Insights Into the Zebrafish Olfactory System: A comprehensive Structural, Lectin- and Immunohistochemical Approach

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Abstract

Fish chemosensory olfactory receptors allow them to detect a wide range of water-soluble chemicals, that mediate fundamental behaviours. Zebrash possess a well-developed sense of smell which governs reproduction, appetite, and fear responses. The spatial organization of functional properties within the olfactory epithelium and bulb are comparable to those of mammals, making this species suitable for studies of olfactory differentiation and regeneration and neuronal representation of olfactory information. The advent of genomic techniques has been decisive for the discovery of specific olfactory cell types and the identification of cell populations expressing vomeronasal receptors. These advances have marched ahead of morphological and neurochemical studies. This study aims to fill the existing gap in specific histological, lectin-histochemical and immunohistochemical studies on the olfactory rosette and the olfactory bulb of the zebrafish. Tissue dissection and microdissection techniques were employed, followed by histological staining techniques, lectin-histochemical labelling (UEA, LEA, BSI-B₄) and immunohistochemistry using antibodies against G proteins subunits ao and a₁₂, growth-associated protein-43, calbindin, calretinin, glial-fibrillary-acidic-protein and luteinizing-hormone-releasing-hormone. The results obtained enrich the available information on the neurochemical patterns of the zebrafish olfactory system, pointing to a greater complexity than the one currently considered, especially when taking into account the peculiarities of the nonsensory epithelium.

Introduction

The olfactory subsystems play a fundamental role in the daily life of all animal species [1], having been extensively studied in mammals, with a fundamental difference between a main olfactory system (MOS) and a vomeronasal or accessory olfactory system (AOS) [2]. While the first one is known for its role in associative behaviours mediated by odorants sensed in the olfactory mucosa [3], the AOS process innate capabilities mediated by pheromones detected by the vomeronasal organ [4].

Regarding fish, extensive information has been accumulated in recent decades about their olfactory capabilities [5, 6]. Their chemosensory receptors allow them to detect a wide range of water-soluble chemicals, that mediate fundamental behaviours. For instance, aminoacids indicate the presence of food [7], nucleotides reveal the freshness of the food [8], bile acids are implicated in migration to spawning sites [9], steroids and prostaglandins excreted in urine, trigger reproductive behaviours [10, 11], and injured skin releases alarm pheromones [12].

In recent decades, zebrafish has become one of the most fruitful model organisms in the field of neurobiology [13, 14]. General aspects of its physiology such as external fertilization or rapid development, together with its rapidly accumulating genome sequence data, have made them a suitable model to deepen into genetic engineering and transcriptomic analyses [15–17]. Zebrafish possess a well-developed sense of smell, which governs a variety of behaviours involved in reproduction, appetite, and fear [18]. Moreover, the functional properties within the sensory epithelium and the olfactory bulb (OB) are comparable to those of mammals. Major aspects determined in mammals, as the so-called rule of one
receptor-one neuron and the convergence of similar axons in the same glomerulus [19], are basically preserved in zebrafish [20]. All this makes the zebrafish a model of vertebrate very suitable for studies of olfactory differentiation and regeneration and neuronal representation of olfactory information [21, 22].

The paired nasal cavity of zebrafish is located at the extremity of the snout, between both eyes. Each cavity is composed of an anterior nostril, through which water enters the cavity, and a posterior nostril, through which water exits the nose. The olfactory epithelium (OE) lies between these two nostrils, arranged in several lamellae that converge in a central raphe, forming a cup-shaped structure known as the rosette [23, 24]. Lamellae are composed of a continuous sensory area, found in the central and medial region of the rosette, as well as a lateral nonsensory epithelium. The sensory area comprises a characteristic pseudostratified columnar epithelium formed primarily by olfactory sensory neurons (OSNs), as well as basal and supporting cells [25, 26].

Regarding the accessory olfactory system, apart from the isolated case of the Dipnoi [27], all fishes, zebrafish included, lack of a chamber or vomeronasal organ and an accessory OB comparable to those present in amphibians, reptiles or mammals [28]. This led to the conclusion that there is no accessory olfactory system in fish. However, studies of morphological features of the olfactory rosette done in zebrafish [29] have revealed a complexity that really corresponds to the overlapping and integrated presence of both the main and olfactory systems. Transgenic lines studied by Sato et al. [30] have unravelled the existence of two segregated neural circuits that originate in the sensory neurons of the OE, each of them featuring specific cell morphology, molecular signatures, and axonal terminations in the OB. Both pathways probably transmit different types of olfactory information (pheromones versus odorants) to higher olfactory centres [31, 32].

In the epithelium of the zebrafish olfactory rosette, four main receptor cell types are differentiated: ciliated, microvillous, crypt, and kappe cells. Ciliated and microvillous are the most numerous neurons and differ from one another for their morphology and relative positions in the OE. The ciliated OSNs are located in the deep layers of the OE, project a long dendrite, and extend several long cilia into the lumen of the rosette cavity. The microvillous OSNs are situated in more superficial layers, bear a short dendrite, and produce short microvilli [33]. The crypt cells are located in the most superficial layer of the OE, have ovoid-shaped cell bodies bearing microvilli and short cilia within the same cell [24, 26, 34]. The complexity of the zebrafish peripheral OS was proven with the recent finding of a fourth olfactory sensory neuron population in this species, named kappe neurons for its characteristic shape. These neurons possess microvilli and show a distinct spatial distribution within the OE, similar to, but significantly different from that of crypt neurons [35]. Finally, scattered among the olfactory sensory neurons are ciliated nonsensory cells, which help to move the mucus covering the OE and basal cells in charge of regenerating the sensory cells [36].

In addition to these morphological differences, a discrimination among cell types can be clearly made according to their molecular expression profiles [37]. In the zebrafish genome there have been identified 140 OR-type genes [38, 39]. The expression of these ORs is observed in the ciliated OSNs [40]. 54 V2R-like
olfactory C family receptor genes were identified by Alioto & Ngai [41] and later on the number has been increased up to 60 [41]. These receptors are found in the microvillous OSNs [42]. Using genome database mining, Saraiva and Korsching [43] identified a new family of 6 V1R-type. Whereas half of the fish V1R genes show a multi-exon structure, all mammalian V1R genes possess a single exon structure [44–46]. It has not been clearly demonstrated which type(s) of OSNs express V1R receptors, although the zV1R1 (ORA1) receptor has been characterized in cells belonging to the apical side of the OE [46] and Oka et al. [48] found that crypt neurons express a single V1R-type receptor, the ORA4 receptor. Finally, 112 trace-amine associated receptors (TAAR) have been identified in zebrafish [49].

A comparative analysis between the olfactory transcriptomes of zebrafish and mouse [50] revealed a high degree of molecular conservation, with orthologs of mouse olfactory cell-specific markers, and all but one of their chemosensory receptor classes expressed in the single zebrafish olfactory organ. All seems to indicate that, despite the remarkable morphological differences between the two classes, Actinopterygii and Mammalia, the molecular mechanisms supporting olfaction in teleost and mammals have similarities despite more than 400 million years of evolutionary divergence.

The large size of zebrafish chemosensory gene families, combined with the high degree of nucleotide identity among their members, make it very difficult to perform comprehensive expression analysis by in-situ hybridization (ISH), Real-time-PCR, or microarray. Although the olfactory system of zebrafish has been subject of sequencing, transcriptomic, and ISH studies, there is a gap regarding immunohistochemical studies that would allow a more comprehensive and structurally precise assessment of its morphofunctional characteristics.

The present study describes the histology, and the lectin-histochemical and immunohistochemical features of the adult zebrafish olfactory rosette and bulb. Three lectins were studied: *Ulex europaeus* agglutinin (UEA), *Bandeiraea simplicifolia* isoelectin B₄ (BSI-B₄), and *Lycopersicon esculentum* agglutinin (LEA). The immunohistochemical study covered a range of antibodies against the G proteins, Gαᵢ2 and Gαo, the calcium-binding proteins, calbindin (CB) and calretinin (CR), growth-associated protein 43 (GAP-43) glial fibrillary acidic protein (GFAP) and luteinizing hormone-releasing hormone (LHRH). Aimed to address the current gaps in our understanding of the immunohistochemical features of both the olfactory rosette and bulb of the adult zebrafish, our study reveals the high complexity of the neurochemical organization of the zebrafish olfactory system and point to the need for more specific morphofunctional studies.

**Material & Methods**

Ten wild-type one-year-old zebrafish (*Danio rerio*, wild-type) were used in this study. They were maintained at 28.5 °C in 30 L aquaria at a rate of 1 fish per liter of dechlorinated water, with reverse osmosis purified, and under a light-dark cycle of 14:10. Fishes were euthanized by tricaine overdose (MS-222, Sigma, St.Louis, MO). Whole heads were promptly immersed in modified Bouin's fixative solution. After 24 hours, the samples were transferred into 70% ethanol. The samples were not decalcified. In all cases paraffin
embedding was used to perform the histological procedures. All samples were cut with a Leica Reichert Jung microtome with a thickness of 4–8 μm. To highlight the different tissue components, we used the following stainings: Haematoxylin-Eosin (HE), 1% Alcian blue (AB) for acid mucopolysaccharides, and Gallego’s Trichrome.

**Gallego’s trichrome:**

This stain allows for the differentiation of components of the connective tissue. It stains erythrocytes green, muscle fibers and collagen light blue, epithelium and glandular tissue red, bone dark blue and cartilage purple. The protocol used was described in detail in [51] as follows: sections were first stained with Ziehl acetic fuchsin for 2 min. After several washes they were introduced into formalin-acetic acid solution for 5 min. After two more washes, the sections were finally introduced into picroindigocarmine for 3–5 min.

**Histochemical and immunohistochemical staining**

The histochemical and immunohistochemical protocols followed by the authors have been fully described in previous contributions [51,52]. Briefly:

**Histochemical labelling (HQ) with lectins:**

We have used (1) a lectin that comes from gorse, the *Ulex europaeus* agglutinin (UEA), α-L-fucose specific, (2) the α-galactose-specific BSI-B₄ that comes from *Bandeiraea simplicifolia*, and (3) *Lycopersicon esculentum* agglutinin (LEA), coming from tomato with a high affinity for N-acetyl-β-D-glucosamine oligomers (Table 1). These stains selectively recognise the different components of the olfactory and vomeronasal pathways in some species [52].

The protocol for the UEA is as follows. (i) blocking the endogenous peroxidase activity of the sample by incubation in 3% H₂O₂ solution for 10 min; (ii) incubation for 30 min in 2% bovine serum albumin (BSA), to prevent nonspecific binding; (iii) incubation with the UEA lectin for 1 h; (iv) 3 x 5 min washes in 0.1 M phosphate buffer (PB, pH 7.2), and (v) incubating for 12 h in a peroxidase-conjugated immunoglobulin against the UEA. Finally, (vi) the sections were washed with PB and developed by (vii) incubation in 0.05% diaminobenzidine (DAB) and 0.003% H₂O₂ for 5 min.

The protocol for the LEA and BSI-B₄ begins with the same two steps. Next, (iii) the incubation of the sections was done overnight in biotinylated lectins diluted in 0.5% BSA. The next day, the samples were (iv) 1.5 h incubation in Vectastain ABC reagent (Vector Laboratories, Burlingame, CA, USA). The samples were finally (v) developed in the same DAB solution as the UEA [51].

**Immunohistochemistry (IHQ) techniques:**

This protocol also began by (i) blocking the endogenous peroxidase. Then, (ii) non-specific binding was blocked with 2.5% horse normal serum from the ImmPRESS reagent kit Anti-mouse IgG/Anti-rabbit IgG
(Vector Laboratories, CA, USA) for 30 min. (iii) The primary antibody was added at the corresponding dilution (Table 1) and incubated overnight. The next day, (iv) the samples were incubated for 20 min with the ImmPRESS VR Polymer HRP Anti-Rabbit IgG Reagent. (v) After rinsing in Tris-buffer (pH 7.61) for 10 minutes, (vi) the samples were finally developed using DAB in the same way as for the lectins [51,52].

All immunohistochemical protocols were checked with the appropriate controls. Samples for which the primary antibody was omitted were used as negative controls. Table 2 gives references to previously published use in zebrafish and other fishes of the antibodies here employed against the same antigens.

**Acquisition of images and digital treatment**

Digital images were captured using the Karl Zeiss MRc5 digital camera attached to a Zeiss Axiophot microscope. Adobe Photoshop CS4 (Adobe Systems, San Jose, CA, USA) was used to adjust parameters such as brightness, contrast and balance light levels for presentation in this work. No features of the image were enhanced in any way, moved, or introduced. Some photomicrographs were formed as a mosaic of several photographs merged with an image-stitching software (PTGui Pro, New House Internet Services BV, The Netherlands).

**Table 1**: Antibodies and lectins used, with species of elaboration, dilution, manufacturer, and catalogue number.
| **Ab/Lectin** | **1st Ab species/dilution** | **1st Ab Catalogue number** | **2nd Ab species/dilution (Catalogue number)** |
|---------------|-----------------------------|----------------------------|-----------------------------------------------|
| Anti-Gao      | Rabbit 1:100                | Sta Cruz Biotechnology     | ImmPRESS VR HRP Anti-Rabbit IgG Reagent MP-6401-15 |
|               |                             | SC-387                     |                                               |
| Anti-Gai2     | Rabbit 1:100                | Sta Cruz Biotechnology     | ImmPRESS VR HRP Anti-Rabbit IgG Reagent MP-6401-15 |
|               |                             | SC-7276                    |                                               |
| Anti-GFAP     | Rabbit 1:400                | Dako Z0334                 | ImmPRESS VR HRP Anti-Rabbit IgG Reagent MP-6401-15 |
| Anti-Calbindin| Rabbit 1:5000               | Swant CB38                 | ImmPRESS VR HRP Anti-Rabbit IgG Reagent MP-6401-15 |
| Anti-GAP-43   | Mouse 1:400-1:4000          | Sigma G9264                | ImmPRESS VR HRP Anti-mouse IgG Reagent MP-6402-15 |
| Anti-Calretinin| Rabbit 1:5000              | Swant 7697                 | ImmPRESS VR HRP Anti-Rabbit IgG Reagent MP-6401-15 |
| Anti-LHRH     | Rabbit 1:500                | Fisher Scientific A235481  | ImmPRESS VR HRP Anti-Rabbit IgG Reagent MP-6401-15 |
| UEA-I*        | 1:10                        | Vector L-1060              | Rabbit 1:50 DAKO P289                        |
| LEA*          | 20 µg/ml                    | Vector B-1175              | Vectastain ABC reagent PK-4000                |
| BSI-B4*       | 100 µg/ml                   | Sigma L-2140               | Vectastain ABC reagent PK-4000                |

**Abbreviations:** Gao: Subunit ao of G protein; Gai2: Subunit ai2 of G protein; OMP: olfactory marker protein; MAP-2: microtubule associated protein-2; GAP-43: growth-associated protein 43; GFAP: glial fibrillary acidic protein; CB: calbindin; CR: calretinin; LHRH: luteinizing hormone-releasing hormone; UEA: *Ulex europaeus* agglutinin; LEA: *Lycopersicum esculentum* agglutinin; BSI-B4: *Bandeiraea simplicifolia* isolectin B4; HRP: horseradish peroxidase; IgG: Immunoglobulin G; ABC: avidin-biotin-complex.

**Table 2:** Previously published use in fishes olfactory system studies of the antibodies employed in this study against the same proteins.
| Antigen   | Host   | Type, clone | Source        | Code  | Fish studied          | Reference |
|-----------|--------|-------------|---------------|-------|-----------------------|-----------|
| Calbindin | Mouse  | Monoclonal  | Swant         | 300   | Acipenser baeri       | [53]      |
| Calbindin | Mouse  | Monoclonal  | Swant         | 300   | Polypterus senegalus  | [54]      |
| Calbindin | Mouse  | Monoclonal  | Swant         | 300   | Polypterus senegalus  | [55]      |
|           |        |             |               |       | Erpetoichthys calabaricus |           |
| Calretinin| Rabbit | Polyclonal  | Chemicon      | AB5054| Poecilia reticulata   | [56]      |
| Calretinin| Rabbit | Polyclonal  | Santa Cruz Biotech | SC-11644| Danio rerio          | [57]      |
| Calretinin| Rabbit | Polyclonal  | Swant         | 7697  | Danio rerio           | [58]      |
| Calretinin| Rabbit | Polyclonal  | Swant         | 7697  | Danio rerio           | [59]      |
| Calretinin| Rabbit | Polyclonal  | Swant         | 7697  | Salmo trutta fario    | [59]      |
| Calretinin| Rabbit | Polyclonal  | Swant         | 7697  | Psetta máxima         | [60]      |
| Calretinin| Rabbit | Polyclonal  | Swant         | 7697  | Danio rerio           | [29]      |
| Calretinin| Rabbit | Polyclonal  | Swant         | 7697  | Danio rerio           | [61]      |
| GAP-43    | Mouse  | Monoclonal  | Sigma         | G9264 | Tilapia mariae        | [62]      |
| GFAP      | Mouse  | Monoclonal  | Sigma         | G3893 | Danio rerio           | [63]      |
| GFAP      | Mouse  | Monoclonal  | ZIRC          | Zrf -1| Danio rerio           | [64]      |
| GFAP      | Rabbit | Polyclonal  | Dako          | Z0334 | Oryzias latipes       | [65]      |
| GFAP      | Rabbit | Polyclonal  | Dako          | Z0334 | Danio rerio           | [66]      |
| GFAP      | Rabbit | Polyclonal  | Dako          | Z0334 | Nothobranchius guentheri | [67]         |
| GFAP      | Rabbit | Polyclonal  | Dako          | Z0334 | Poecilia reticulata   | [68]      |
|           |        |             |               |       | Carassius auratus     |           |
| GFAP      | Rabbit | Polyclonal  | Dako          | Z0334 | Danio rerio           | [69]      |
| GFAP      | Rabbit | Polyclonal  | Dako          | Z0334 | Astatotilapia burtoni | [70]      |
| GFAP      | Rabbit | Polyclonal  | Sigma         | G9269 | Danio rerio           | [71]      |
| Gao       | Rabbit | Polyclonal  | Santa Cruz Biotech | SC-387| Tenualosa ilisha     | [72]      |
| Gao       | Rabbit | Polyclonal  | Santa Cruz Biotech | SC-387| Tenualosa ilisha     | [72]      |
Results

The zebrafish olfactory rosette occupies an anterodorsal position, slightly rostral to the orbits (Fig. 1, 2A, 2C). The most relevant microscopic aspects are shown by the histological stains employed by us: hematoxylin-eosin (Fig. 2,3C,D,E,G,H), alcian Blue (Fig. 3F), trichromic of Gallego (Fig. 3A,B). In a parasagittal section at orbital level, its topographic relationship with the eye can be seen, as well as the localization of the outlet nostril, which coincide with a zone of lesser development of the lamellae (Fig. 2C). In a sagittal section that includes the central part of the OB (Fig. 2B, 3B) the rosette can be seen at its maximum expression, divided into two symmetrical zones by the presence of a wide raphe that serves to support the lamellae. A section in a median plane through the branches of the olfactory nerve show how they are associated with each lamella and how the nerve constitute a single branch that reaches the OB from the ventromedial side (Fig. 2D).

A thin bone capsule delimits the nasal cavity, but this bone tissue is not part of the lamella skeleton (Fig. 2B,D,3B). The organization of the sensory pseudostratified epithelium stained by hematoxylin-eosin allows for the differentiation of basal cells from the olfactory sensory cells. The three main olfactory cell types are densely intermingled, but can be distinguished by their characteristic shape and spatial position: a slender dendrite and a basal soma for ciliated neurons, a rounded cell body and an intermediate soma position for microvillous neurons and a large globose soma in an apical position for crypt neurons (Fig. 3C). The luminal surface of the nonsensory epithelium is covered by cilia (Fig. 3D,E). Alcian Blue staining shows the presence of acidic mucopolysaccharides on the epithelial surface of the lamella, mainly in the luminal surface of the sensory area, whereas the nonsensory epithelium border is free of acid mucins (Fig. 3F).

The zebrafish OB is diffusely laminated, but four layers can be identified from the periphery towards the centre: olfactory nerve layer, glomerular layer, mitral cell layer and granular layer. (Fig. 3G-H). The outermost layer of this structure corresponds to the olfactory nerve layer and it is formed by the axonal endings of olfactory receptor neurons (ORNs). The glomeruli that comprise the glomerular layer do not resemble distinct spheres as they do in mammals, probably due to the few number of periglomerular cells and glial elements found in zebrafish. These glomeruli are formed by both the contribution of the
olfactory nerve endings and the dendrites of the mitral cells, whose cell bodies are located in the subjacent layer. Mitral cells are identified by their large pale nuclei. Finally, the deeper layer corresponds to the granular layer and shows the biggest, where granular cell clumps can be found (Fig. 3H).

**Immunohistochemical staining of the olfactory rosette**

The immunohistochemical study with anti-Gαi2 and anti-Gαo produce two differentiated patterns (Fig. 4). Intense immunoreactivity was noticed in the central and medial portions of the OE when employing Anti-Gαi2 (Fig. 4A-D). The immunopositive cells are distributed across the entire thickness of the epithelium, but appear mostly concentrated in its superficial half. Contrastly, anti-Gαo produces a diffuse immunoreactivity circumscribed to cells present on the apical surface of the OE (Fig. 4E-F). Interestingly, we also found large isolated cells with stronger anti-Gαo immunopositivity in the OE (Fig. 4F). Additionally, the anti-Gαo labelling also stains the apical surface of the nonsensory area of the hair cells (Fig. 4G). Both markers, anti-Gαi2 and anti-Gαo label the olfactory nerves (Fig. 4B,G).

The results of the immunohistochemical study with anti-calbindin (anti-CB) and anti-calretinin (anti-CR) are depicted in Fig. 5A,B,D,E and 5C,F respectively. Both markers label a subpopulation of olfactory sensory neurons. Sagittal and transverse sections of the rosette show how the anti-CB immunolabelling is mostly located in the deeper part of the neurosensorial epithelium (Fig. 5D), whereas the nonsensory epithelium is Anti-CB immunonegative (Fig. 5B). Anti-CR immunolabelling produces a stronger labelling than anti-CB in neuroepithelial cells, which is mostly concentrated in the medial part of the lamellae (Fig. 5C), and their deeper neuroepithelial layers (Fig. 5F). Very rarely superficial cells are immunolabelled. The nonsensory cripts do not show anti-CR labelling.

Anti-GFAP immunolabels isolated big cell bodies in the apical part of the whole OE (Fig. 5G-J). These cell bodies are present in both sagittal and transverse sections. Anti-GAP-43 immunolabelling is located in the apical part of the nonsensory epithelium and in individual cell bodies in the cripts. (Fig. 5K,L). Anti-LHRH produces a light immunolabelling, mainly located in the cell processes of both the sensory and nonsensory epithelia (Fig. 5M,N).

**Lectin histochemical staining of the olfactory rosette**

The three lectins employed in this study labelled the olfactory system following individual patterns (Fig. 6). The labelling with LEA of the whole OE clearly delineates the limits between the olfactory and the nonsensory epithelium -this latter unstained- (Fig. 6A,B). Additionally, big oval neuron-like cell bodies in the cripts of the nonsensory epithelium are also stained, and in some of them thin dendritic processes can be clearly appreciated (Fig. 6C,D). UEA only labelled secretory material, mainly in the cripts, but also in the sensory epithelium but in lesser extent (Fig. 6E,F). BSI-B4 marks individual cells in the cripts and in the nonsensory part of the lamella and very occasionally cells belonging to the sensory part of the OE. In all cases they are scattered cells, smaller than those marked by LEA (Fig. 6G,H).
Immunohistochemical and lectin histochemical staining of the olfactory bulb

The immunohistochemical labelling of the olfactory bulb (Fig. 7), shows mainly two patterns. In the case of calcium-binding proteins (CB and CR), the labelling is mainly concentrated in the dorsal and lateral parts. Additionally, anti-calbindin stains glomeruli belonging to the medial part of the bulb and ventrolateral parts of the bulb, this latter with less intensity. Regarding G-proteins, both antibodies show a similar pattern, with a higher concentration of the labelling in ventrolateral areas, especially anti-Gao. Additionally Gai2 marks glomeruli in dorsomedial position. Finally, LEA shows a labelling circumscribed to the ventrolateral portion. This lectin also labels the dorsolateral area but with less intensity. LHRH is expressed mainly in the dorsolateral glomeruli. Anti-GAP-43 and the other two lectins employed by us, did not show significant results in the OB.

Discussion

Under an appearance of simplicity, in recent decades the olfactory system of fish has revealed a very high structural complexity, according to cell types and receptors involved. The advent of genomic techniques such as RT-PCR, ISH, genomic and transcriptomic analyses, has been decisive for the discovery in fish of specific olfactory sensorial cell types such as kappe or crypt cells. These techniques have also played a significant role in the identification and characterization of large cell populations expressing vomeronasal receptors [40, 42], thus putting an end to the longstanding controversy about the existence of an AOS in fish [32].

In zebrafish, genomic advances have been so rapid that they have unavoidably marched ahead of morphological and neurochemical studies. Instead, studies in mammals, have formed a solid basis on which to build knowledge of the olfactory and vomeronasal systems [78, 79]. For this reason, there is a lack of specific histological, lectin-histochemical and immunohistochemical studies of the olfactory rosette and OB of the zebrafish. To discuss our results, we must therefore contextualize them in a higher taxonomic order including other fish families.

Although the presence of a vomeronasal organ is a tetrapod evolutive innovation, the vomeronasal receptor genes have been identified in fish and even in the lamprey [80]. Taking into account that each vomeronasal type receptor, V1R and V2R, is associated in mammals to a unique G protein, Gai2 and Gao respectively, the present study aimed to determine whether there is a correlation between such G-proteins and the zebrafish olfactory cell morphology. It is known from the literature that the receptor molecules and the G-protein specific for each receptor are detectable not only in the dendritic process of the neuroreceptor cell, but also along the axons and their termination in the glomeruli of the OB [75]. For this reason, we have extended the study of these molecules of the chain transduction to the olfactory bulb.

Regarding Gao, Hansen et al. [75] correlated in goldfish Carassius auratus the receptor cell morphology and the cell types distribution, with the expression of G-proteins, demonstrating that anti-Gao
immunoreactivity was present on microvillar ORNs located in the upper half of the OE. This happens similarly in the Gao neurons identified by us in the zebrafish, pointing to the reliability of anti-Gao as a marker of microvillar V2R-like cells in this species. Other studies in different fish species, such as the case of Chondrichthyes, support this view. Thus, immunohistochemical studies of G protein α subunits in the olfactory organ of *Scyliorhinus canicula* (Elasmobranch) and *Chimaera monstrosa* (Holocephali) found the presence of Gao in virtually all ORNs, which was consistent with the presence of V2Rs [73, 74].

In our case, additionally to the profuse microvillar Gao-positive neurons, we have also found a subpopulation of large, oval-shaped Gao positive cells, always located on the apical surface of the OE. Although their morphology is reminiscent of crypt cells, the study by Ahuja et al. [35] in zebrafish demonstrated that these cells constitute a new olfactory cell type, the kappe cells. Their immunofluorescence study showed that kappe neurons are identified by their anti-Gao immunoreactivity, demonstrating a scattered spatial distribution within the OE, similar to, but significantly different from that of crypt neurons. Our study is the first immunohistochemical report that confirms the presence of kappe cells in fish.

Ahuja et al. [35] also found that kappe neurons project to a single identified target glomerulus within the OB, belonging to the mediodorsal cluster. This observation coincides with the anti-Gao pattern of labelling found by us in the OB, showing immunopositivity to Gao in the mediodorsal part of the bulb. Additionally, we have also found an intense immunoreactivity to Gao in the ventrolateral glomeruli, an area which has been attributed to an important projection of fibers from microvillar cells [30, 81].

Our observations confirm the validity of the anti-Gao antibody as a reliable marker of V2R-like receptor cells in zebrafish, pointing to the presence in zebrafish of a large population of microvillar and kappe cells whose transduction chain is analogous to that present in mammals V2R vomeronasal cells. Therefore, this appears to be an ancient trait conserved through the vertebrate evolution [75].

Regarding crypt olfactory receptor cells, none of the antibodies and lectins employed by us have labelled specifically these cells. Nonetheless, the study by Catania et al. [82] characterized immunohistochemically these crypt cells in zebrafish employing an antibody against the neurotrophin receptor Trk-A.

To our knowledge, the inhibitory subunit Gai2 has not been studied in the fish olfactory system. This fact is surprising since Gai2 is part of the vomeronasal V1R receptor transduction chain in mammals. Mammalian Vmn1r genes show a rather dynamic evolution, in striking contrast to the highly conserved fish orthologous, the ORA gene family. In zebrafish, six ORA receptors have been identified [43], and only in one of them, the ORA4, the precise location of its expression has been studied. Thus, ORA4 receptor was found to be expressed in crypt neurons, but not associated with Gai2 but with the inhibitory G protein, Gi1b [48]. As for its ligands, it is only known that the ORA1 gene recognizes with high specificity and sensitivity the 4-hydroxyphenylacetic acid [83], which might function as a pheromone for reproductive behaviour in zebrafish. ORA1 is ancestral to mammalian V1Rs, and its putative function as pheromone receptor is reminiscent of the role of several mammalian V1Rs as pheromone receptors.
The anti-Gai2 pattern of labelling is very different from that found with anti-Gao, as it covers a wider thickness of the epithelium, although it rarely reaches the deepest cell layers. Moreover, unlike anti-Gao immunolabelling, it comprises the entire extension of the olfactory epithelium clearly demarcating it from the nonsensory epithelium. The immunohistochemistry of the olfactory bulb using anti-Gai2 results in the labelling of a glomeruli subpopulation confirming that the Gai2-positive cells in the olfactory rosette are sensory neurons that convey information to the brain. If ORA receptors coincide with the V1Rs in having the protein subunit Gai2 in their transduction chain, it is surprising that such a small number of receptors are expressed on such a high number of olfactory cells as those detected in our zebrafish olfactory rosette and bulb immunolabelling.

Calcium-binding proteins contribute to calcium homeostasis by buffering the intracellular free calcium concentration [84]. Both CR and CB protect sensory neurons against calcium increases during periods of high frequency discharge as well as in pathological conditions [85]. Moreover, calretinin and calbindin immunoreactive (CR-ir and CB-ir, respectively) neurons in the cerebral cortex are resistant to degenerative processes in Alzheimer’s disease [86].

The distribution of CR in the olfactory system of the zebrafish was investigated for the first time by Castro et al. [59], by using immunocytochemical techniques. Our CR immunoreactivity coincide essentially with their own observations. Accordingly, it is remarkable the presence of numerous CR-ir bipolar cells in the neuroepithelium and an intense immunopositivity in the olfactory nerve. Parisi et al. [61] performed immunofluorescence against CR on zebrafish crypt cells, finding immunolabelling in the OE, primarily in the intermediate cells, but also in the superficial layer. Morphologically, immunopositive cells resembled to them both microvillous and crypt cells. However, our light microscopy anti-CR immunopositive cells featured a slender dendrite and an elongated soma; a morphology closer to olfactory than to microvillous cells. Our observations agree with the immuno-electron microscopy investigation by Gayoso et al. [29] who found a consistent anti-CR immunopositivity in the ciliated cells and only very rarely in the microvillar cells. Additionally, neither Castro et al. [59], Gayoso et al. [29] nor us have found anti-CR immunopositive crypt cells. Studies in other fish species such as the one carried out in the olfactory rosette of guppy [56] have proved similar observations to those found by us in zebrafish.

Regarding the OB, Castro et al. [59] and Braubach et al. [58] in a comprehensive study of the whole population of olfactory glomeruli found that the dorsolateral, ventrolateral, and ventromedial glomerular fields exhibited strong anti-CR labelling. Instead, the dorsomedial area exhibited only faintly CR-ir fibers. Our observations are mostly comparable to those found by them, with the exception that we did not find immunopositive ventromedial glomeruli, likely due to a difference in the levels chosen for each study.

Regarding the expression of calbindin in the olfactory system of the zebrafish, there is a lack of information. However, there have been studies in other fish species such as the chondrostean, Acipenser baerii [53] and the cladistian fish Polypterus senegalus [54], in both cases finding a very faint expression in the olfactory rosette. Our study shows a very high expression of calbindin in the zebrafish olfactory organ, which is accompanied by a parallel expression in the OB. The immunolabelling is comparable to
that produced by anti-calretinin, but wider in the case of calbindin, as it is extensible to the ventrolateral glomeruli.

The expression of GFAP has been very little studied in the olfactory system of fish. Notwithstanding, there is a specific study in zebrafish by Lazzari et al. [69] about olfactory ensheathing cells (OECs) with employed different markers (antibodies against GFAP, S100, NCAM, p75). OECs are unique glial cells with axonal growth-promoting properties, involved in the regenerating capability of ORNs throughout life. These cells sustain the continuous axon extension and successful topographic targeting of the olfactory receptor neurons. They are present in the OE and the OB and are also expressed along the entire length of the olfactory nerve. Lazzari et al. [69] found in zebrafish a slight immunostaining in the OE and moderate immunolabelling in the nerve layer of the bulb. In our case, we have found a stronger labelling in both structures. Although we have used the same fixative and commercial antibody as the one used by Lazzari et al. [69] their samples suffered decalcification with EDTA, whereas we did not subject the tissue to any kind of chelating treatment. This fact might explain the differences in the immunolabelling intensity found between the two studies. Remarkably, we found the presence of immunopositive cells in the apical surface of the epithelium, which vary in shape and size, but they are predominantly globose. The meaning of such immunopositive cell bodies, previously undescribed, should be further studied.

Anti-LHRH has been used in fish and mammals to characterize the terminal nerve [77]; a ganglionated extrabulbar nerve, independent of the olfactory nerve, but close enough to be identified by classical histological methods. Its function is uncertain, although its fibres facilitate migration of LHRH cells to the hypothalamus, thus participating in the development of the hypothalamic-gonadal axis [87]. Although extrabulbar elements have been characterized in the forebrain of the zebrafish [29], they differed from the terminal nerve as they were not immunoreactive to the most widely employed marker for this nerve, FMRFamide [88]. We were not able to identify LHRH fibers in the olfactory rosette, but we observed immunoreactivity in neuronal elements of the dorsolateral OB. This result is consistent with that observed in fish by Münz & Class [89] and mammals by Witkin & Silverman [90].

Histochemical labelling with lectins has been widely used in fish, but to a lesser extent in the olfactory system, and with only a few references to the specific case of zebrafish. UEA-I, specific for L-fucose, has been studied in trout [91] finding intense labelling in cell processes located in the apical region of the OE, in some elements of the basal layer and a few cells in the nonsensory epithelium. However, regarding the OB the authors only found positive glomerular fields in five of seven trouts, and in a heterogeneous shape. Pastor et al. [92], studied two Teleostei, Sparus auratus and Dicentrarchus labrax finding negative reaction in the olfactory rosette. Our own findings highlight the interspecific diversity in UEA marking, as we have found a positive reaction on the luminal surface of the neuroepithelium and a high concentration of L-fucose in the crypts of nonsensory epithelium, probably due to the mucosal secretion of these cells. Regarding the OB we did not find labelling in our specimens.

LEA has been more widely used to characterize the olfactory system of fish than UEA, but surprisingly there are no specific studies in zebrafish, where we have seen that it is an excellent marker of the sensory
epithelium. LEA labels all its cellular elements, establishing a clear border with the unlabelled nonsensory epithelium. The striking presence in the crypts of individualized cells with a clear neuronal morphology, oval shape and fine dendritic processes, has not previously described and should be object of future studies. Interestingly, the neuronal features of these cells, would coincide with the observations by Amato et al. [93] who found TRPV4 immunoreactive “unknown cells” in the nonsensory epithelium of the zebrafish olfactory rosette. TRPV4 is a nonselective cation channel that belongs to the vanilloid subfamily of transient receptor potential ion channels. These authors suggest that these TRPV4 cells might be involved in olfactory sensation. Moreover, Parisi et al. [61] verified that TRPV4 cells did not colocalized with calretinin, which is consistent with the lack of calretinin immunopositive cells in the nonsensory crypts, reported by us. Moreover, our immunolabelling with anti-GAP-43 produced a similar pattern in the crypt epithelium when compared to LEA. All these results together point for the first time to a chemosensory nature of the crypts LEA positive cells.

All mammal and fish species studied till date have shown positivity to LEA in their olfactory sensory epithelia, apart from the case of Pleuronectiformes [94], in which surprisingly and as an exception, LEA negative staining in the OE was reported. Regarding the OB, our results are consistent with those observed in the lungfish Protopterus annectens [95] where LEA positivity was found in the ventral part of the OB, a region associated with reproductive behaviour [96].

Our marking with BSI-B₄ stained both the olfactory and the nonsensory epithelium, respectively producing a striking pattern containing scattered cells in the olfactory sensory epithelium, and a widespread labelling of neuronal-like cells similar to those identified with LEA in the nonsensory epithelium. There are no references to compare this striking result in zebrafish, since studies with this lectin existing in other fish species, such as eels and sharks [97, 98] have been restricted to the sensory epithelium, where these authors described a diffuse labelling.

As a whole, we have for the first time exhaustively characterized the olfactory system of the zebrafish at a morphological and immunohistochemical level expanding the results obtained by other authors in specific studies with some of the antibodies and lectins employed by us. These results enrich the information available on the neurochemical profile of the olfactory system of zebrafish and point to a greater complexity than the one currently considered, especially if the peculiarities of the nonsensory epithelium are taken into account.

**Declarations**

**AUTHORS CONTRIBUTION**

P.R.V., P.S.Q., L.S. designed the research., P.R.V., A.J.A., C.C., M.V.T., I.O.L., P.S.Q., L.S. performed the work, P.R.V., P.S.Q., L.S. analyzed and discussed the results and wrote the paper.

**COMPLIANCE OF ETHICAL STANDARDS**
Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval

The care, use and treatment of zebrafish were performed in agreement with the Animal Care and Use Committee of the University of Santiago de Compostela and the standard protocols of Spain (Directive 2012-63-UE). The protocol was approved by the Animal Care and Use Committee of the University of Santiago de Compostela. Xunta de Galicia Code AE-LU003.

Informed consent

No human subject was used in this study.

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