-occurrence of piscidin 1 and 5HT in the polymorphous granular cells (PGC) along with the presence of the neuroepithelial-like cells in the lung of *Polypterus senegalus*. The authors have also correlated the capacity of PGCs to produce nitric oxide (NO), the piscidin peptide and neuroactive substances, with a primitive form of immunoregulation of the innate immunity. Almost all the immune cells in vertebrates express at least one 5-hydroxytryptamine (5-HT) component, and in recent years a number of immunoregulatory functions have been ascribed to serotonin (5-HT). Immune cells express 5-HT receptors of the 5-HT1, 5-HT2, 5-HT3, 5-HT4 and 5-HT7 classes, 5-HT transporters, as well as the key enzymes for 5HT synthesis and its degradation (monoamine oxidase) [9]. Most of the body's 5-HT is circulating in the bloodstream, transported by blood platelets and is released upon activation. The functions of 5-HT are mediated by members of the receptor subtype classes, the serotonin transporter (SERT), and by covalent binding of 5-HT to different effector proteins in immune processes. The 5-HT transporter (SERT) function in platelets is critical for maintaining adequate 5-HT concentrations in the circulatory system and in specific leukocyte functions required for chemiotaxis or cytokine secretion. In monocytes/macrophages 5-HT modulates cytokine secretion. The neutrophil recruitment and T-cell activation can both be mediated by 5-HT [10]. Recent evidence expands the biological role of 5-HT2B that acts not only as a neurotransmitter, but also as an important modulator of both innate and adaptative immune responses [11].

The respiratory tract in vertebrates is densely populated by neurons and immune cells that sense and respond to environmental challenges [12]. Besides being the main neurotransmitter in the parasympathetic nervous system, acetylcholine (Ach) can act as a signaling molecule in non-neuronal tissue [13] via its muscarinic and nicotinic Ach receptors that have been identified in mast cells [14]. At mucosal glands and epithelial cells, acetylcholine regulates mucus secretion and via ciliary beat frequency, mucus clearance. The nicotinic acetylcholine receptor, a key player in neuronal communication, converts neurotransmitter binding into membrane electrical depolarization. This protein combines binding sites for neurotransmitter acetylcholine (Ach) and a cationic transmembrane ion channel. Muscarinic receptors are transmembrane proteins that mediate the signals through the G proteins. Increasing evidence of the involvement of nicotinic Ach receptors in the modulation of inflammation and homeostasis has been reported. Moreover it is involved in cholinergic modulation of the immune cells [14].

The presence and function of the granulocyte lineages in fish is still unclear, but recent morphologic, ultrastructural and molecular studies have allowed the characterization of eosinophils in zebrafish [15]. These cells are a distinct lineage of granulocytes that provide innate immune surveillance with T lymphocyte-mediated humoral immune response. They often co-localize with mast cells and their clustering around the parasympathetic nerves in the mammalian airways suggesting that eosinophils increase the release of acetylcholine from the cholinergic nerves terminals [16]. Previous studies have also reported the presence of nicotinic Ach receptors in these immune cells.

Neuroepithelial cells (NECs) in fish gills are described as the primary O_2 chemoreceptors [1,17], although recent studies by Zachar et al [18] provided evidence for the presence of cholinergic cells that immunoreacted for the vesicular acetylcholine transporter (VAChT) and are negative for 5HT in the zebrafish gill.

Here we investigate for the first time the expression of acetylcholine and its alpha 2 subunit of Ach nicotinic receptor, including localization of the antimicrobial peptide piscidin 1 in the gills and its associated immune cells in the African butterfly fish, *Pantodon buchholzi*. This fish is an obligate air-breather and makes aerial gas exchanges using its respiratory gas bladders. Their gills are strongly populated by a rich variety of immune cells and contains a lower number of NECs. While the gill epithelial cells produce Ach and express nicotinic receptors. Based on these results, we suggest here, with caution, that the lifestyle and the adverse environmental factors such as water oxygen oscillations and major climatic variations could have stimulated *Pantodon* immune system.

2. Materials and Methods

2.1. Ethical Statement

Handling and care of animals were conducted in accordance with the ethical principles indicated by the European Union Directive (63/2010/EU) on the use of animals for scientific purposes.

2.2. Animals and preparation of gill sections

Ten adult specimens (5 males and 5 females) of the African butterflyfish *Pantodon buchholzi* were obtained from commercial suppliers, transferred to the laboratory and euthanized by an overdose via immersion MS 222 (according to [19]). All the gill arches from both left and right branchial chambers were excised and immersed in 4% (w/v) paraformaldehyde (PFA) in phosphate buffer saline (PBS) pH 7.4, for 6-8 h, dehydrated in graded ethanol, cleared in xylene, embedded in Paraplast (Mc Cormick-Scientific, St Louis, MO, USA) and cut in to 5-10 micron sections using a rotatory microtome (Leica, RM2135, Nussloch, Germany) and collected on gelatin-coated microscope slides. Light microscopic observations were made using gill sections that were stained with hematoxylin and eosin (Thermo Fisher Scientific), periodic acid-Schiff/Alcian blue and toluidine blue according to the manifacturer's instructions (Sigma-Aldrich).

2.3. Immunohistochemistry and Confocal Immunofluorescence Microscopy

Techniques for immunolabeling were similar to those previously described for a wide variety of tissues from fish visceral organs and skin [20–27].
Double immunostainings were carried out at room temperature. Samples were rinsed in PBS between each incubation step unless indicated otherwise. The primary and secondary antibodies used in this study, as well as the dilutions, are listed in Table 1. The antigens in the double-label experiments with primary antisera raised in different species, were simultaneously detected by means of indirect immunofluorescence. Sections of 5-10 μm were rinsed three times with double distilled water and transferred for 1 h to a blocking solution with PBS containing 0.5% (v/v) Triton-100 (Sigma-Aldrich), 0.2% (w/v) bovine serum albumin (BSA, Jackson, Immunoresearch, West Grove, PA, USA), 1% (v/v) dimethyl-sulphoxide, 0.02% sodium azide (w/v?) and 5% (v/v?) 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. The sections were analyzed, and images acquired using a Zeiss LSMDUO confocal laser scanning microscope with META module (Carl Zeiss, Micro Imaging, GmbH, Germany).

2.4. Image analysis

Zen 2011 (LSM 700 Zeiss software) built in "colocalization view" was used to highlight the expression of both antibody signals in order to produce a "colabeling" signal. Digital images were cropped, and the figure montage prepared using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA).

2.5. Primary antibodies and antibody specificity

All primary antibodies were chosen based on previous morphological studies performed in zebrafish and other teleost fish species. The characterization, specificity and reliability of the antibodies and their application in morphological studies of the skin, the gill and the air breathing organs (ABOs, Graham 1997) in fish have already been described [17,18,20–26].

5HT, VACHT, both nicotinic and muscarinic acetylcholine receptors and the antimicrobial peptide piscidin 1 are widely occurring neurochemicals in fish (See Table 2). The antibodies directed against these neuroimmune markers are reported previously by Zaccone et al. [24–26] and Ruangsri et al. [28]. See Table 2.

The monoclonal 5HT antibody (Code MO 758; Dako/Agilent Pathology Solutions, Santa Clara, CA, USA) has been used previously to characterize the serotonergic NECs of several teleost fish [17,24–26,29]. It was raised in mouse against 5-hydroxytryptamine hydrochloride, used at a dilution range 1:50 to 1:100, and localized with goat-antimouse secondary antibody conjugated with Alexa 594 (red) (1:100, Invitrogen, Burlington, ON, Canada). The specificity of this antibody is assumed to be similar in all the vertebrate species [30] and used to detect serotonergic neurons in the enteric nervous system in the zebrafish [30,31], the catfish *Clarias batrachus* and the gar *Lepisosteus oculatus* [22]; in the gill neurons and filament, in the muscle innervation, the NECs in the gill and skin of the giant mudskipper *Periophthalmodon schlosseri*, the NECs and ABOs of the catfish *Clarias gariepinus* [24,32], and in the NECs of the gill and the glottis of the air breathing fish *Arapaima gigas* [26]. We used this antibody in a colabeling procedure with the antibodies against VAChT to reveal the occurrence of 5HT and the co-occurrence of 5HT and VAChT in the pavement cells of the gill epithelium, immune cell types and cholinergic nerves in the gill of *P. bucholz.* Furthermore, dendritic-like cells present in the middle layer of *P. schlosseri's* epidermis co-express 5-HT and VAChT immunostaining [33]. The polyclonal VAChT antibody V5387 (Sigma-Aldrich, dilution 1:250, raised in rabbit) has been used to characterize the distribution of acetylcholine in the chain and proximal neurons and extrinsic innervation in the gill filament of trout, goldfish, the gill NECs of the giant mudskipper and the air breathing fish *Arapaima gigas*, the gill neurons of the catfish *Heteropneustes fossilis* and *C. gariepinus* and the innervation of the zebrafish heart [24–26,34,35].

Vertebrate nAChRs are widely expressed in the CNS, while in the periphery they mediate transmission at neuromuscular junctions. nAChRs are found in non-neuronal tissues such as epithelia and in their macrophages and keratinocytes. Five zebrafish neuronal nAChR cDNAs (alpha 2, 3, 4, 6, 7) and zebrafish muscle nAChR subunit cDNAs were recently cloned [36] and detected by hybridization in situ [37]. These are largely expressed in regions analogous to structures in mammals. In our study we have used a polyclonal antibody (Alomone Labs) directed against cholinergic receptor nicotinic alpha 2 (CHRNA2). This antibody has homology with zebrafish nAChR cDNA (alpha 2). The nAChR antibody has also been used in the terrestrial snail *Helix pomatia* to label nerve varicosities innervating flexor muscles [38]. For control, the CHRNA2 antibody was pre-treated with the specific blocking peptide nAChR alpha 2 (1:100, BDS II(B-450), Alomone Labs) for 48 h at 4 C before the application of the antibody to the tissue for 2 h at room temperature. This procedure eliminates the CHRNA2 immunolabeling. The CHRNA2 was used to detect the eosinophils, the mast cells and their nerve interaction, the localization of varicose nerve fibers running in the pillar capillary compartment, and those in close vicinity of the eosinophils in the gill of *P. buchholzi*.

In our study we have employed an affinity purified polyclonal antibody specific for Piscidin 1 (Pis 1). The peptide cod antigen corresponding to C-FIHHIIGWISHGVRAIHRAIHG was used for polyclonal production by GeneScript [28]. Two piscidin paralogues (pis 1 and pis 2) and a novel alternative slice variant of pis 2 were previously reported by Fernandes et al. [39] and Ruangsri et al. [28]. The specificity of the Pis 1 antibody was confirmed by Western blot analysis using synthetic piscidins and tissue extracts as test samples according

to Ruangsri et al. [28]. In tissue sections the antibody specificity was determined by pre-incubating the serial sections with the cod Pis 1 peptide according to the manufacturer instructions and those reported by Ruangsri et al. [28] i.e. the pre-absorbed antibody was later diluted in dilution buffer to make the final concentration of 14.5 $\mu g.ml^{-1}$ before being used for immunohistochemistry. The specificity of the Pis 1 antibody was confirmed by blocking experiments i.e. the ability to specifically and totally neutralize the response by pre-incubating the antibody with Piscidin 1 peptide. The pis 1 antibody was used in Atlantic cod *Gadus morhua* to detect blood leucocytes and phagocytic cells, hematopoietic cells, neural tissues, intestinal and gill epithelial cells, swim bladder innervation and skin. We have used the pis 1 antibody to label the mast cells associated with eosinophils, the mucous cells lining the epithelium of branchial arches and perivascular innervation of the efferent filament arteries in the gill of *P. buchholzi*.

2.6. Transmission electron microscopy (TEM)

Small tissue samples fixed in 3% (v/v) glutaraldehyde were postfixed in 1% (w/v) osmium tetroxide, dehydrated in graded acetone and embedded in Araldite (Fluka, Bucks, Switzerland), following routine procedures [40]. Semithin sections for assessment of tissue structure were cut with an LKB III ultratome, stained with 1% toluidine blue and observed with a Zeiss Axioskop 2 plus microscope equipped with an AxioCam HRc digital camera. Ultrathin sections were cut with a Leica ultracut UCT, stained with uranyl acetate and lead citrate and examined with a Jeol-JEM-1011 working at 80 KV and equipped with a Gatan ORISUS SC 1000 CCD camera.

3. Results

3.1. VAChT and 5HT

VAChT and 5HT occupy different cell types in the gill of *P. buchholzi*. In the distal halves of the gill filament and interfilamentous areas one group of larger and rounded 5HT-immunopositive cells that mimic the distribution patterns of the so-called neuroepithelial cells (NECs) in fish airways were seen. In fact, these cells were found in the efferent aspects of the gill filaments and proximal lamellar epithelium and sometimes in gill lamellae. They were stained with hematoxylin eosin, but several granules in the cytoplasm were empty (Fig. 1a), and the eosinophilic material was seen in the cytoplasmic matrix. The same cells were not stained with basic dyes such as toluidine blue but showed an intense PAS positivity (Fig.1b). The PAS reaction demonstrated a specific intergranular or perigranular coloration, and the granules appeared just coarse vacuoles. The cell types displayed the morphological and histochemical characteristics of eosinophils [15,41].

Observations from confocal study have indicated that eosinophils contain 5-HT (Fig. 2a) and frequently formed clusters associated sometimes with cholinergic nerve fibers (Figs. 2 b-d). Intraepithelial mast cells immunoreactive for both 5HT and VAChT were seen in very close association with the eosinophils (Fig. 2 b). The double immunolabeling method with 5HT and VAChT antibodies revealed that often granules expressing the VAChT protein were seen in the cytoplasm of mast cells as opposed to the peripheral cytoplasm of the eosinophils (Fig. 2 b).
VAChT positive cholinergic epithelial cells were present in the filament and lamellar epithelium like those found by Zachar et al. [18] in the gill filament of zebrafish where a high density of 5HT oxygen-sensitive NEC populations were located. The VAChT dome shaped epithelial cells (Fig. 3 a) in the lamellar epithelium were often observed in the tip of both proximal and distal lamellae or may occupy a great proportion of the lamellar epithelium and did not contain 5HT (Fig. 3 a). Our observations showed that the epithelial cells of the lamellae appeared to come in contact with cholinergic nerve fibers (Fig. 3 b-d) that are seen sometimes surrounding the blood channels of the lamellae.

3.2. Alpha 2 subunit of Ach nicotinic receptor and 5HT.

Double labeling with 5HT and alpha 2 subunit of nAChR showed the presence of many 5HT clustered positive eosinophils scattered in the apical end of the gill filament epithelium and in the interlamellar filament epithelium of *P. buchholzi*. Co-expression of the two neurochemicals was observed in some cells. A rich supply of nerves containing alpha 2 subunit of nAChR is detected in axons that adhere the 5HT positive eosinophils (Figs. 4 d-e), suggesting its involvement in a possible feedback control of Ach release from the nerve terminals.

A prominent feature of the eosinophils in the gill of *P. buchholzi* is the coexistence with mast cells. Colocalization studies using antibodies to 5HT and alpha 2nAChR showed that mast cells expressing the cholinergic nicotinic receptor were often observed in very close proximity to the eosinophils showing 5HT immunoreactivity (Fig. 4 g). Notably mast cells expressing alpha 2 subunit of nAChR had both epithelial and subepithelial localization in the gill lamellae. Analysis of nerve fibers revealed that a diffuse network of bundles of nerves showing immunoreactivity for alpha 2 subunit of nAChR in close contact with the pilaster cells of the endothelial lining (pillar capillary compartment) was also seen (Fig. 4 d). Alpha 2 subunit of nAChR-positive mast cells were also seen at the points of contact with the nerves.

Cholinergic epithelial cells of gill lamellae were labelled by alpha 2nAChR antibodies and carried nicotinic receptors (Fig. 4 f), and these findings are correlated with those obtained with VAChT antibodies (see above).

3.3. 5HT and Piscidin 1 (Pis1)

Double immunostaining with 5HT and Pis1 antibodies revealed that the two neurochemicals are colocalized in mast cells. In contrast, eosinophils showed 5HT immunoreactivity and a negative response to Pis1 antibody (Figs. 5 a-b). A prominent feature reported in this study is the closeness of the Pis1-positive mast cells to eosinophils (Figs. 5 a-b-e).

Double labelling with 5HT and Pis1 antibodies showed the presence of mast cells in the apical gill filament and lamellar epithelium, the thicker epithelium lining the branchial arches, the submucosal layer and the connective tissue (Figs. 5 a-b). Most mast cells were strongly immunostained with the Pis1 antibody, but several mast cells showed an overlap of both 5HT and Pis1 immunoreactivity. Mast cells were also seen in the lumen of larger vessels (efferent filament artery) including the basal blood channels of the gill lamellae (Fig. 5 c). Notably, nerve fibers innervating the vascular walls of the efferent filament arteries showed Pis1 immunoreactivity (Figs. 5 a-b). Mucous cells in the stratified epithelium of the branchial arches and a few ones in the simple epithelium of the gill lamellae were labelled by the Pis1 antibody (Figs. 5 c-d). The peripheral cytoplasm of the superficial epithelial (pavement) cells of the gill lamellae was immunostained by the Pis1 antibody (Fig. 5 f). It labeled the cellular coat surrounding the external boundary surface.

3.4 Structure of eosinophils and epithelial gill pavement cells

The gill filaments and the lamellae appeared covered by squamous, pavement cells. Pavement cells were joined by tight junctions (Fig. 6a). These junctions were long and complex in the filaments where the junctional complexes could include several desmosomes (Fig. 6a). In contrast, tight junctions appeared simpler, shorter and mostly perpendicular to the cell surface in the lamellae (Fig. 6a, inset). In the two cases, pavement cells contained numerous clathrin-coated-like vesicles (Fig. 6a) that are probably involved in endocytosis and intracellular trafficking. Curiously, these vesicles were mostly restricted to the junctional areas. Pavement cells also contained membrane-bound, small round granules with a uniform, dense content (Fig. 6b). These granules could appear very regular (Fig. 6b, inset) or could show irregular shape and content distribution, suggesting different steps of a degranulation process.

Under the surface epithelium the gill filaments showed, in addition to other structural cell types, eosinophils and mast cells. Eosinophils had an irregularly lobed nucleus and numerous cytoplasmic granules (Fig. 6c). The granules contained a disc-shaped, dense crystalline inclusion embedded in a clear matrix. *Pantodon* eosinophils have been characterized morphologically in a recent paper [42]. They could be found within the vessels or in the extracellular space. In the latter case, eosinophils were frequently observed in the proximity of mast cells (Fig. 6c). Mast cells showed a small nucleus and a large cytoplasm that was mostly occupied by a short number of very dense granules. Mast cells appeared to be restricted to the filament tissue, but eosinophils could be found in the vascular spaces of the lamellae.

4. Discussion

4.1. Cholinergic and serotonergic pathways are present in Pantodon's gill filament

The 5-HT positive cell types observed in the distal halves and interlamellar areas of the gill filament and their distribution patterns are surprinsingly very similar to the NECs found in the teleost gill and their neurochemical contents including the presence of Ach nicotinic receptor (Lauriano et al., submitted) and Ach muscarinic receptor on these cells in fishes [26].In these cells a PAS positivity of the intergranular substance is observed.These characteristics and their ultrastructure are confirmed in the eosinophils of lower vertebrates [43,44].

Intraepithelial mast cells showing co-localization of 5-HT and VAChT were seen in close association with eosinophils. While Ach labeled with VAChT antibody is reported for the first time in the mast cells of fish, the presence of 5-HT has also been reported in other fish species and their mammalian counterpart [45].

Varicose nerve fibers showing immunoreactivity to alpha 2 subunit of nicotinic Ach receptor were observed in close association with pilaster cells. Nervous innervation of cholinergic epithelial cells was reported by Zachar [18] using different antibodies in the efferent aspect of the gill filament of the zebrafish. Acetylcholine produced by nAChR is a vasoactive substance in fish gills (reviewed [46], and its release from the cholinergic pavement cells of the gill lamellae, as also demonstrated by the presence of dense cored vesicles, may also affect the constriction of the pillar capillary compartment that is actively controlled by innervated sphincters located in the gill filament [44].The pillar cells consisting of an actinomyosin substance are thought to play a key role in lamellar blood flow [44]. Also, Ach was implicated in lamellar recruitment whereby the number of perfused respiratory lamellae (that represent the primary site for gas exchange) is increased during periods of hypoxia. In fact, acetylcholine as vasoactive substance [46] causes the vasoconstriction of the arterioles that supply blood to lamellae. Another possibility may be that the cholinergic cells of the lamellae like those of the efferent epithelium [18] may function as O_2 chemoreceptors based on the absence of the NEcs in the gill filaments of the species here studied,although currently there are no direct data supporting their role in O_2 or CO_2 chemoreception. Finally, an autocrine/paracrine role of acetylcholine released by cholinergic cells of the lamellae could be to regulate various aspects of the innate mucosal defense. Such a role is supported by the evidence reported for the epithelial cholinergic system of the lung, since the epithelial cholinergic cells of the gill lamellae carry nicotinic receptors for the release of Ach like those found in the airway epithelial cells of the lung [47] and are involved in production of antimicrobial peptide such as piscidin 1(see below).

Our results provide morphological evidence of a cholinergic cell system in the gill lamellae of the freshwater butterflyfish and is in agreement with previous studies focused on the hyperventilatory response to hypoxia and gill sites such as proximal and distal lamellae associated with ventilation and perfusion [18,48,49]. NECs, within the fish gill there are numerous potential sites for oxygen sensing. Sites capable of monitoring arterial and venous oxygen levels are the efferent filament artery, the central venous sinus or the afferent filament artery; aquatic hypoxia may be detected when the lamellar, filament or gill raker epithelia are exposed to lower oxygen concentrations. Oxygen sensing cells could be either chemoreceptive or paracrine [50].

An important aspect of this cholinergic system is also the presence of cholinergic nerve terminals in close proximity to the clustering of eosinophils that are sometimes seen accumulating around the nerve bundles, as shown by the VAChT antibody. The neural control of parasympathetic nerves in airway smooth muscle is in reported by Costello et al. [51]. An immunomodulatory function of lung neuroepithelial bodies (NEBs) was established and included the induction of eosinophil chemotaxis [52]. The M2 muscarinic receptors on the parasympathetic nerves in the lung regulate the release of acetylcholine. Eosinophils in the species studied express 5-HT and alpha 2 subunit of nAChR are seen in close proximity to mast cells (see below). nAChRs are involved in excitation neurotransmission and neuro-muscle junction through autonomic nerve ganglia [53]. 5-HT like CGRP have been found to be eosinophil chemoattractants [52]. These results suggest that both eosinophils and NEBs share in common immunomodulatory substances, such as 5-HT. Thus, it is theoretically possible that mast cells that lie in close vicinity to eosinophils respond to the chemoattractant stimuli emanating from the eosinophils as a consequence of antigenic stimulus reception, as in the mammalian model.

4.2. Eosinophilic-cholinergic mast cells and nerve interaction. The role of acetylcholine in O_2 sensing and immune-related mechanisms

Eosinophils have been identified and characterized in all the vertebrate species studied, but their morphology, repertoire of cell surface receptors like nicotinic receptors and intracellular contents can vary significantly from one another [15]. A prominent feature of the eosinophils in the gill of *P. bucholzi* is the coexistence with mast cells. These results agree with those reported in mammalian vertebrates where both types of immune cells were found under homeostatic conditions (see [54] for review). Eosinophils and mast cells were also seen coexisting in large numbers, although their interaction remain to be fully elucidated [55].

A diffuse network of nerves containing immunoreactivity for alpha 2 subunit of nAChR is seen in close contact with pillar capillary compartment in the gill lamellae. This accounts for the key role of nicotinic receptors in the release of acetylcholine and their interaction with the vasomotor control and recruitment of the lamellae.

nAChR alpha 2 subunit -immunopositve mast cells are seen in close contact with nerves. These results are in agreement with the parasympathetic regulation of mast cells from the findings of the contacts of the vagal afferent terminals with the mast cells in the intestine, and the expression of acetylcholine nicotinic receptors in mast cells [14].

Cholinergic pavement cells of the gill lamellae were also immunostained with the antibodies against the alpha 2 subunit of nAChR. This accounts for the presumptive role regarding the involvement of nicotinic receptors in O_2 sensing processes in the epithelial cells of the gill lamellae, and the NEC function in O_2 chemoreception in fish, where acetylcholine and ATP are implicated in excitatory neurotransmission [56].

The multiple roles of acetylcholine expression in the gill of *P. bucholzi* suggest that, besides a cholinergic hypothesis of O_2 chemoreception relying on the release of acetylcholine by the pavement cells, the presence of acetylcholine and nAChR in mast cells is correlated with the role of these cells in triggering the innate immune system.

4.3. Association of eosinophils with Pis 1 immunopositive mast cells. A presumptive role of eosinophils in mediating cell functions. Pis 1 immunoreactivity in the blood channels of the gill lamellae and vascular innervation

Mast cells in the species studied co-localize 5-HT and Pis 1, but are immunonegative to Pis 1 antibody. To our knowledge no studies are available in literature regarding the localization of 5-HT in the eosinophils of fish. New data demonstrated a broad capacity for 5-HT synthesis and transport in immune cells [57]. 5-HT is

chemotactic for eosinophils and contributes to eosinophil recruitment [57]. Mast cells have the capacity to modulate eosinophil functions and vice versa. Both cell types synthesize a plethora of distinct mediators and display different surface receptors. We report for the first time the close association of Pis 1 positive mast cells with eosinophils. The local accumulation of mast cells and the association of these cells with eosinophils may be consistent with the production of chemical compounds, namely chemoattractants, such as 5-HT by the eosinophils. Mast cells also produce immunoreactive 5-HT. However, their ability to recognize appropriate chemotactic stimuli by eosinophils is not demonstrated. It is also not known the presumptive signaling pathways that both cell types may use for proper cooperation among receptors triggered with chemoattractants [58].

The use of Pis 1 antibodies have exploited the expression of this antimicrobial peptide in the nerve bundles running to efferent filament arteries for their identification, but questions remain to be addressed concerning the interaction of the Pis 1 with the branchial vascular system.

Both mucous cells in the filaments and branchial arches, and the cellular coat surrounding the external boundary surface of the gill pavement cells contain Pis1 immunoreactivity. This coat consisting of different carbohydrate residues is secreted by both the distant mucous cells, or in situ by the superficial pavement cells themselves, as suggested by the number of mucous vesicles concentrated beneath the apical membrane [59,60]. The presence of Pis 1 in mucous secretions may be implicated in local immune response and defenses.

4.4. Evolutionary interpretations

One of the biggest challenges in paleontology is the incompleteness of fossil record. Due to the rarity of non-mineralized tissue preservation in fossils, some evolutionary interpretations may be based on the extant phylogenetic bracket. Although these interpretations are not direct, they represent an important clue to understand the evolutionary history of anatomical complexes and taxa.

In this context, accepting its remarkable morphological conservatism [61], *Pantodon* is an outstanding key taxon to make inferences on the evolutionary processes of groups closely related to it. *Pantodon* is pointed to have the slowest rate of morphological divergence in comparison with most invertebrates and vertebrate taxa, contrasting with the temporal divergence of 50 Myr between its two populations [61]. *P. buchholzi*, the freshwater butterfly fish, inhabit ponds and rivers of tropical central Congo, lower Niger basins (West Africa), and few adjacent coastal drainages [61] (Fig. 7). It is an obligate air breather and makes gas exchanges through a large and functional gas bladder [40,62,63]. The low rate of NECs (primary O_2 chemoreceptors) in the gills of *P. buchholzi* and the strong presence of a rich variety of immune cells described here could be indicative of adverse aquatic factors, marked by environmental hypoxia and climatic oscillations, that would affect its immune system and sensory biology. Another hypothesis would be the loss of gill functionality, considering that the most important oxygen supplier of this species is the gas bladder. In this context, a functional gas bladder may have favored the survival of *Pantodon* in environments with marked oxygen fluctuations, compensating the lack of oxygen supply through aerial gas exchanges.

Accepting the fossil *Singida* from the Eocene lacustrine beds of Tanzania [64] as the sister taxon of *Pantodon* [61,65–67] (Fig. 7), a functional gas bladder was, most probably, already preserved in these genera. However, direct observations on *Singida* gas bladder and gills are not possible, due to its poor preservation by impressions [65]. The possible presence of a functional gas bladder in *Singida* can be inferred based on the presence of a functional respiratory gas bladder in other extant osteoglossoidei, such as *Pantodon*, *Arapaima* and *Heterotis*.

5. Conclusion and significance

In addition to its presence in neurons, ACh is expressed in pro- and eukaryotic non-neuronal cells. Thus, ACh works as a signaling molecule in non-neuronal cells and tissues, before its neuronal spans. For this reason,

Wessler et al. [68] coined the term “Non-neuronal Cholinergic System (nNCS)” to indicate the presence of ACh in cells independent of neurons. Also, Grando [69] called ACh as “universal cytotransmitter” for its involvement in the regulation of basic and nervous-independent cell functions such as proliferation, differentiation, secretion, and local release of mediators (i.e. nitric oxide and pro-inflammatory cytokines). Evidence for the synthesis and release of ACh is available in mammalian cells and even invertebrates, but it has not been reported in lower vertebrate epithelia, such as fish gill epithelia. The activation of the non-neuronal cholinergic system is involved in regulation of epithelial layer integrity, as well as in the interaction between airways and immune cells.

Regarding the cholinergic system, both mAChRs and nAChRs were studied in fish. Their functions were related to neurotransmission, neuromodulation, olfactory mechanisms, glutamate release and memory construction (see for review [70]). Also, Zaccone et al. [26] reported very recently the presence of mAChR in the neuroepithelial cells of the gills and the glottis of the air-breathing fish, *Arapaima gigas*. Also evidence of the effect of serotonin on the immune system in teleost fish and that of the cholinergic system on the immune responses of these lower vertebrates are very limited as well as the mechanisms of neuromodulation for the cholinergic system [70].

Our investigations show that there is a cholinergic circuit in the gill of the species studied that could mediate intrinsic sensory and secretory reflexes. This may be due to the capacity of ACh to regulate multiple sites of neuronal (cholinergic nerve terminals) and non-neuronal release (ACh release from mast cells and pavement cells of gill lamellae) within the gill; these may be consistent with the multifunctional role of ACh in O_2 chemo sensing and immunomodulation. This information provided here will serve as a basis for future studies investigating the function of the acetylcholine in the gill of the species studied.

A prominent feature in this study is the association of eosinophils with mast cells, as well as the interaction of eosinophils with cholinergic nerves. Much remains to clarify the concerted action of both types of immune cells and if the eosinophil-nerve interaction can modulate both acetylcholine release and receptor (nicotinic and muscarinic receptor) expression. Future studies should attempt to sequence the acetylcholine receptors in teleost, since they represent a high variable group within vertebrates. Next investigations are required for a better understanding of basic issues in neuroimmunology in this species and in lower vertebrates, such teleost fish.

A proposed mechanism of morphological stasis in *Pantodon* was correlated by Lavoué et al. [61] with stabilizing selection, ecological niche, conservatism and genetic and developmental constraints [71]. Many higher specialized aspects of form, sensory biology and behavior in *Pantodon* were tightly interconnected such that selection has constrained phenotypic divergence in this lineage [61]. The peculiar cytological aspects we have found in the structural organization of the gill in this species may also offer opportunities to assess the relative roles of intrinsic and extrinsic constraints in stabilizing an efficient innate immune system, the strategies of cholinergic pavement cells of the lamellae for dealing with potential sites for O_2 chemoreception following hypoxia, and finally a complex and unusual morphology.

Table 1. Primary and secondary antibodies used in the present study.

Primary antibodies	Clonality	Manufacturer	Dilution	ID Number
5-HT	Monoclonal	Agilent Technologies	1:50-1:100	AB_2341169
Anti-VAChT (C-terminal)	Polyclonal	Sigma-Aldrich	1:500	AB_261875
Anti-Nicotinic Acetylcholine Receptor alpha 2 (CHRNA2)	Polyclonal	Alomone Labs	1:100	AB_2340998
Anti-Piscidin 1(Pis1) antibody	Polyclonal	GeneScript	1:100	Produced on demand
BDS II (B-450) Kv 3.4 specific peptide blocker		Alomone Labs	1:100	

Secondary antibodies	Manufacturer	Dilution	ID Number
Alexa fluor 488 Donkey anti-mouse	Invitrogen	1:300	AB_141607
Alexa fluor 594 Donkey anti-rabbit	Invitrogen	1:300	AB_141637

Table 2. Summary of antibodies against neuroimmune markers found in fish species studied to date.

Antibody	**References**
5-HT	Jonz and Nurse (2003) [72] Jonz et al., (2016) [17] Rahbar et al., (2016) [56] Saltys et al., (2006) [29] Shakarchi et al., (2013) [73] Zaccone et al., (2017, 2018, 2019, 2020) [25,26,74,75]
VAChT	Porteus et al., (2013)[76] Regan et al., (2011) [77] Shakarchi et al., (2013) [73] Stoyek et al., (2015) [35] Zaccone et al., (2017,2018,2019,2020) [25,26,74,75] Zachar et al., (2016) [18]
mAChR	Zaccone et al., (2020) [26]
nAChRs	Ackerman and Boyd (2020) [37]
Alpha 7 nAChR	Papke et al., 2012 [78]
Piscidin 1	Silphaduang et al., (2006) [79] Mulero et al., (2007) [45] Ruangsri et al., (2012) [28]

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Captions to figures

Fig. 1. A, B. A Distribution of eosinophils in the gill filament epithelium of *Pantodon buchholzi* stained with haematoxylin-eosin structural method. Olympus BX51; UPlanFI 20x/0.50 JAPAN B. A region of the image in A shown at higher magnification reveals pronounced changes in morphology of the granules with a marked loss of their core contents. B Longitudinal section of the gill showing a high concentration of PAS positive granules in the eosinophils (arrows) found along the basal lamina of the interlamellar region and the leading edge of the gill filament (GF). E, Eosinophils, GL, Gill lamella, GF, Gill filament. Olympus BX51; PlanAPo 40x/0.95 ∞/0.11-0.23. Scale bars: 30 μm (left), 20 μm (right)

Fig. 2. Eosinophils (E) in the gill of *P. buchholzi* immunostained with antibodies to 5HT and VAChT. a. Confocal imaging of eosinophils in the distal end of gill filament epithelium displaying 5HT immunoreactivity. MC, Mucous cell; EFA, Efferent filament artery. b. A region of the distal gill filament showing the presence of clustered eosinophils (arrows) and their close association with 5HT-VAChT positive mast cells (arrows) and nerve fibers (NB and in inset). MC Mucous cell. c, d Confocal imaging of eosinophils in the apical gill filament showing cell clustering and labelling with antibodies against 5HT and VAChT. Note the location in the merged image (c) of 5HT eosinophils with nearby 5HT-VAChT positive mast cells (M) and in the red color channel (in d) an eosinophil in close contact with a nerve bundle (NB). LSM 700 Axio Observer; Objective EC Plan-Neofluar 40x/1.30 Oil DIC M27; pinhole airy 1. Scale bars: 20 μm

Fig. 3. Confocal images of the gill lamellae of *P. buchholzi* immunolabelled with antibodies against 5HT and VAChT. a. Dome-shaped pavement cells (arrow) in the tips of the gill lamellae are densely immunostained by the antibody against VAChT. Note also the presence of eosinophils (E) in cell clusters in the gill filament epithelium. EFA Efferent filament artery. LSM 700 Axio Observer; Objective EC Plan-Neofluar 20x/0.50 M27; pinhole airy 1. b-d. Most of the lamellar pavement cells are intensely immunostained by the VAChT antibody. Note the large nuclei (N) of the pavement cells and the labeling in cell perimeter (arrowheads) and also the basement membranes (arrows). Varicose nerve fibers are seen coursing in the basal pole of the pavement cells (long arrow) and the pilaster cells (P). Inset reveals nervous innervation of pavement cells. Numerous degranulated eosinophils (E) are seen in the gill filament epithelium. LSM 700 Axio Observer; Objective EC Plan-Neofluar 40x/1.30 Oil DIC M27; pinhole airy 1. Scale bars: 20 μm

Fig. 4. Confocal images of the gill epithelium immunolabeled with antibodies against 5HT and alpha 2 subunit of nAChR. a-c. Confocal imaging of eosinophils (E) in the apical end of filament epithelium showing the coexpression of the neurochemical markers in the same cells. EFA, efferent filament artery.
d. Longitudinal section of gill filament showing the close association between a 5HT positive eosinophil (E) and nerve bundles (NB) labelled by the alpha 2 nAChR antibody. Nerve varicosities and nerve bundles (arrows) are also seen along the pillar cell bodies (P) and blood spaces of the gill lamellae. e. Eosinophils (E) display 5HT immunoreactivity and also nerve bundles (arrows) make contact with these. The outer surface of the pavement cells as well also a plexus of nerve fibers (arrows) around the pillar cell bodies (arrowheads and in inset) are immunolabelled by the antibody against alpha 2 nAChR. f. Pavement cells of the gill lamellae are labelled by the alpha 2 nAChR (arrows). g. Mast cells (arrows) displaying immunoreactity to alpha 2 subunit nAChR are seen in close proximity to a cluster of 5HT positive eosinophils (E). LSM 700 Axio Observer; Objective EC Plan-Neofluar 40x/1.30 Oil DIC M27; pinhole airy 1. Scale bars: 20 μm

Fig. 5. Confocal images of the gill filament of *P. buchholzi*. Double immunostaining with antibodies against 5HT and Piscidin 1(Pis 1). a, b. Eosinophil (E) in the distal region of the filament, labelled with antibody with 5HT, and Pis 1 negative. Mast cells (arrows) showing colocalization of 5HT and Pis1 are seen in very close vicinity to eosinophils. In the red color channel (b) the mast cells display the highest immunoreactivity for Pis1(arrows and in inset). Bundled nerve fibers (NB) are seen surrounding the vascular wall of efferent filament artery (EFA). Pis 1 immunopositive mast cells (arrowheads) are seen in subepithelial localization and blood channels (B) of gill lamellae. c. Pis 1 immunoreactity is found in a mucous cell (MC) and mast cells in basal blood channels of the gill lamellae. d. Mucous cells (MC) discharging their products showing high

immunoreactivity to Pis 1 are seen in the surface epithelial layer lining the branchial arches. e. Region of distal gill filament showing a 5HT eosinophil (E) with closely associated 5HT-Pis 1 positive mast cells (arrows). Pis 1 immunoreactity is also seen in the apical surface of pavement cells (arrows) and mast cells (arrowheads) in the gill lamellae. EFA, efferent filament artery. LSM 700 Axio Observer; Objective EC Plan-Neofluar 40x/1.30 Oil DIC M27; pinhole airy 1. Scale bars: 20 μm

Fig. 6. TEM micrographs of the *P. buchholzi* gills. a: Gill filament. Overlapping pavement cells appear joined by a junctional complex that includes long tight junctions (arrows) and desmosomes (arrowheads). Note the presence of numerous clathrin-coated-like vesicles in the rounded junctional area. Inset of a: Gill lamellae. Pavement cells in the lamellae are joined by short tight junctions (arrow). Numerous clathrin-coated-like vesicles are evident. b: Gill filament. Pavement cells contain small, dense granules (arrows), which appear to be degranulating. Note the absence of clathrin-coated-like vesicles. The vascular endothelium exhibits numerous, small dense granules (arrowhead). Inset of b: Gill filament. Detail of regular dense granules. c: Gill filament. Eosinophils (Eo) and mast cells (MC) appear in close proximity. Tangential section of the mast cell does not allow to see the nucleus. Arrowhead, granules in the vascular endothelium. E, erythrocyte. Scale bars: a, 1 μm; inset of a, 250 nm; b, 500 nm; inset of b, 300 nm; c, 1 μm.

Fig. 7. Geographical distribution of the extant *Pantodon buchholzi* from West Africa (yellow ellipses) and the fossil *Singida jacksonoides* from the Eocene of East Africa (red ellipse). Phylogeny of osteoglossomorphs from the maximum-parsimony analysis of Wilson and Murray (2008), revealing that *Pantodon buchholzi* and *Singida jacksonoides* are sister taxa (orange rectangle).

Highlights

Immunocytochemical markers of acetylcholine and nAChR in the gill of butterfly fish

Eosinophils and mast cells and their nerve interaction

Absence of NECs is compensated by the cholinergic epithelial cells

Ach signaling during evolutionary history

Figure 1

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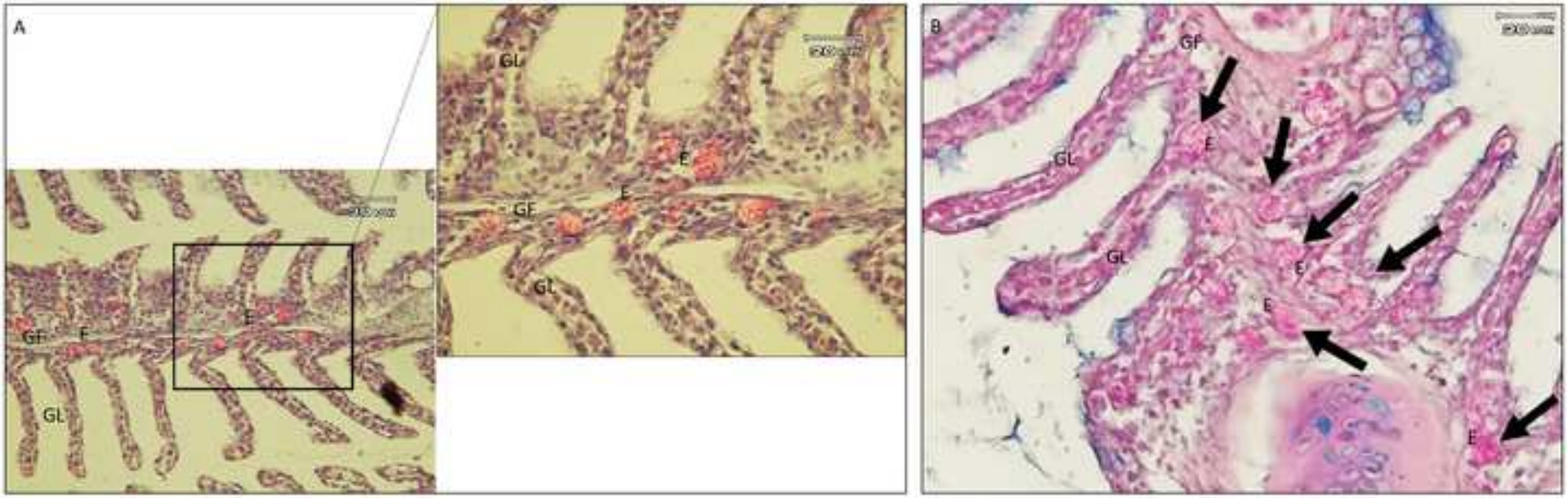

Figure 2

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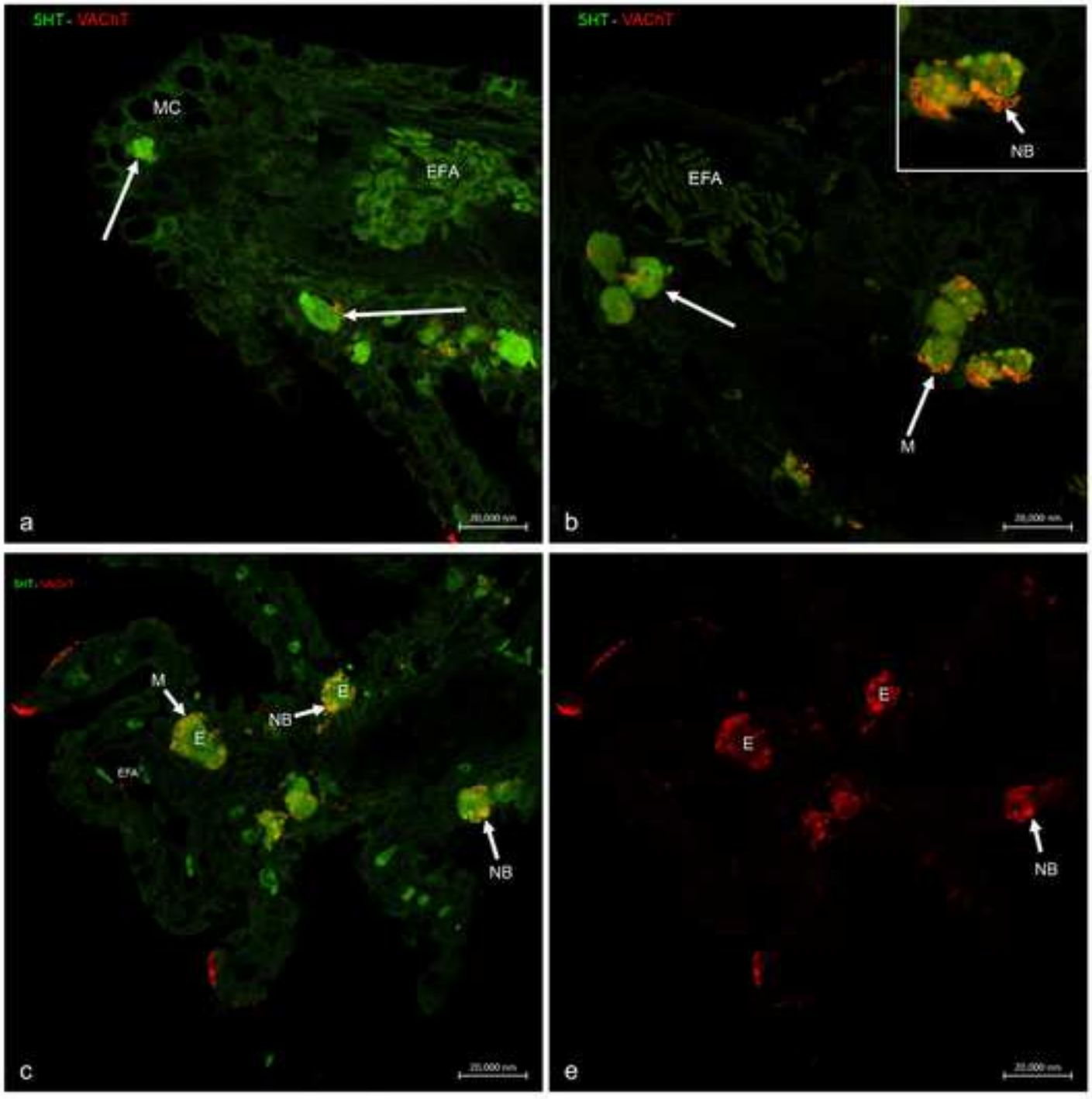

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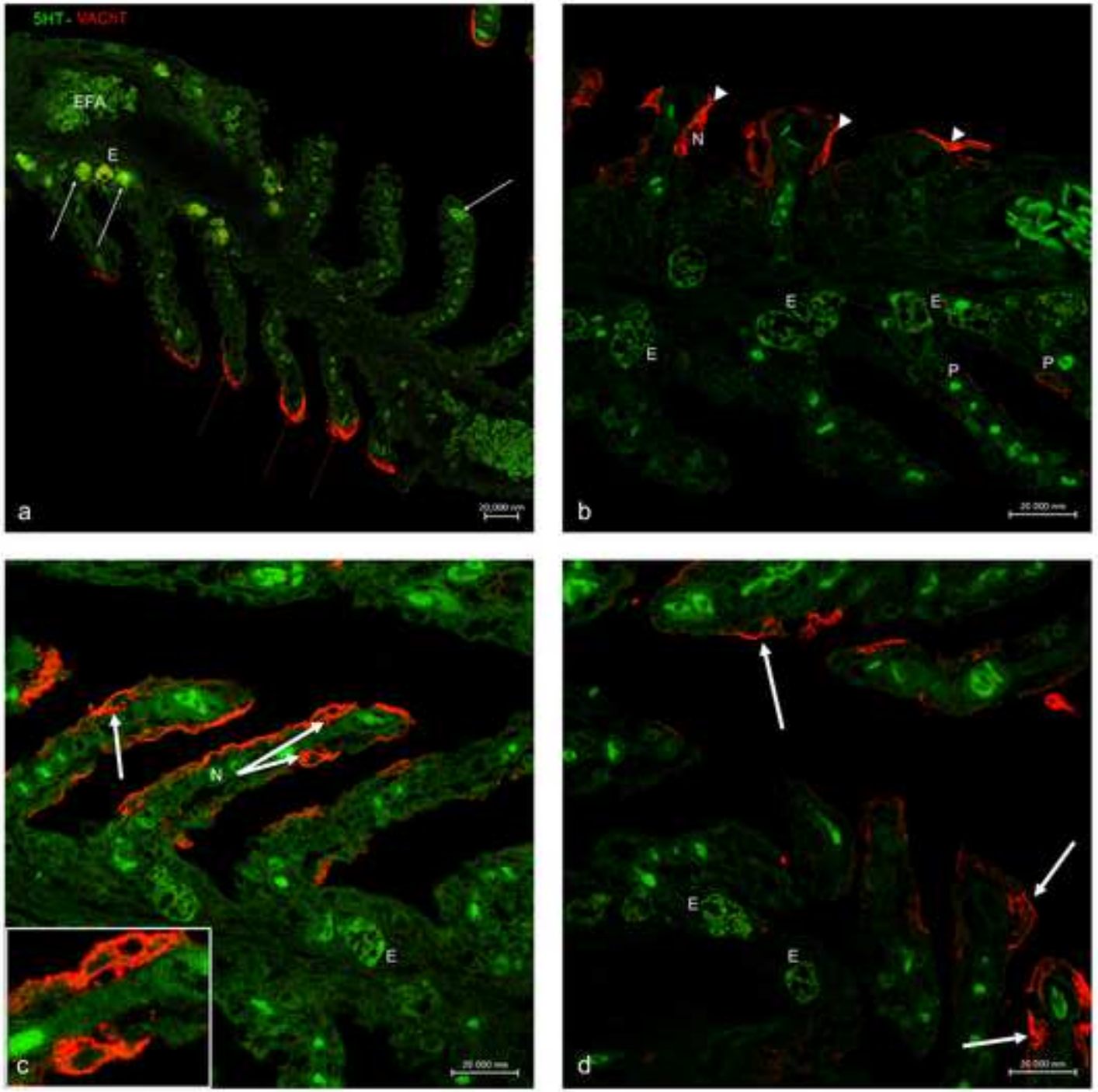

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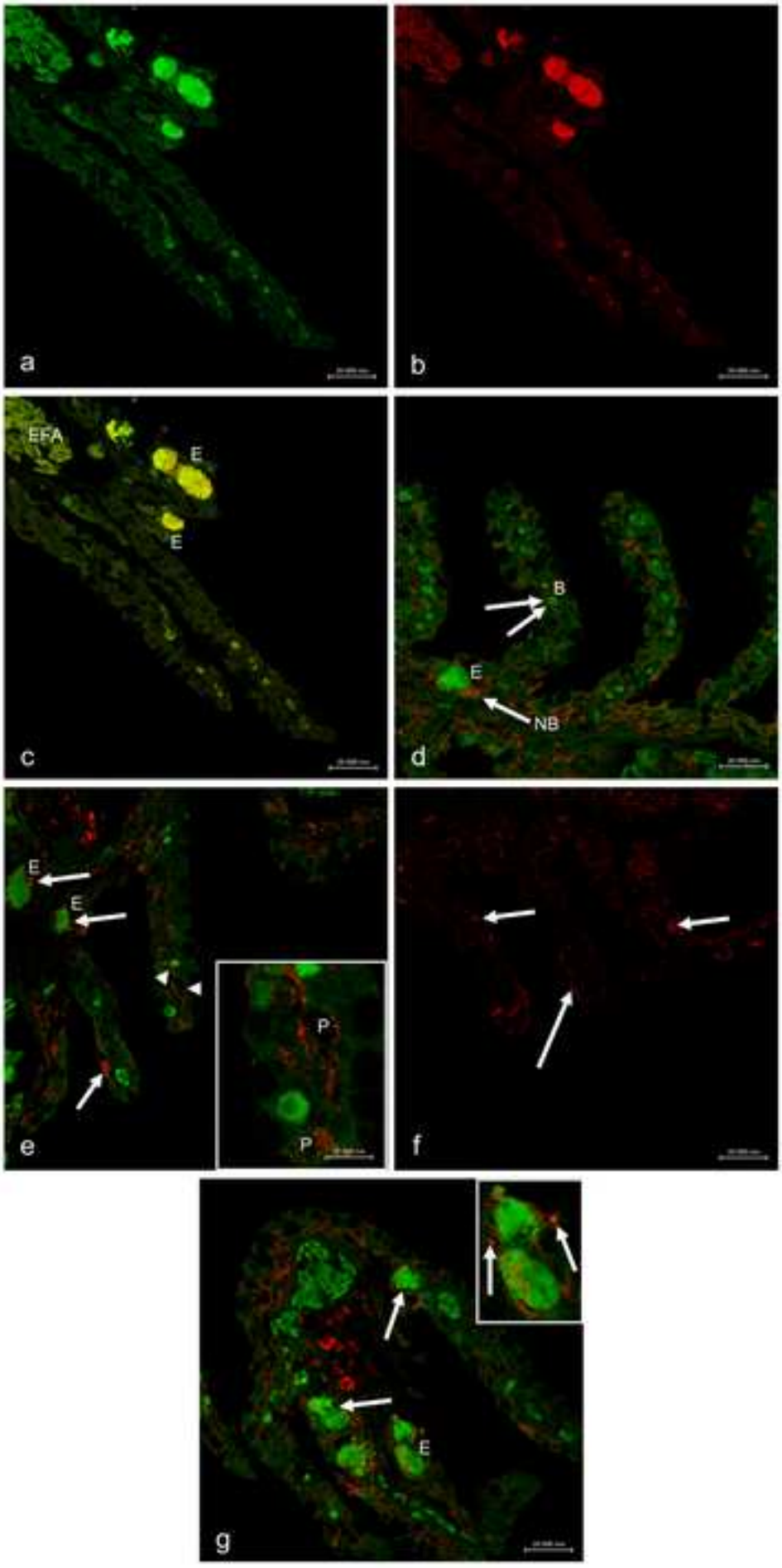

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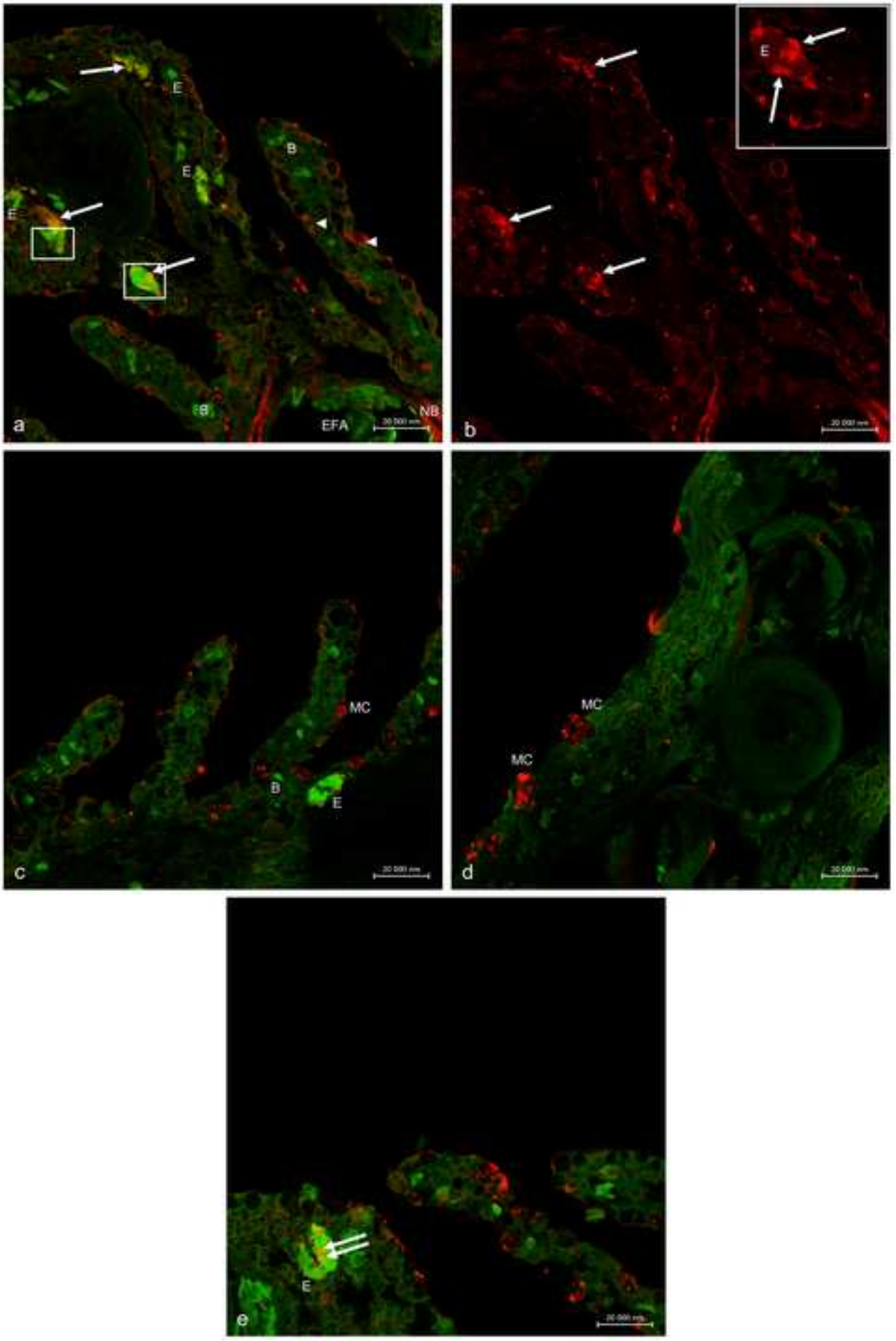

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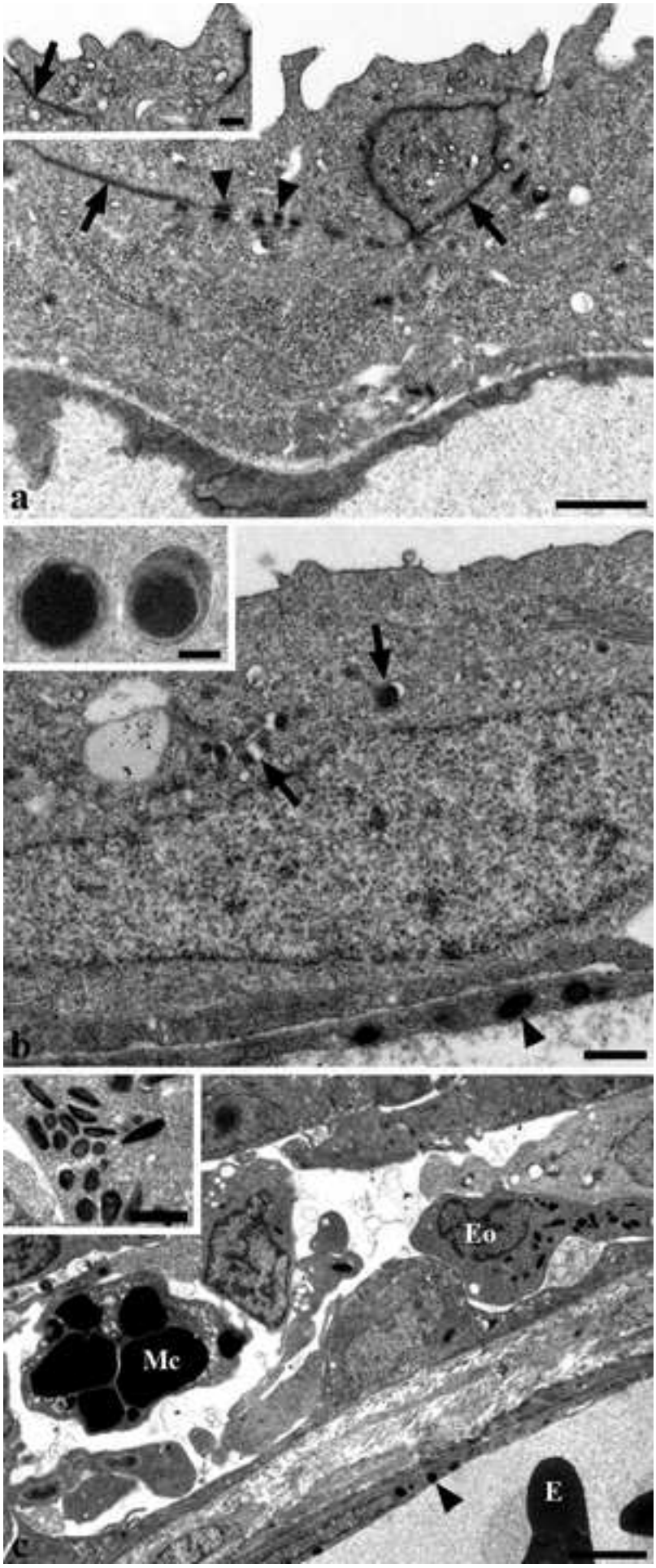

Figure 7

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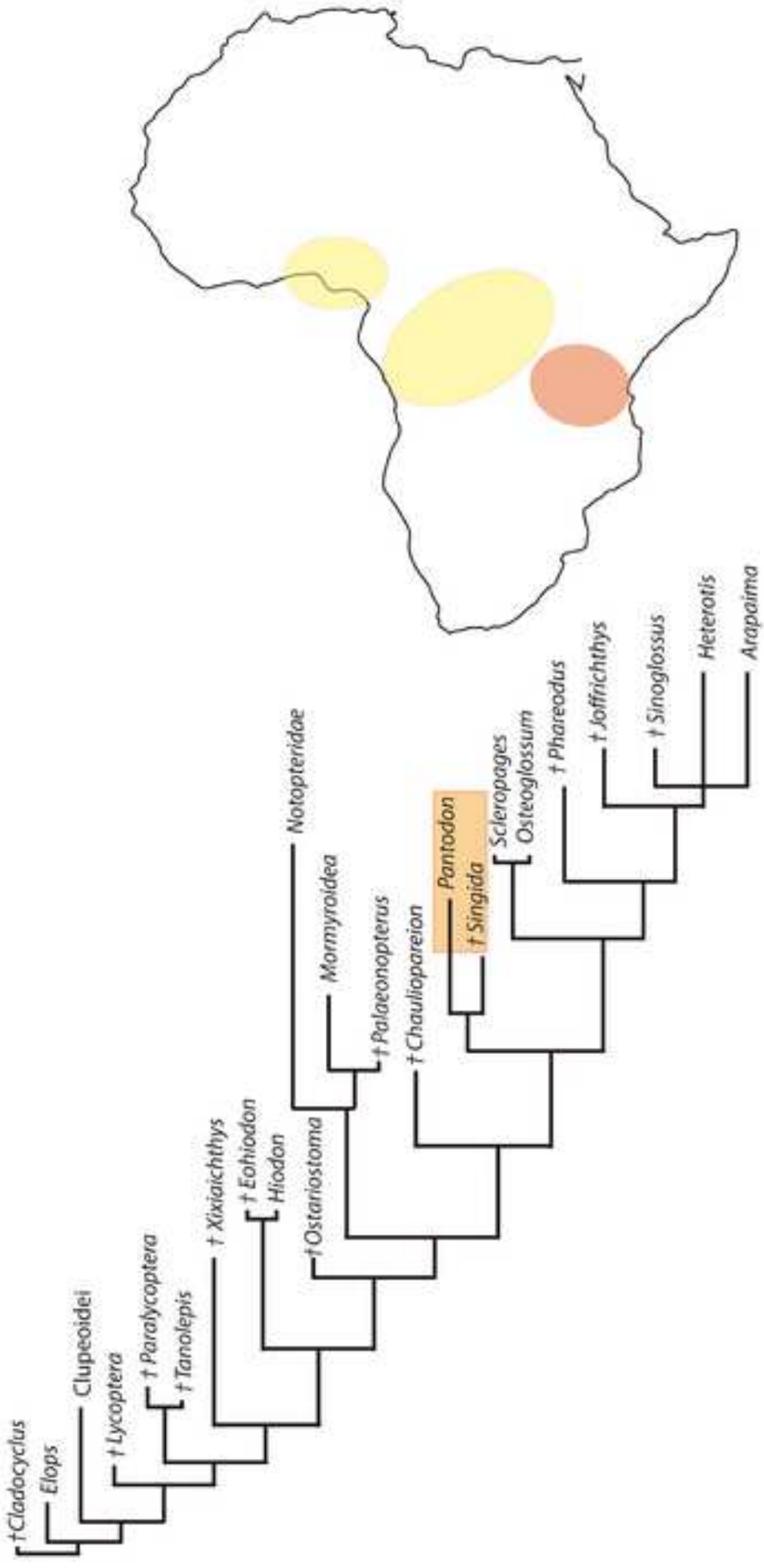

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