

# First description of an olfactory neuroblastoma in goldfish *Carassius auratus*: a case report

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**ABSTRACT:** An external pinkish growing mass that emerged from the right nostril of an adult goldfish *Carassius auratus* L. was evaluated by means of light microscopy and immunohistochemistry. The neoplasm presented a well-developed fibrovascular stroma associated with solid cell nests and a large number of Flexner-Wintersteiner rosettes. Myelinated fibres were observed around them. Neoplastic cells showed a prominent degree of nuclear atypia and low mitotic activity. The latter was in agreement with the low reactivity of tumour cells to anti-proliferating cell nuclear antigen antibody. Immunohistochemistry also revealed anti-neuronal nitric oxide synthase, anti-S100 protein, anti-neuropeptide Y, and anti-cytokeratin immunoreactivity in tumour cells as well as in normal olfactory epithelium of goldfish control sections. Histopathological and immunohistochemical findings strongly suggest a diagnosis of an olfactory neuroblastoma (ONB). To our knowledge this is the first description of ONB in goldfish.

**KEY WORDS:** Tumour · Olfactory neuroblastoma · Goldfish · *Carassius auratus* · Histopathology · Immunohistochemistry

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## INTRODUCTION

In comparison with mammals, the literature on the occurrence, biology and etiopathogenesis of fish tumours is scarce (Groff 2004). In addition, the great interspecific variability observed in fishes justifies further study of this issue, especially the use of diagnostic tools such as antibodies in order to set standards for fish tumour classification (Nairn et al. 2001).

Olfactory neuroblastoma (ONB) or esthesioneuroblastoma is an uncommon malignant neoplasm which arises from the olfactory epithelium and shows an expansive, partly submucosal growth and may infiltrate adjacent structures (Constantinidis et al. 2004). Since its first description in Berger & Luc (1924), nearly 1000 cases of ONB have been reported in humans (Broich et al. 1997), while only a few have been observed in other animals (Herrold & Dunham 1963,

Brunst & Roque 1967, Correa et al. 1975, Anderson & Cordy 1981, Vollrath et al. 1986, Cox & Powers 1989, Schrenzel et al. 1990, Hara et al. 2002, Zwart et al. 2002, Döpke et al. 2005, Yamate et al. 2006, Ueno et al. 2007).

In fish, ONB has previously been reported in *Sparus aurata* L. (Thomas 1932, cited by Ishikawa et al. 1978), *Coregonus hoyi* (Milner) (Dawe & Harshbarger 1975, cited by Ishikawa et al. 1978), *Cyprinus carpio* L. (Ishikawa et al. 1978), and *Oryzias latipes* (Temminck & Schlegel) (Torikata et al. 1989). Although Torikata et al. (1989) mentioned the occurrence of 2 cases of ONB in goldfish, no description of the tumour is documented. Here we describe a case of ONB in goldfish *Carassius auratus* L. by means of histopathology and immunohistochemistry, with the purpose of adding to the information available for future standardization of fish tumour classification.

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## MATERIALS AND METHODS

An adult goldfish *Carassius auratus* L. was referred to a private veterinary clinic located in Rosario City, Argentina. The specimen showed an external pinkish growing mass emerging from the right nostril. The roughly shaped mass of approximately 0.5 cm in diameter was excised, fixed in 10% v/v buffered formaldehyde solution and submitted to the Diagnostic Pathology Service of the School of Veterinary Sciences-National University of Rosario, Argentina. The sample was dehydrated in a graded series of ethanol and embedded in paraffin wax. Sections of 3 to 5 µm in thickness were stained using haematoxylin and eosin, combined periodic acid-Schiff (PAS)/Alcian blue, the Grimelius technique and Tolivia staining.

In order to define the origin of the presumptive tumour, an immunohistochemical study was carried

out. Unless otherwise stated, all incubations were performed at room temperature in a humid chamber, and all washing procedures consisted of 3 successive 5 min immersions in 0.1 M phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked by incubation in Peroxidase Blocking Reagent (Dako) for 30 min, and after a rinse in PBS, antigens were exposed by heating under pressure in different buffer solutions or by incubating the samples with proteolytic enzymes depending on the primary antibody employed (Table 1). The sections were subsequently washed again with PBS, treated with 3% skimmed milk powder for 15 min to block non-specific antibody binding, incubated with the primary antibody as indicated in Table 1, washed with PBS, and incubated for 30 min with anti-mouse or anti-rabbit EnVision+ System Labelled Polymer-HRP (DakoCytomation). After further rinsing, the sections were finally developed using

Table 1. Antibodies employed in this study. RT: room temperature; ON: overnight. Heating was carried out in a pressure cooker

Antibodies	Antigen retrieval method	Antibody working dilution	Incubation parameters	Source code	Positive control tissue
Monoclonal anti-neurofilament protein (NFP)	Heating in 10 mM Tris base/1 mM EDTA buffer (pH 9.0), 20 min	1:100	2 h, RT	DakoCytomation (M-0762)	Rat brain
Monoclonal anti-proliferating cell nuclear antigen (PCNA)	Heating in 10 mM sodium citrate buffer (pH 6.0), 15 min	1:100	2 h, RT	DakoCytomation (M-0879)	Swine testicle
Monoclonal anti-synaptophysin (SYN)	Heating in 10 mM sodium citrate buffer (pH 6.0), 15 min	1:250	30 min, RT	DakoCytomation (M-0776)	Swine brain
Polyclonal anti-calcitonin-gene-related peptide (CGRP)	–	1:800	ON, 4°C	Peninsula Labs (T-4032)	Swine gut
Polyclonal anti-cytokeratin (CYT)	Incubation with Proteinase K solution (10 µg ml <sup>-1</sup> ), 5 min	1:800	2 h, RT	DakoCytomation (Z0622)	Cow skin
Polyclonal anti-glial fibrillary acidic protein (GFAP)	Incubation with Proteinase K solution (10 µg ml <sup>-1</sup> ), 5 min	1:5000	ON, 4°C	DakoCytomation (Z0334)	Swine brain
Polyclonal anti-leucine-enkephalin (L-ENK)	–	1:2000	3 h, RT	Peninsula Labs (T-4290)	Swine gut
Polyclonal anti-neuronal nitric oxide synthase (nNOS)	–	1:200	2 h, RT	Serotec (AHP-477)	Rat brain
Polyclonal anti-neuronal specific enolase (NSE)	Heating in 10 mM sodium citrate buffer (pH 6.0), 15 min	1:15	2 h, RT	Biomeda (215M)	Swine brain
Polyclonal anti-neuropeptide Y (NPY)	–	1:1200	ON, 4°C	Peninsula Labs (T-4454)	Swine gut
Polyclonal anti-S100 Protein (S100)	Heating in 10 mM sodium citrate buffer (pH 6.0), 15 min	1:300	ON, 4°C	Thermo Scientific (RB-044)	Cow brain
Polyclonal anti-vasoactive intestinal polypeptide (VIP)	–	1:300	ON, 4°C	Peninsula Labs (T-4246)	Swine gut

3,3-diaminobenzidine tetrahydrochloride (Dako), immersed in deionised water to stop the reaction, counterstained with haematoxylin, dehydrated and coverslipped. In each series of stained sections, positive and negative controls were included to assess the specificity of the assay. The organs employed as positive control are listed in Table 1. The negative control slides were sections in which the primary antibody was replaced with PBS. Sections of normal tissues of goldfish (gut and a transverse section of the head through the olfactory organ) were included, in order to determine if the absence of immunostaining in tumour sections was due to the deficiency of antigen expression or lack of interspecific cross-reactivity.

## RESULTS

Histologically, the mass showed a lobular architecture and a well-developed fibrovascular stroma where a large number of nests of neoplastic cells were present (Fig. 1). Although some of them were solid, the majority of the nests were formed from cells arranged around a clear lumen typical of Flexner-Wintersteiner (FW) type rosettes (Figs. 1 & 2). On some occasions, the rosettes exhibited cell debris and an eosinophilic and PAS-positive material within the lumen, while no cilia were observed (Figs. 1 & 3). A large amount of myelinated fibres closely associated with the rosettes were observed in the fibrovascular stroma (Fig. 4). Tumour cells showed a marked anisokaryosis and were pleomorphic depending on their location. FW rosettes were formed from columnar cells whereas cuboidal cells occurred in the solid nests. Between the columnar cells of the FW rosettes, either basal cubical cells or slender cells with basal nuclei could be observed. Moreover, the nuclear/cytoplasmic ratio was higher in those cells from the solid nests. The nuclei were pale and euchromatic with granular chromatin and conspicuous nucleoli. The number of mitotic figures was scant, particularly in neoplastic nests. The cytoplasm was eosinophilic and some columnar neoplastic cells showed PAS-positive granules in the apical region (Fig. 3). Tumour cells were negative for Grimelius stain. A few discrete areas of necrosis were seen in the deeper zones of the tumour.

Immunohistochemistry revealed immunopositivity for neuronal nitric oxide synthase (nNOS) in some neoplastic cells. In FW rosettes, a weak reaction was observed in the perinuclear cytoplasm of columnar cells, whereas the immunoreaction was stronger in basal cuboidal cells (Fig. 5). In solid nests, some cells with cytoplasmic projections resembling those of receptor neurons exhibited an intense reaction to anti-nNOS antibody (Fig. 6). The S100 immunosignal was

diffuse and mainly located in columnar neoplastic cells of rosettes (Fig. 7). In addition, the majority of cuboidal cells of the solid nests and rosettes were also immunostained with the antibodies against cytokeratin (CYT) and neuropeptide Y (NPY) (Figs. 8 & 9). The elongated cells located in FW rosettes were morphologically similar to supporting cells of the olfactory epithelium and showed immunoreactivity to the anti-CYT antibody (Fig. 8). A low number of either cuboidal or columnar neoplastic cells, situated mainly in FW rosettes, exhibited proliferating cell nuclear antigen (PCNA) immunoreactivity with a nuclear or cytoplasmic staining pattern, depending on the cell cycle phase (Fig. 10). On the other hand, tumour cells did not show immunoreactivity to leucine-enkephalin (L-ENK) and vasoactive intestinal polypeptide (VIP), while cross-reactivity to these antisera was detected in goldfish control sections.

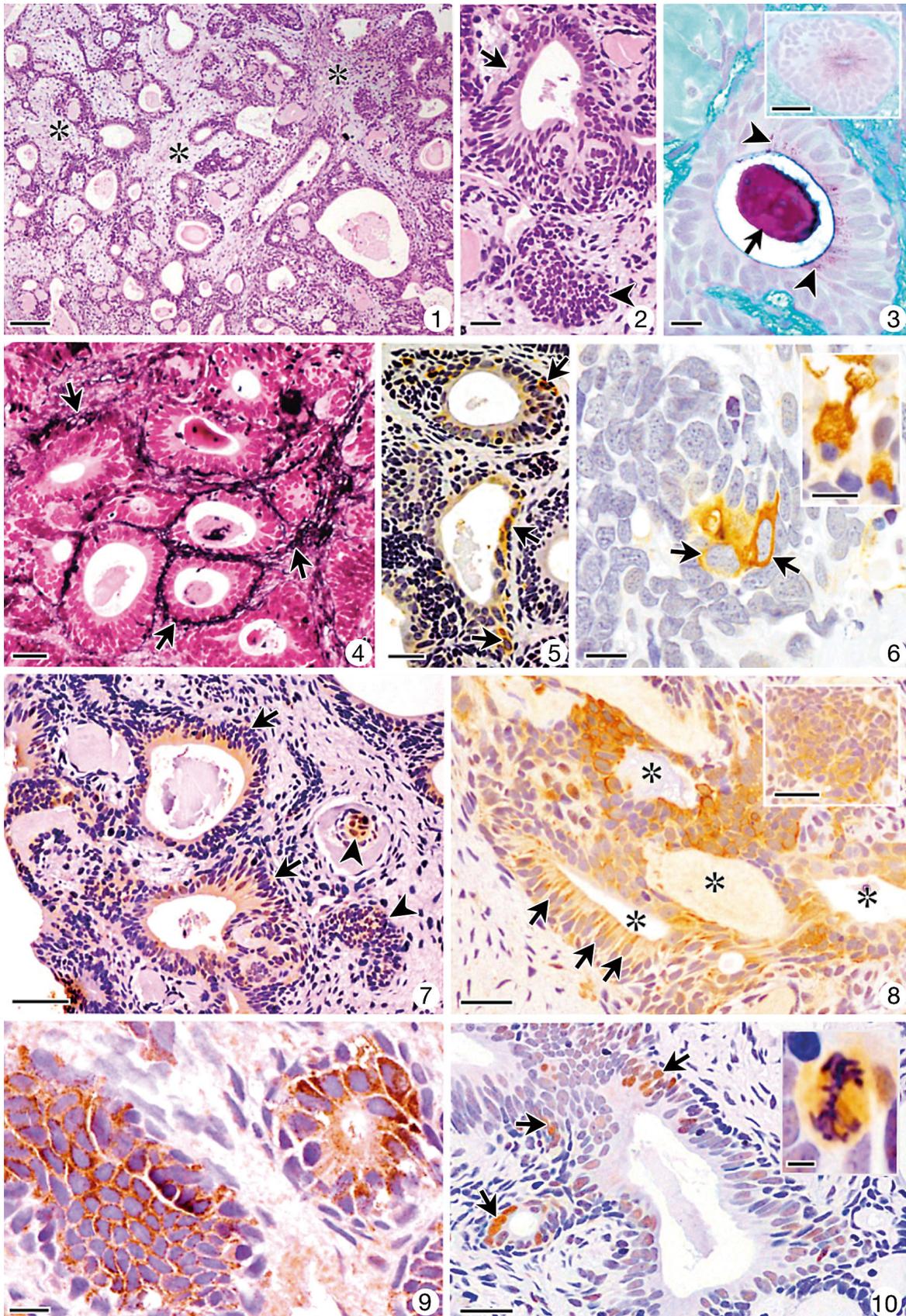
In control sections of the normal olfactory organ of *Carassius auratus*, immunoreactivity to all antisera that immunomarked neoplastic cells, i.e. anti-nNOS, anti-S100, anti-CYT, and anti-NPY antibodies, was also seen in cells of the olfactory epithelium.

No interspecific cross-reactivity was observed for neuronal-specific enolase (NSE), synaptophysin (SYN), neurofilament protein (NFP), glial fibrillary acidic protein (GFAP), and calcitonin-gene-related peptide (CGRP), either in the neoplasm or in the goldfish control sections.

The tissues were processed for transmission electron microscopy (TEM) study. However, since the sample was not initially fixed in a glutaraldehyde solution, the image quality was poor and did not provide useful diagnostic information.

## DISCUSSION

ONB is an uncommon malignant neoplasm which arises from the olfactory epithelium. The structure of the olfactory organ in fish varies widely depending on the degree of development and ecological habitats. In teleosts, the paired olfactory organs are usually located on the dorsal side of the head and are arranged in 2 oval rosettes. Each rosette is constituted by a series of folds or olfactory lamellae that rise from the floor of the nasal cavity and extend radially from a central raphe. Both sides of the lamellae are lined with the olfactory epithelium (Hara 2000a). This epithelium is separated into 2 regions, sensory and non-sensory (Hara 2000b). The former exhibits a pseudostratified epithelium which in goldfish is constituted by 8 different cell types (Hansen et al. 1999). Three of them, the ciliated and microvillus receptor cells and crypt cells, are bipolar primary neurons that play a role in the reception of



Figs. 1 to 10. Fig. 1. Panoramic view of the tumour in which the fibrovascular trabeculae (asterisks) surrounding the nest can be clearly seen. Bar = 200  $\mu\text{m}$ . Haematoxylin-eosin. Fig. 2. Detailed microphotography of the 2 types of nest observed in the neoplasm, i.e. solid nest (arrowhead) and Flexner-Wintersteiner (FW) rosette (arrow). Bar = 50  $\mu\text{m}$ . Haematoxylin-eosin. Fig. 3. A FW rosette showing periodic acid-Schiff (PAS)-positive material (arrow) in its lumen. Note the presence of PAS-positive granules (arrowheads) in the apical cytoplasm of some neoplastic cells. Bar = 20  $\mu\text{m}$ . Inset: Large number of PAS-positive granules in tumour cells of a developing rosette. Bar = 50  $\mu\text{m}$ . PAS-Alcian blue. Fig. 4. Myelinated fibres (arrows) extending from the base of rosettes and spreading into the fibrovascular stroma. Bar = 60  $\mu\text{m}$ . Tolivia staining. Fig. 5. Neural nitric oxide synthase (nNOS)-immunoreactive cells (arrows) in FW rosettes. Bar = 50  $\mu\text{m}$ . Anti-nNOS immunohistochemistry. Fig. 6. Strong staining with nNOS antibody in cells (arrows) of a solid nest. Bar = 20  $\mu\text{m}$ . Inset: A well-differentiated ciliated olfactory neuron showing nNOS reactivity in the epithelium. Bar = 20  $\mu\text{m}$ . Anti-nNOS immunohistochemistry. Fig. 7. Diffuse pattern of immunostaining for S100 protein (S100) in FW rosettes (arrows) and in solid nests (arrowheads). Bar = 50  $\mu\text{m}$ . Anti-S100 immunohistochemistry. Fig. 8. Cytokeratin (CYT)-immunoreactive neoplastic cells broadly distributed in FW rosettes (asterisks, main figure) as well as in a solid nest (inset). Note the presence of immunoreaction to anti-CYT antibody in the apical cytoplasm of elongate cells (arrows) in FW rosettes. Bar = 50  $\mu\text{m}$ . Anti-CYT immunohistochemistry. Fig. 9. A granular cytoplasmic staining with the antibody against neuropeptide Y (NPY) in tumour cells. Bar = 20  $\mu\text{m}$ . Anti-NPY immunohistochemistry. Fig. 10. Nuclear immunostaining with anti-proliferating cell nuclear antigen (PCNA) antibody in some neoplastic cells (arrows) localised in FW rosettes. Bar = 50  $\mu\text{m}$ . Inset: A mitotic cell in metaphase exhibiting cytoplasmic PCNA immunoreactivity. Bar = 5  $\mu\text{m}$ . Anti-PCNA immunohistochemistry

chemosensory signals that are conducted through the olfactory nerve to the olfactory bulb. Solitary chemosensory cells, that are not part of the olfactory system, show a similar morphology to receptor cells, but the axon is absent. Other cell types founded in the olfactory epithelium are supporting cells, basal cells, goblet cells, and ciliated non-sensory cells (Hansen et al. 1999).

As yet, in spite of the extensive literature on ONB in humans (for a recent review see Faragalla & Weinreb 2009), the only other animals for which this tumour has been described are rodents (Herrold & Dunham 1963, Vollrath et al. 1986), cats (Cox & Powers 1989, Schrenzel et al. 1990, Parker et al. 2010), dogs (Hara et al. 2002, Ueno et al. 2007), horses (Döpke et al. 2005, Yamate et al. 2006), a cow (Anderson & Cordy 1981), a monkey (Correa et al. 1975), a blue-tongued skink (Zwart et al. 2002), an axolotl (Brunst & Roque 1967), and also a few fish species (Ishikawa et al. 1978, Torikata et al. 1989).

The histopathological features of the tumour observed in this study, such as a fibrovascular stroma associated with FW rosettes, indicate a presumptive

diagnosis of ONB. Rosettes are not pathognomonic for neuroblastomas since they can be present in other neoplasms, such as neuroendocrine carcinomas, which also occur in the olfactory region. However, in some species such as cats, the presence of prominent rosettes has been considered to be a useful indicator of ONB (Wilson & Dungworth 2002). In addition, the close association between rosettes and myelinated nerve fibres demonstrated by Tolivia staining strongly suggests that the origin of these neoplastic structures might be the olfactory epithelium.

In accordance with the histological classification for ONB proposed by Hyams (1988), the mass described in this study was evaluated as grade II or III, since it showed some morphological features of both categories, i.e. high frequency of FW rosettes, occurrence of discrete areas of necrosis and prominent degree of nuclear atypia, as well as lobular growth pattern, presence of fibrovascular stroma and low mitotic activity. The latter characteristic was compatible with the results obtained by PCNA immunohistochemistry, since only a few number of neoplastic cells were immunoreactive to this antibody indicating the low proliferative rate of the tumour. Notwithstanding, in another 2 cases of ONB in fish, a moderate to high number of mitosis was observed, especially in those zones of the neoplasm showing a great percentage of undifferentiated cells (Ishikawa et al. 1978, Torikata et al. 1989). The histology of ONB in *Cyprinus carpio* (Ishikawa et al. 1978) and *Oryzias latipes* (Torikata et al. 1989) was similar to that described in our study, mainly in the presence of FW rosettes, separated by a delicate fibrovascular stroma, which were associated with bundles of axons identified by TEM. On the other hand, the rosettes observed in ONB of *Carassius auratus* and *O. latipes* were devoid of cilia, in contrast to that reported for *C. carpio* (Ishikawa et al. 1978). These authors stated that ONB in cold-blooded species are well-differentiated tumours and are characterised by the presence of cilia in rosettes, whereas in mammals the absence of this cell specialisation is also constant (Ishikawa et al. 1978). However, this may be a diagnostic oversimplification since cilia are present in some cases of ONB in mammals (Takahashi et al. 1987, Cox & Powers 1989), and the ONB in *C. auratus* described in this study and that in *O. latipes* described by Torikata et al. (1989) lacked these structures. In contrast to Torikata et al. (1989), we failed to identify Grimelius-positive granules in tumour cells in the goldfish ONB.

Although ONB cells can be positive for several neural and glial immunomarkers, the immunohistochemical profile reported for ONB differs among species (Cox & Powers 1989, Hara et al. 2002, Döpke et al. 2005, Yamate et al. 2006, Lee & Kim 2007, Ueno et

al. 2007). Even within the same species, ONB does not reveal a consistent pattern useful for diagnosis (Wilson & Dungworth 2002).

Among the neural markers employed in this study, only nNOS and S100 showed cross-reaction with goldfish tissues. nNOS immunostaining was observed in the neoplastic cells located at both FW rosettes and solid nests, with a stronger reaction at the latter site. In addition, ciliated olfactory neurons of the normal epithelium were immunoreactive to nNOS. Previous reports have also described nNOS immunoreactivity in receptor neurons from olfactory epithelium in *Oreochromis mossambicus* (Peters) (Singru et al. 2003) and *Clarias batrachus* (L.) (Gaikwad et al. 2009). Thus, immunoreactivity of the goldfish ONB cells with this antibody is plausible. Due to the scarcity of specific antibodies against fish antigens, nNOS antibody could be of great interest, at least for *Carassius auratus* and the above-mentioned species, as a diagnostic tool for tumours suspected to be of neural origin.

Some neoplastic cells of solid nests and mainly those in FW rosettes of the goldfish ONB also exhibited S100 immunoreactivity. This has previously been reported for ONB in a dog (Ueno et al. 2007), cats (Cox & Powers 1989), horses (Döpke et al. 2005, Yamate et al. 2006), humans (Fragalla & Weinreb 2009) and domestic medaka (Torikata et al. 1989). Although immunoreactivity against anti-S100 antisera was a common feature in both the ONB reported in *Oryzias latipes* and *Carassius auratus*, the number of positive cells was lower and the intensity of immunoreaction was higher in *O. latipes* (Torikata et al. 1989). Moreover, S100 antibodies immunolabelled crypt neurons of normal olfactory epithelium in control sections of goldfish, as has also been observed for other fish such as *Danio rerio* (Hamilton) (Germana et al. 2004, 2007) and *Poecilia reticulata* Peters (Bettini et al. 2009). Recently, another 2 types of tumour reported in goldfish also showed immunoreaction to anti-S100 antibodies: schwannomas (Marino et al. 2007) and a presumptive medulloepithelioma (Bartlett et al. 2010). However, the histopathology of ONB is distinctive and allows differentiation from these neoplasms. The main morphological features of ONB in goldfish were the presence of cuboidal or columnar eosinophilic cells arranged in solid nests and FW rosettes closely associated with myelinated fibres in the fibrovascular stroma, as was corroborated by Tolivia staining. Although medulloepitheliomas in mammals usually exhibit FW rosettes (Dubielzig 2002, Vajaranant et al. 2005, Schoeniger et al. 2006), in the case report documented for *C. auratus*, only Homer-Wright rosettes were occasionally observed (Bartlett et al. 2010). On the other hand, the schwannomas described in goldfish were composed of elongated pale cells densely packed and organised in

parallel ranks with distinctive palisading of their nuclei (Marino et al. 2007).

NPY and CYT antibodies strongly labelled neoplastic cells of the goldfish ONB. Although NPY has not previously been reported as an ONB marker, some studies have indicated the presence of anti-NPY immunoreactivity in olfactory receptor neurons in fish (Gaikwad et al. 2004) and in olfactory microvillar cells in mammals (Montani et al. 2006), as was observed in the goldfish control sections. The ability of NPY to stimulate neuroproliferation of olfactory neuronal precursor cells demonstrated in rats (Hansel et al. 2001) could explain the high degree of NPY immunolabelling in tumour cells of the goldfish ONB.

The anti-CYT immunoreactivity in neoplastic cells of ONB reported in other studies varies greatly. Although ONB are negative for CYT in some reports (Döpke et al. 2005, Yamate et al. 2006, Lee & Kim 2007), CYT-positive cells have been described in some canine, feline and human cases (Takahashi et al. 1987, Koestner & Higgins 2002). In addition, embryonic neurons arising from the olfactory placode can be labelled with anti-CYT antibodies (Okabe et al. 1997). Therefore, it should be considered that cytokeratin may be expressed in neuroblastomas, and the identification of cytokeratin should not be regarded as evidence to ruling out neuroblastic differentiation in craniofacial neoplasms (Okabe et al. 1997).

The differential diagnosis of ONB should exclude other neoplasms found in the olfactory chamber, such as sinonasal undifferentiated carcinomas (SNUC), amelanotic melanomas, lymphoid tumours and neuroendocrine carcinomas (NEC) (Wilson & Dungworth 2002, Thompson 2009), although none of these entities have so far been reported in fish at this location. The existence of FW rosettes in the neoplasm described in our study rules out SNUC and lymphoid tumours, since neither type of neoplasm shows these structures (Thompson 2009). In addition, lymphoid tumours are negative for anti-CYT and anti-S100 antibodies (Thompson 2009), in contrast to the immunophenotype of the goldfish ONB. Immunohistochemistry also aids in the distinction between ONB in goldfish and SNUC because, even though both neoplasms are positive for anti-CYT staining, SNUC lacks S100 immunoreactivity (Mills 2002). On the contrary, amelanotic melanomas are positive for S100 but do not react with anti-CYT antibodies (Yu et al. 2005, Thompson 2009), which immunohistochemically differentiates them from the goldfish ONB. Among all the neoplasms mentioned above, NEC has the greatest overlap in the immunohistochemical profile in comparison with ONB, at least with the antibodies employed in our study, since both tumours are positive for anti-CYT and anti-S100 antibodies (Koestner

& Higgins 2002, Wilson & Dungworth 2002, Thompson 2009). However, no immunoreactivity to antibodies that react with NEC, such as L-ENK or VIP (Wilson & Dungworth 2002), was observed in the goldfish tumour. Finally, although NEC might also present rosette formation, the identification of myelinated nerve fibres associated with the FW rosettes in the neoplasm described in *Carassius auratus* exclude the possibility of SNUC, amelanotic melanomas, lymphoid tumours or NEC, since none of these neoplasms show a neurofibrillary stroma (Thompson 2009).

In conclusion, this is the first ONB described in *Carassius auratus*. This diagnosis is supported by (1) the histopathological features of the tumour, (2) the large amount of myelinated fibres associated with rosettes, and (3) the anti-nNOS, anti-S100, anti-NPY, and anti-CYT immunoreactivity observed both in neoplastic cells and normal olfactory epithelium. This sustains the hypothesis that the ONB reported in *C. auratus* arose from the olfactory epithelium.

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