Parasitology

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The impact of *Anguillicoloides crassus* (Nematoda) on European eel swimbladder: histopathology and relationship between neuroendocrine and immune cells

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Research Article

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Abstract

The swimbladder functions as a hydrostatic organ in most bony fishes, including the European eel, *Anguilla anguilla*. Infection by the nematode *Anguillicoloides crassus* impairs swimbladder function, significantly compromising the success of the eel spawning migration. Swimbladders from 32 yellow eels taken from Lake Trasimeno (Central Italy) were analysed by histopathology- and electron microscopy-based techniques. Sixteen eels (50%) harboured *A. crassus* in their swimbladders and intensity of infection ranged from 2 to 17 adult nematodes per organ (6.9 ± 1.6, mean ± S.E.). Gross observations of heavily infected swimbladders showed opacity and histological analysis found a papillose aspect to the mucosa and hyperplasia of the *lamina propria*, *muscularis mucosae* and *submucosa*. Inflammation, haemorrhages, dilation of blood vessels and epithelial erosion were common in infected swimbladders. In the epithelium of parasitized swimbladders, many empty spaces and lack of apical junctional complexes were frequent among the gas gland cells. In heavily infected swimbladders, we observed hyperplasia, cellular swelling and abundant vacuolization in the apical portion of the gas gland cells. Numerous mast cells and several macrophage aggregates were noticed in the mucosal layer of infected swimbladders. We found more nervous and endocrine elements immunoreactive to a panel of six rabbit polyclonal antibodies in infected swimbladders compared to uninfected.

Introduction

The European eel *Anguilla anguilla* is an economically important species in Europe and globally (De Charleroy *et al*., 1990; Dekker, 2003). During the past three decades, the European eel has declined drastically throughout its distribution (Dekker, 2003). Habitat loss, climatic and oceanic changes, pollution, mortality due to river obstacles, overexploitation and parasites have been suggested as possible reasons for the decline (Dekker, 2003; Kirk, 2003; Vogel, 2010; Pelster, 2015; Frisch *et al*., 2016).

European eel reproduction depends on a spawning migration of 5000–7000 km from the European coast to the Sargasso Sea in about 5 months. This long-distance journey causes great physiological stress on the eels including the swimbladder, a hydrostatic organ that provides neutral buoyancy (Sjöberg *et al*., 2009; Pelster, 2015). Thus, any damage to swimbladder function could potentially interfere with the eel's ability to migrate and reproduce.

The nematode *Anguillicoloides crassus* (Kuwahara *et al*., 1974) parasitizes the swimbladder of at least five eel species in addition to the European eel (Pratt *et al*., 2019) and is the most extensively studied parasite of anguillid eels (Lefebvre *et al*., 2012). This nematode was accidentally introduced to Europe at the beginning of the 1980s by the importation of infected Japanese eels to Germany (Køie, 1991). Transportation of eels for the global aquaculture trade has spread *A. crassus* throughout the world (ICES, 2010).

Numerous authors have suggested that the nematode *A. crassus* impairs swimbladder function and significantly compromises the success of the eels' spawning migration (Kirk, 2003; Pelster, 2015). Known pathological changes in eel swimbladder induced by *A. crassus* include lesions, haemorrhaging, infiltration of inflammatory cells, dilation of blood vessels, fibrosis, which increases the thickness of the swimbladder wall, the presence of granulocytes and macrophages around encysted parasite larvae, changes in epithelial cells and alteration of gas composition (Haenen *et al*., 1989, 1994; Van Banning and Haenen, 1990; Molnár *et al*., 1993; Molnár, 1994; Molnár and Szèkely, 1995; Würtz and Taraschewski, 2000; Knopf, 2006; Lefebvre *et al*., 2011, 2012). Others have reported on the effects of *A. crassus* on the cellular and humoral immune response in *A. anguilla* swimbladder (Knopf *et al*., 2000; Knopf, 2006; Terech-Majewska *et al*., 2015) and on the physiological status of *A. anguilla* swimbladder in the presence of the nematode (Kelly *et al*., 2000; Sures *et al*., 2001). The relationship between eel swimbladder and *A. crassus* infection has also been investigated by radiodiagnostic

Table 1. Rabbit polyclonal antibodies used in the present study, their source, dilution and results in eel swimbladders uninfected and infected with *Anguillicoloides crassus*

			Intramural nervous elements		Epithelial endocrine cells	
Antibody anti-	Source and code	Dilution	UN	INF	UN	INF
CGRP	Peninsula Labs., [Belmont, CA, USA], T4239	1:100	+	+++	–	–
NPY	Peninsula Labs., [Belmont, CA, USA], IHC7180	1:100	–	–	–	+++
nNOS	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, sc.648	1:50	+	+++	–	–
5-HT	Chemicon [MerckMillipore, Damstadt, D], AB938	1:100	–	++	–	+++
SP	Peninsula Labs., [Belmont, CA, USA], T4170	1:200	–	–	–	–
VIP	Peninsula Labs., [Belmont, CA, USA], T4246	1:500	+	+++	–	–

CGRP, calcitonin gene-related peptide; NPY, neuropeptide Y; nNOS, neuronal-nitric oxide synthase; 5-HT, 5-hydroxytriptamine (serotonin); SP, substance P; VIP, vasoactive intestinal peptide; UN, uninfected; INF, infected.
Frequencies of endocrine cells and nerve cell bodies and fibres immunoreactive to the indicated antibodies were relatively quantified by the symbols: +++: high presence, ++: medium presence, +: low presence, and –: no presence.

methods (Beregi *et al.*, 1998; Székely *et al.*, 2005), X-ray computerized tomography (Székely *et al.*, 2004) and ultrasound scanning techniques (Frisch *et al.*, 2016).

In addition to pathological changes caused by parasitization, the presence of *A. crassus* has been associated with a reduction in the distribution of oxygen to the swimbladder and changes in the mechanism of gas deposition, impeding swimbladder function (Würtz *et al.*, 1996; Nimeth *et al.*, 2000; Pelster, 2015). Consequently, the nematode affects the vertical movement of its eel host and directly stresses the eel throughout migration, which could reduce the number of eels that reach the Sargasso Sea to spawn (Sprengel and Luchtenberg, 1991; Kirk, 2003; Palstra *et al.*, 2007; Barry *et al.*, 2014; Pelster, 2015). While parasitization by *A. crassus* is thought to be one reason for the decline of *A. anguilla* populations (Pelster, 2015), the mechanisms underlying the collapse are still uncertain, with other factors also likely contributing (Dekker, 2003; Van Ginneken and Maes, 2005).

Helminths are extraordinarily successful parasites because they can modulate the host immune response (Maizels *et al.*, 2018). In fish, the epithelia of gills, skin and the digestive tract form a physical barrier providing the first line of defence against infection (Koshio, 2016; Wang *et al.*, 2019). When this mucosal barrier is disturbed, resident immune cells are activated and tissue-specific leucocytes are recruited from the blood (Shi and Pamer, 2011; Tafalla *et al.*, 2016; Salinas and Magadán, 2017; Dezfuli *et al.*, 2020). One of the most important sites of damage induced by adult and larval (L2) *A. crassus* is towards the inner-most layer of the swimbladder, which is formed by gas gland cells (Pelster, 1995; Maina, 2000). This report includes a comparison between gas gland cells in infected *vs* uninfected swimbladders.

In many fishes, macrophages (Mulero *et al.*, 2008; Grayfer *et al.*, 2018; Mosberian-Tanha *et al.*, 2018) and mast cells (MCs) (Buchmann, 2012; Secombes and Ellis, 2012; Galindo-Villegas *et al.*, 2016; Dezfuli *et al.*, 2020) are active in the immune response against parasites. Several authors have reported the occurrence of granulocytes and macrophages/macrophage aggregates (MAs) in eel swimbladders infected with *A. crassus* (Molnár *et al.*, 1993; Molnár, 1994; Molnár and Szèkely, 1995; Würtz and Taraschewski, 2000; Knopf, 2006) but no details have been provided previously about the type of granulocyte.

A set of extrinsic and intrinsic ganglia regulates the inflation/deflation of gas in the swimbladder, changing its volume in response to the fish's movements in the water column (Finney *et al.*, 2006; Robertson *et al.*, 2007; Nilsson, 2009). In addition to adrenergic and cholinergic pathways, other neurotransmitters have been documented in the nervous system components of the swimbladder, including met-enkephalin, substance P (SP), vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), neuronal nitric oxide synthase (nNOS) and serotonin (5-hydroxytryptamine, 5-HT) (Lundin and Holmgren, 1984, 1989; Lundin, 1991, 1999; Schwerte *et al.*, 1999; Finney *et al.*, 2006; Robertson *et al.*, 2007; Nilsson, 2009). This account represents the first immunohistochemistry survey on neuroendocrine components of eel swimbladders infected with *A. crassus*.

Materials and methods

A subpopulation of 32 *A. anguilla* with total length ranging from 34.5 to 63.6 cm (44.7 ± 1.1 cm, mean ± S.E.) and weighing from 60 to 500 g (162.1 ± 15. 2 g, mean ± S.E.) were collected in July 2019 in Lake Trasimeno (Central Italy, 43°9′11″N and 12°15′E) by professional fishermen of a local consortium using fyke nets. Trasimeno Lake is the largest lake in Italy, due to its shallowness. The eels were transferred alive to the consortium's facilities where they were euthanized with an overdose of MS222 (125 mg L^{-1}, tricaine methanesulfonate, Sandoz, Basel, Switzerland). Once euthanized, the spinal cords were severed before the fish were dissected ventrally. The alimentary canal was removed to allow for the removal of the intact swimbladder from the eel's body.

Each swimbladder was opened longitudinally and several 15 × 15 mm pieces were excised and fixed in 10% neutral buffered formalin for 24 h. Thereafter, the samples were dehydrated through an alcohol series and then paraffin wax-embedded using a Shandon Citadel 2000 tissue processor. Multiple 5 μM sections were taken from each tissue block, stained with Alcian Blue, or Haematoxylin and Eosin and/or Giemsa, and examined and photographed using a Nikon Microscope ECLIPSE 80i.

A panel of six rabbit polyclonal antibodies was chosen to study the neuroendocrine structures in swimbladders infected with *A. crassus* (Table 1). In our lab, we have frequently and successfully employed these antibodies to reveal nerve cell bodies and fibres (Dezfuli *et al.*, 2018) and endocrine cells in the gut of numerous fish species (Dezfuli *et al.*, 2000, 2002; Bosi *et al.*, 2005*a*, *b*). The immunohistochemical reactions were made on sections of uninfected and infected eel swimbladder as reported in our previous work (Dezfuli *et al.*, 2018). Negative controls were performed by incubating sections with: (a) phosphate-buffered saline instead of the primary antibody; or (b) preadsorbed antibody with its corresponding antigen as reported in Table 2. Sections were examined and photographed using a digital camera

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Table 2. Blocking peptides and amine used for preabsorption in the negative control reactions.

Blocking peptides and amine	Source and code	Concentration	Preabsorption
CGRP	Bachem AG [Bubendorf, CH], H4924	10 μg mL^{-1}	24 h at 4°C
NPY	Bachem AG [Bubendorf, CH], H6375	10 μg mL^{-1}	24 h at 4°C
nNOS	Santa Cruz Biotechnology, Inc. [Santa Cruz, CA, USA], sc648P	100 μg mL^{-1}	16–18 h at R. T.
5-HT	Sigma-Aldrich [St. Louis, MO, USA], H9523	100 μg mL^{-1}	16–18 h at R. T.
SP	Bachem AG [Bubendorf, CH], H1890	10 μg mL^{-1}	24 h at 4°C
VIP	Sigma-Aldrich [St. Louis, MO, USA], V3628	100 μg mL^{-1}	16-18 h at R. T.

CGRP, calcitonin gene-related peptide; NPY, neuropeptide Y; nNOS, neuronal nitric oxide synthase; 5-HT, 5-hydroxytriptamine (serotonin); SP, substance P; VIP, vasoactive intestinal peptide; R. T., room temperature.

(Olympus Camedia C-5060, 5.1 Mp) and image analysis software (DP-software, Olympus, Milan, Italy). Evaluation of the frequency of the immunoreactive nervous and endocrine elements was based on subjective estimates after the examination of three sections from different regions for each uninfected and parasitized swimbladder.

For transmission electron microscopy (TEM), numerous 7 × 7 mm pieces of infected and uninfected swimbladders were fixed and embedded according to the methods reported previously (Dezfuli *et al.*, 2018). Portions of 10 infected and five uninfected swimbladders were fixed in cold 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2.5 h and routinely processed for scanning electron microscopy (SEM). Observation was done with a Cambridge Stereoscan 360 at an acceleration voltage of 20 kV.

Results

Sixteen eels out of 32 (50%) harboured *A. crassus* in their swimbladder and the intensity of infection ranged from 2 to 17 adult nematodes per organ (6.9 ± 1.6, mean ± S.E.). In six heavily infected swimbladders, adults and over 200 L2 larvae co-occurred (see further). During autopsy, uninfected swimbladders had thin walls and appeared transparent with different regions of the organ easily recognizable (Fig. 1A). Swimbladders with adult *A. crassus* were darker and less transparent, with large nematodes visible through the organ's wall (Fig. 1B). In very heavily parasitized swimbladders (Fig. 1C), the organ was even darker and the surface in some parts was distended by large nematodes that occupied the entire luminal space of the organ (Fig. 1C). Upon excision of infected swimbladders, long (several millimetres), black worms inside the lumen were visible to the naked eye.

The eel swimbladder consists of four distinct layers (Fig. 2A), with wall architecture similar to the anterior intestine, from which the swimbladder is derived. We use the terminology suggested by Fänge (1953) for swimbladder layers. The innermost layer facing the swimbladder lumen is the mucosa; it has a simple cuboidal epithelium containing gas gland cells, which form folds and the *lamina propria*, rich in blood vessels. External to the mucosa is the *muscularis mucosae*. The third layer is the *lamina submucosa* with its loose network of connective elements, and the fourth layer is the *lamina serosa*, which forms the external covering of the swimbladder.

Observations of histological sections of uninfected (Fig. 2A) and infected (Fig. 2B) swimbladders revealed remarkable structural differences. In uninfected organs, the epithelium was very thin (Fig. 2A). In contrast, swimbladders harbouring *A. crassus* showed hyperplasia of the *lamina mucosa*, *submucosa* and *muscularis mucosae* (Figs 2B and C). One of the most evident changes was the proliferation of epithelial cells and swimbladder folds that assumed a papillose aspect (Figs 2B and C). Dilation of blood vessels in the mucosal layer was common, far from any nematodes

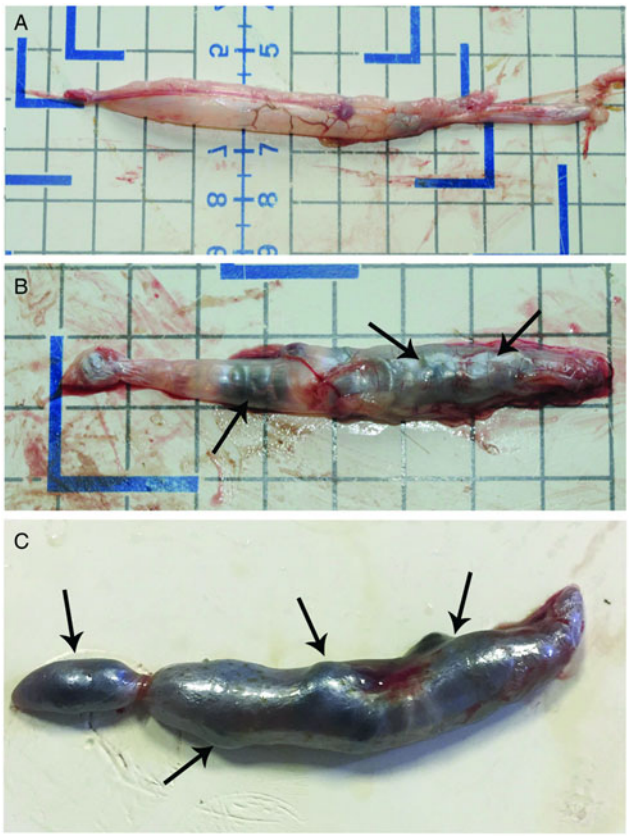

Fig. 1. Photos after necropsy of *Anguilla anguilla*. (A) Uninfected swimbladder, note transparency allowing one to see inside the organ. (B) Swimbladder with some pre-adult and adult *Anguillicoloides crassus*, worms (arrows) occupied most of the organ's lumen. (C) Very heavily infected swimbladder appeared very dark with a thick wall, large adult *A. crassus* occupied the entire luminal space and distended some parts of the organ surface (arrows).

(Fig. 2C) and near parasite eggs (Fig. 2B) and larvae (Fig. 2D). In sections of some infected swimbladders, L2 and/or L3 larvae were observed in the *lamina submucosa*; often the larva was surrounded by loose connective tissue and host mononuclear cells (Fig. 2C). The intestines of adult nematodes were frequently filled with host erythrocytes and the adult worms often occupied most of the luminal space of the swimbladder (Fig. 2E). The presence and feeding activity of numerous larvae induced epithelial erosion; the residue of gas gland cells was seen among the larvae (Fig. 2F).

The adult nematodes induced inflammation in the swimbladder; numerous MCs were seen scattered among the gas gland cells and in the *lamina propria* near the blood vessels (Fig. 3A). Frequently, in the *lamina propria*, MCs were in degranulation (Fig. 3B); indeed, these cells were found around encapsulated

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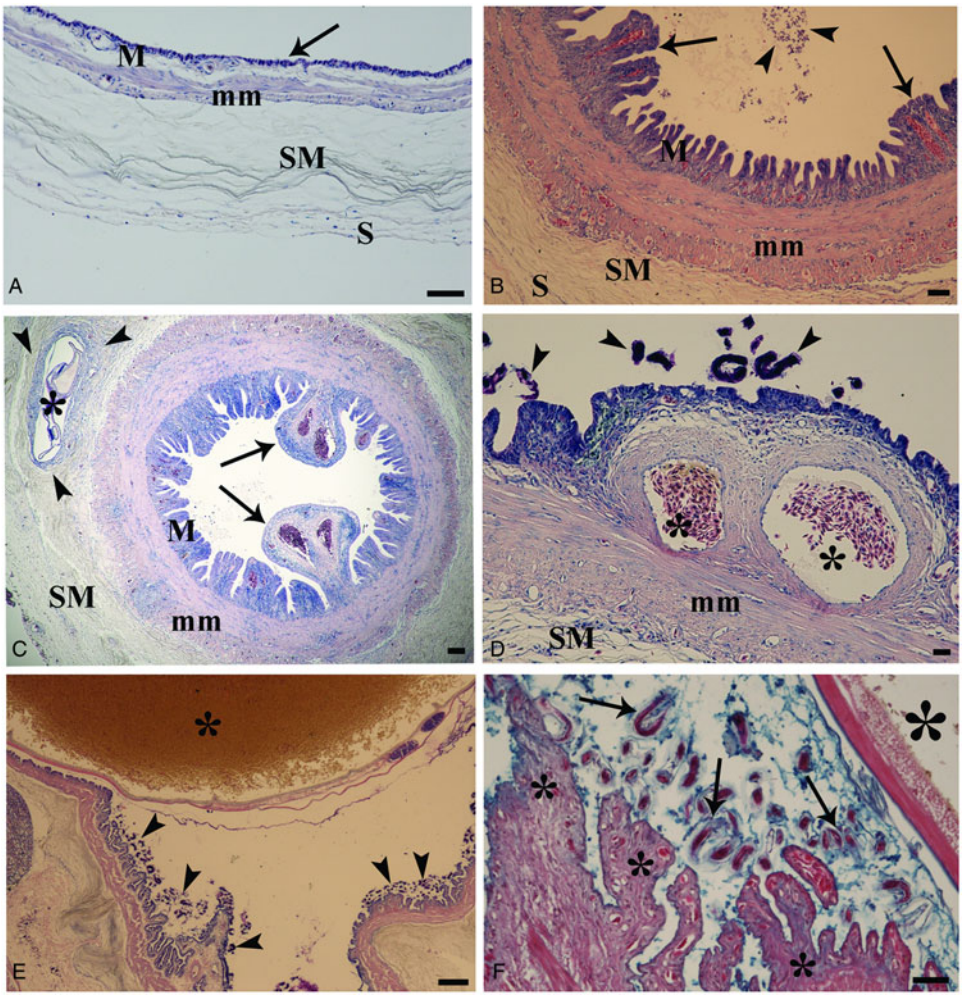

Fig. 2. Histological sections of *A. anguilla* swimbladder stained with Giemsa. (A) Sagittal section of uninfected swimbladder, note thin epithelium (arrow), M = *lamina mucosa*, mm = *muscularis mucosae*, SM = *lamina submucosa*, S = *lamina serosa*, Giemsa stain, scale bar = 50 μM. (B) Transverse section of heavily infected swimbladder, hyperplasia of the main layers is remarkable, papillose aspect of folds (arrows) and nematode eggs in the lumen are evident (arrowheads), M = *lamina mucosa*, mm = *muscularis mucosae*, SM = *lamina submucosa*, scale bar = 50 μM. (C) Infected swimbladder, abnormal dilation of blood vessels (arrows) visible far from nematode, an L3 (asterisk) in submucosa is surrounded with loose connective tissue (arrow heads), M = *lamina mucosa*, mm = *muscularis mucosae*, SM = *lamina submucosa*, scale bar = 100 μM. (D) Higher magnification of dilated vessels (asterisks) of mucosal layer, *A. crassus* larvae (arrow heads), mm = *muscularis mucosae*, SM = *lamina submucosa*, scale bar = 20 μM. (E) Intestine of an adult *A. crassus* is filled with eel erythrocytes (asterisk), numerous nematode larvae (arrow heads) are near the swimbladder epithelium, scale bar = 100 μM. (F) An adult nematode (big asterisk), feeding activity of numerous larvae (arrows) eroded epithelium of the swimbladder folds (small asterisks) and residues of gas gland cells among larvae are visible, Giemsa stain, scale bar = 50 μM.

larvae in the submucosal layer. MAs occurred among gas gland cells (Fig. 3C) and in the *lamina propria* close to the MCs and blood vessels (Fig. 3D). MAs contained azure-brownish pigments (Figs 3C and d) and in some cases were observed in the *lamina serosa*. In very heavily infected swimbladders, the supranuclear region of the gas gland cells was vacuolized (Figs 3B and D).

Sections of infected/uninfected swimbladders were observed also by SEM. Figure 4A shows a luminal space occupied by a single adult female. Often adult worms were accompanied by numerous L2 larvae attached to the epithelium and/or free in the lumen and surrounded by secretion and host erythrocytes (Fig. 4B). In infected swimbladder, dilation of the blood vessels increased the wall thickness (Figs 4C and D) which ranged from 290 to 392 μM (Fig. 4D). The walls of swimbladders not infected with *A. crassus* varied in thickness from 120 to 170 μM (Fig. 4E).

TEM revealed ultrastructural differences between infected and uninfected swimbladders. In organs with no *A. crassus*, gas gland cells were intact, cuboidal in shape, with the nucleus with euchromatin and heterochromatin apposed to the nuclear envelope, with short microvilli on the luminal side, well-developed basolateral labyrinths (Fig. 5A) and clear apical junctional complexes (Fig. 5B). Within the cytoplasm, very few electron-dense vesicles and some round-oval mitochondria with normal cristae (not shown) were observed. In lightly infected swimbladders, short microvilli persisted, translucid vesicles were frequent within the cytoplasm of gas gland cells, and empty spaces were common between the cells; nonetheless, basolateral labyrinths assumed a vertical position between cells (Fig. 5C). Moreover, the presence of MCs among gas gland cells and within the *lamina propria* was common (respectively Figs 5C and D). In heavily infected swimbladders, epithelial dysplasia was noticed, gas gland cells assumed a bubble-shaped aspect and their supranuclear region showed intense vacuolization (Figs 5E and F). In swimbladders with several worms, the nuclei changed shape, had more heterochromatin and some were necrotic, and the mitochondria were much more electron-dense with short undefined cristae (not shown). Between gas gland cells, abundant empty spaces and the lack of a distinct apical junctional complex were common and labyrinths moved to the top of the epithelium (Fig. 5F). Indeed, in heavily parasitized swimbladders, gas gland cell degeneration was remarkable and the MCs were scattered among the epithelial cells residue (Fig. 5F).

In uninfected swimbladders, the nervous components were not immunoreactive to antibodies to NPY, 5-HT and SP (Table 1,

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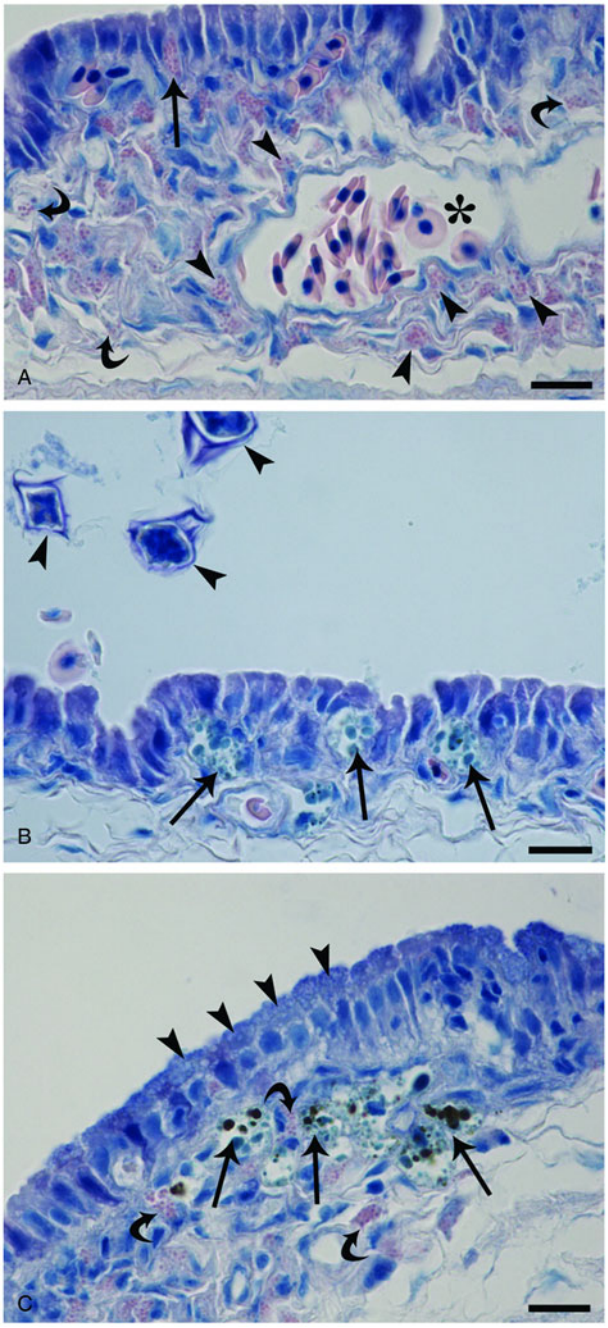

Fig. 3. Histological sections of *A. anguilla* swimbladder stained with Giemsa. (A) Arrow shows a mast cell among gas gland cells, numerous mast cells (arrow heads) around dilated blood vessel (asterisk), some mast cells are in degranulation (curved arrows), scale bar = 50 μM. (B) Occurrence of macrophage aggregates (arrows) among gas gland cells, sections of larvae of *A. crassus* (arrow heads) in the lumen, scale bar = 100 μM. (C) Macrophage aggregates in *lamina propria* (arrows) near mast cells (curved arrows), see vacuolization of apical part of gas gland cells (arrow heads), scale bar = 5 μM.

Figs 6A and b). In contrast, they showed a positive reaction to antibodies to calcitonin gene-related peptide (CGRP), nNOS and VIP (Table 1, Fig. 6C) in single small ganglia and slender nerve fibres interspersed in the *submucosa* (Fig. 6C). In infected organs, we observed large ganglia with several anti-CGRP immunoreactive neurons, and a high number of positive nerve fibres (Figs 6D and E). Several neurons and nerve bundles were immunoreactive to the antibody to NOS, both under the mucosa (Fig. 6F) and close to the blood vessels (Fig. 6G). Ganglia with anti-5-HT immunoreactive neurons were seen in the walls of infected swimbladders (Fig. 6H). Anti-VIP immunoreactive neurons in large ganglia and numerous nerve fibres were observed in the connective tissue of the *submucosa* layer (Fig. 6I) and close to the blood capillaries (Fig. 6J) of the swimbladder. In the epithelium of uninfected swimbladders, endocrine cells were not observed to react to any of the antibodies used (Table 1). Conversely, a high number of endocrine cells immunoreactive to the antibodies to 5-HT and NPY were observed in the epithelium of very heavily infected swimbladders (Table 1, Fig. 7).

Discussion

In this study, 50% of eels sampled from Lake Trasimeno were infected with the nematode *A. crassus*, with some swimbladders completely filled with an impressive number of large adults and a few hundred larvae. Under natural conditions, the presence of so many *A. crassus* larvae in the swimbladder could be a considerable stressor for eels (Sures *et al.*, 2001).

In eels, gas gland cells are responsible for initiating gas secretion and play a crucial role in the metabolism of swimbladder tissue (Pelster, 1995; Würtz *et al.*, 1996; Barry *et al.*, 2014). Thus, understanding the effects of parasites on these critical cells will contribute to our understanding of the subsequent effects on migration. Previously, Würtz and Taraschewski (2000) had provided the only ultrastructural survey of the effects of *A. crassus* on eel swimbladder gas gland cells. The results reported here support those earlier findings and further our understanding with several new observations, including vacuolization of the apical part of the gas gland cells and thus their disintegration, necrotic nuclei of some disintegrating cells, displacement of the basal labyrinth towards the top of the epithelium, the presence of many empty spaces among gas gland cells and a lack of defined apical junctional complexes. The combination of larval *A. crassus* feeding on tissue and inducing degeneration of the gas gland cells (Kirk, 2003, current study) and the presence of adult worms in the lumen reduce the gas-secreting capacity of gas gland cells and swimbladder wall elasticity (respectively Würtz *et al.*, 1996; Würtz and Taraschewski, 2000; Barry *et al.*, 2014).

Most previous reports on the cellular immune response in the eel swimbladder–*A. crassus* system dealt with the presence of granulocytes and macrophages around larvae in the wall of the stomach, intestine or swimbladder (Haenen *et al.*, 1989; Molnár *et al.*, 1993; Molnár, 1994; Würtz and Taraschewski, 2000; Knopf, 2006). Two very important components of the innate immune system are MAs and MCs; in this report, we have documented high numbers of the above components in the mucosal layer of heavily infected swimbladders. Therefore, below we will examine in turn MAs and MCs.

Phagocytosis is the primary defence mechanism of all metazoan organisms (Grayfer *et al.*, 2018). MAs are groups of pigmented phagocytes primarily found in kidney, spleen and liver; MAs are reported in more than 130 fish species and are active in immune defences as well as in normal physiological processes (Steinel and Bolnick, 2017). The intestine possesses the largest pool of MAs, which is essential for epithelial renewal (Bain and Mowat, 2014). The presence of MAs in infected eel swimbladders has been reported previously (Molnár, 1994; Würtz and Taraschewski, 2000; Knopf, 2006; Lefebvre *et al.*, 2012), but no insight was provided on a reason for their occurrence in this organ and Munoz *et al.* (2015) reported a decrease in the number of MAs in eel swimbladders harbouring *A. crassus*, compared with uninfected controls. In the current study, we saw numerous MAs in the mucosal layer of infected swimbladders, and one interesting finding of this study was the presence of intraepithelial MAs. In some cases, intraepithelial MAs occurred very close to nematode larvae and eggs adhered to the gas gland cell surface. It has been reported that turtle MAs are aggressive phagocytes, attacking bacteria, fungi and helminth eggs *in vitro* (Johnson

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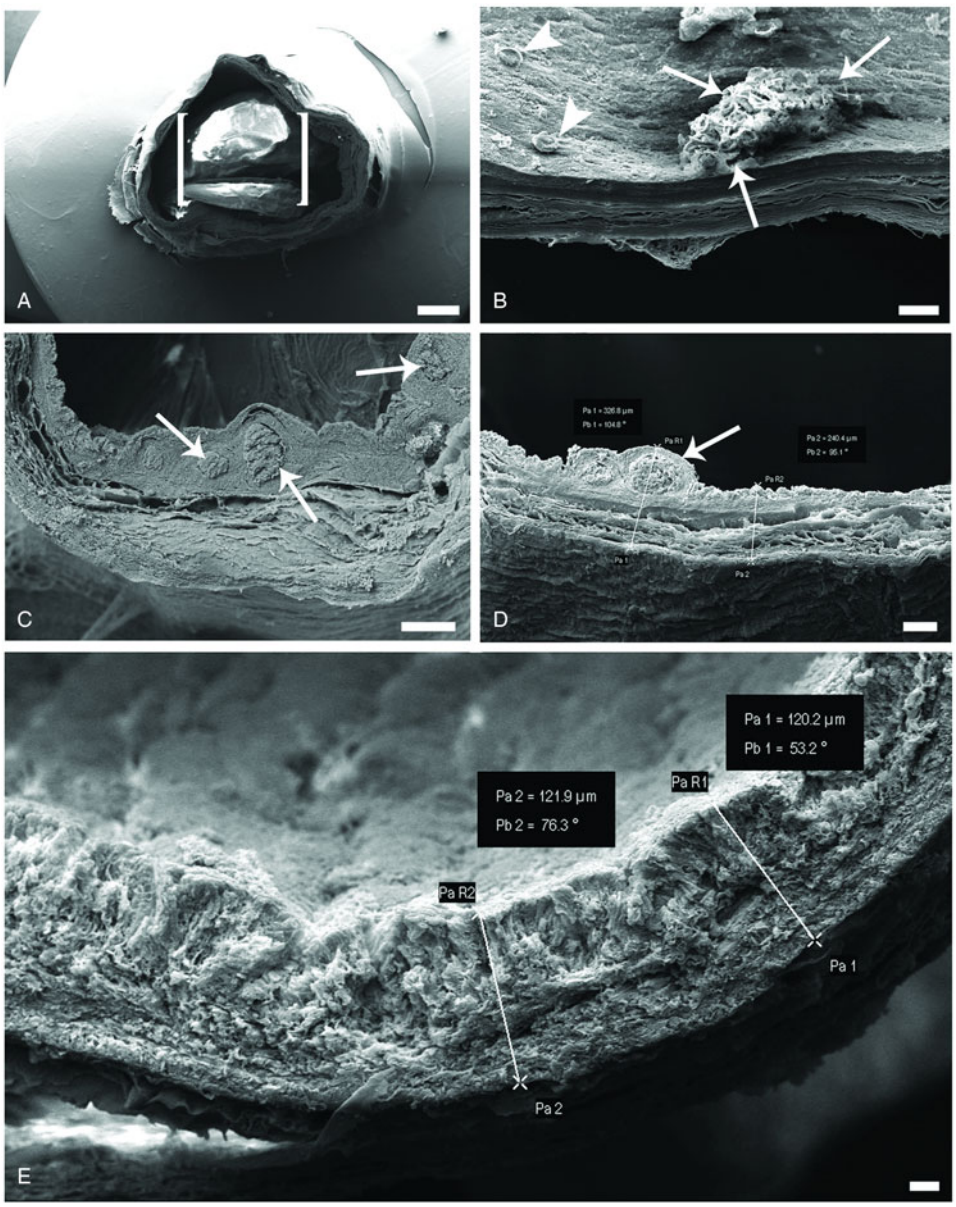

Fig. 4. Scanning electron micrographs of *A. anguilla* swimbladder. (A) Image of about 7 mm in length of infected swimbladder, one single adult *A. crassus* (parenthesis) occupied most luminal space of this portion, scale bar = 1 mm. (B) Some single larvae (arrow heads) and numerous larvae covered with secretion (arrows) are attached to the epithelium of swimbladder, scale bar = 100 μM. (C) Heavily infected swimbladder, image of dilated blood vessels (arrows), scale bar = 200 μM. (D) Micrograph shows piece of infected swimbladder, with dilated vessels (arrows) and increased thickness of swimbladder wall, scale bar = 100 μM. (E) Image of uninfected swimbladder, note thinner wall, scale bar = 20 μM.

et al., 1999). Recently in the intestine of *Chelon ramada*, a high number of intraepithelial macrophages engulfing spores of *Myxobolus mugchelo* (Myxozoa) was observed by Dezfuli *et al.* (2020). MAs regulate inflammatory response and protect mucosa against pathogens and scavenge dead cells and foreign debris (Estensoro *et al.*, 2014). Because feeding activity of larval and adult *A. crassus* damage epithelial cells of eel swimbladder, intraepithelial MAs might be necessary to phagocytose the residues of damaged gas gland cells.

In heavily infected eel swimbladders, numerous MCs were encountered in the mucosal layer, among gas gland cells and in *lamina propria* close to blood vessels. To regulate inflammation and coordinate an appropriate response against pathogens, in all vertebrates MCs are strategically placed at perivascular sites (Secombes and Ellis, 2012; Dezfuli *et al.*, 2018). By degranulation, MCs release their granules (Bosi *et al.*, 2018; Dezfuli *et al.*, 2020), which contain a wide spectrum of inflammatory and immunomodulatory mediators such as α-N-acetyl-galactosamine (Dezfuli *et al.*, 2015), piscidins (Salger *et al.*, 2017), histamine (Gomez Gonzalez *et al.*, 2017) and serotonin (Da Silva *et al.*, 2017). There is a dearth of reports on the co-occurrence of MCs and MAs in the epithelium of infected organs of fish. In this survey, we documented a high number of MCs and several MAs in the epithelium and in the rest of the mucosal layer of highly infected eel swimbladders. The same phenomenon was encountered in the intestines of *Silurus glanis* harbouring an acanthocephalan (Dezfuli *et al.*, 2017) and in the gut of mullet heavily infected with a myxozoan (Dezfuli *et al.*, 2020). Our previous and current results strongly favour the hypothesis that eels use these two types of immune cells to face the extensive attack by *A. crassus* on the swimbladder mucosal layer.

The autonomic nervous system regulates the inflation/deflation of gas in the swimbladder (Finney *et al.*, 2006; Robertson *et al.*, 2007; Nilsson, 2009; Dumbarton *et al.*, 2010). In uninfected swimbladders, nerve fibres were thin and few in number and intramural ganglia were small, consisting of one to three neurons, and they were sometimes negative and in several occasions immunoreactive to anti-CGRP, -VIP and -nNOS. Accounts on the positive reaction of the nerve cell bodies of the fish swimbladder to VIP and nNOS have been reported previously (Lundin and Holmgren, 1984, 1989; Lundin, 1991; Schwerte *et al.*, 1999; Finney *et al.*, 2006; Robertson *et al.*, 2007). The immunoreactivity to anti-CGRP antibody reported here is the first for nerve cell bodies and fibres in a fish swimbladder. In fish, CGRP is involved

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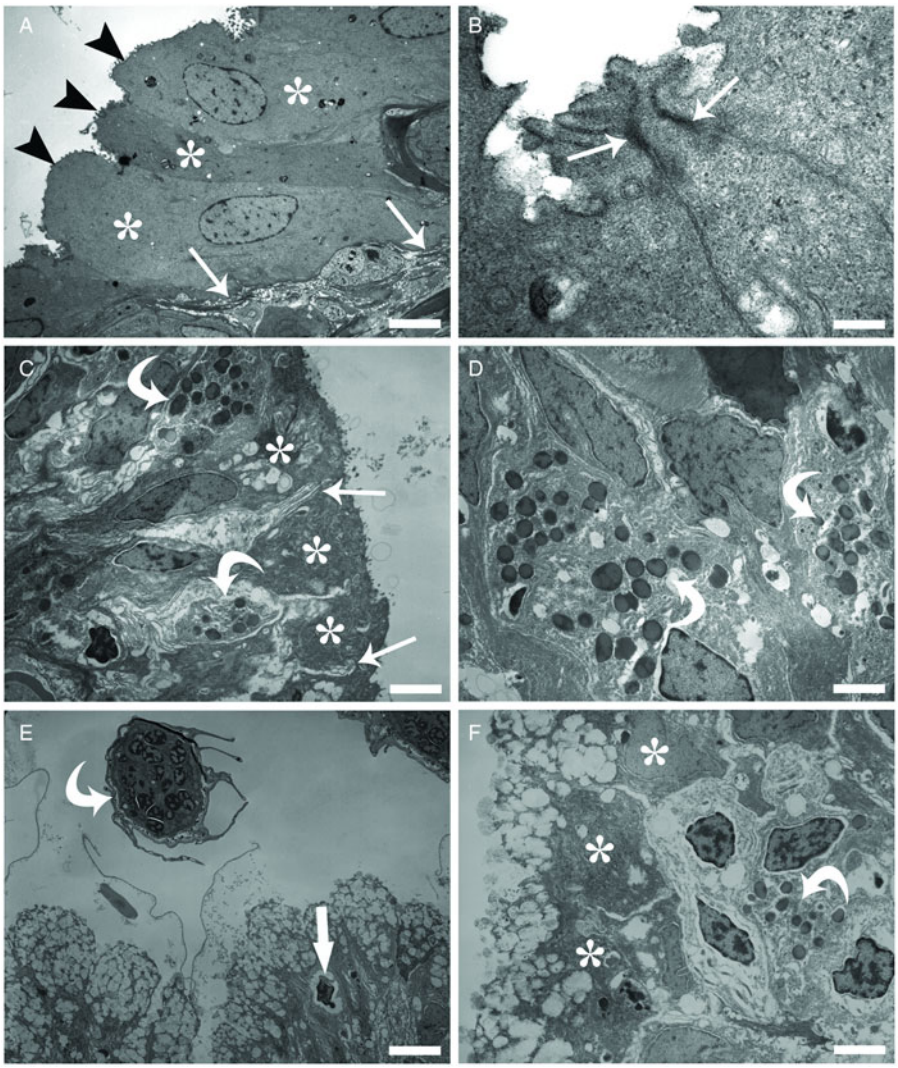

Fig. 5. Transmission electron microscopy micrographs of *A. anguilla* swimbladder. (A) Uninfected swimbladder, note normal aspect of three gas gland cells (asterisks), short microvilli (arrow heads) and lack of empty spaces between cells, arrows show basolateral labyrinths, scale bar = 3 μm. (B) Uninfected swimbladder, high magnification of apical junctional complex (arrows) between adjacent gas gland cells, scale bar = 0.2 μm. (C) Slightly infected swimbladder, between gas gland cells (asterisks), empty spaces and translucid vesicles in cytoplasm are visible, displacement of basolateral labyrinths (arrows) in vertical position and two mast cells (curved arrows) among gas gland cells are evident, scale bar = 2.6 μm. (D) Occurrence of mast cells with electron-dense granules within the *lamina propria*, degranulation (curved arrows) is visible, scale bar = 2 μm. (E) Epithelium of heavily infected swimbladder, note intense vacuolization-degeneration of apical part of gas gland cells, necrotic nucleus (arrow) and *A. crassus* larva (curved arrow) in swimbladder lumen, scale bar = 5.2 μm. (F) Epithelium of heavily infected swimbladder, gas gland cells (asterisks) vacuolization-degeneration, absence of distinct apical junctional complex and basolateral labyrinths are visible, a mast cell (curved arrow) among the residue of gas gland cells, scale bar = 2.7 μm.

in the control of gut peristalsis, by an inhibitory effect on the intestinal smooth muscle (Ceccotti *et al.*, 2018), in vasodilatation (Shahbazi *et al.*, 2009) and in the regulation of body fluid homoeostasis. Several studies have shown the immunoreactivity of the nervous components of the fish swimbladder to NPY, 5-HT and SP as neurotransmitters (Lundin and Holmgren, 1984, 1989; Lundin, 1991; Finney *et al.*, 2006; Robertson *et al.*, 2007; Pereira *et al.*, 2017). We failed to find these neuromodulators in neurons of the uninfected swimbladders. Regarding the absence of NPY in swimbladders of adult zebrafish, Robertson *et al.* (2007) stated that, '….cells noted in this study had either disappeared by adulthood, or that they continued to be present but did not contain detectable levels of NPY'. Thus, the above suggestion could be true also for uninfected eel swimbladders, that is, probably in the absence of the parasite, the eel swimbladder does not produce a detectable quantity of NPY.

In infected swimbladders, large ganglia were noticed in the submucosa and close to smooth muscle fibres, with neurons immunoreactive to anti-CGRP, -nNOS, -VIP and -5-HT antibodies. The network of nerve fibres immunoreactive to these antibodies was dense, especially in layers rich with blood vessels. The increase in the number of nNOS-immunoreactive neurons was related to the synthesis of nitric oxide, and it was reported as a strong vasodilator of the blood vessels in the eel swimbladder (Schwerte *et al.*, 1999). With regard to VIP, it has a vasodilatory effect, increasing the flow through the gas gland cells (Lundin and Holmgren, 1984; Schwerte *et al.*, 1999; Finney *et al.*, 2006) and thus increasing the swimbladder volume. Moreover, VIP causes the relaxation of smooth muscle in swimbladders of eel and zebrafish (respectively, Lundin, 1991; Finney *et al.*, 2006), whereas 5-HT activated smooth muscle contraction in the eel swimbladder (Lundin, 1991). Herein, in swimbladders harbouring *A. crassus*, changes to the pattern of intramural nervous system with contrasting signals were reported for the first time. Based on the known roles of these compounds in swimbladders of eels and other bony fish, we hypothesize that the observed differences are attributable to the occurrence of *A. crassus*.

In eel swimbladders harbouring several adults and numerous larvae of *A. crassus*, many endocrine cells immunoreactive to anti-NPY and -5-HT antibodies were observed within the epithelium. In contrast, no endocrine cells positive to the above antibodies were observed in the epithelium of swimbladders with very few adults and with no larvae in the lumen. About 90% of the body's 5-HT is produced by enterochromaffin cells in the gut, which reveals the importance of this amine in gut function (Wang *et al.*, 2018). Lundin and Holmgren (1989) reported the expression of 5-HT in the swimbladder epithelial cells of four fish species. Serotonin is one of the most important mediator molecules in inflammation caused by helminths (Dezfuli *et al.*, 2000, 2002; Bosi *et al.*, 2005*a*; Wang *et al.*, 2018). Recently, Gonzáles-Stegmaier *et al.* (2017) have demonstrated the pro-inflammatory effects of NPY in the SHK-1 cell line and head kidney of the Atlantic salmon *Salmo salar*. In heavily infected swimbladder of eels, the occurrence of numerous positive

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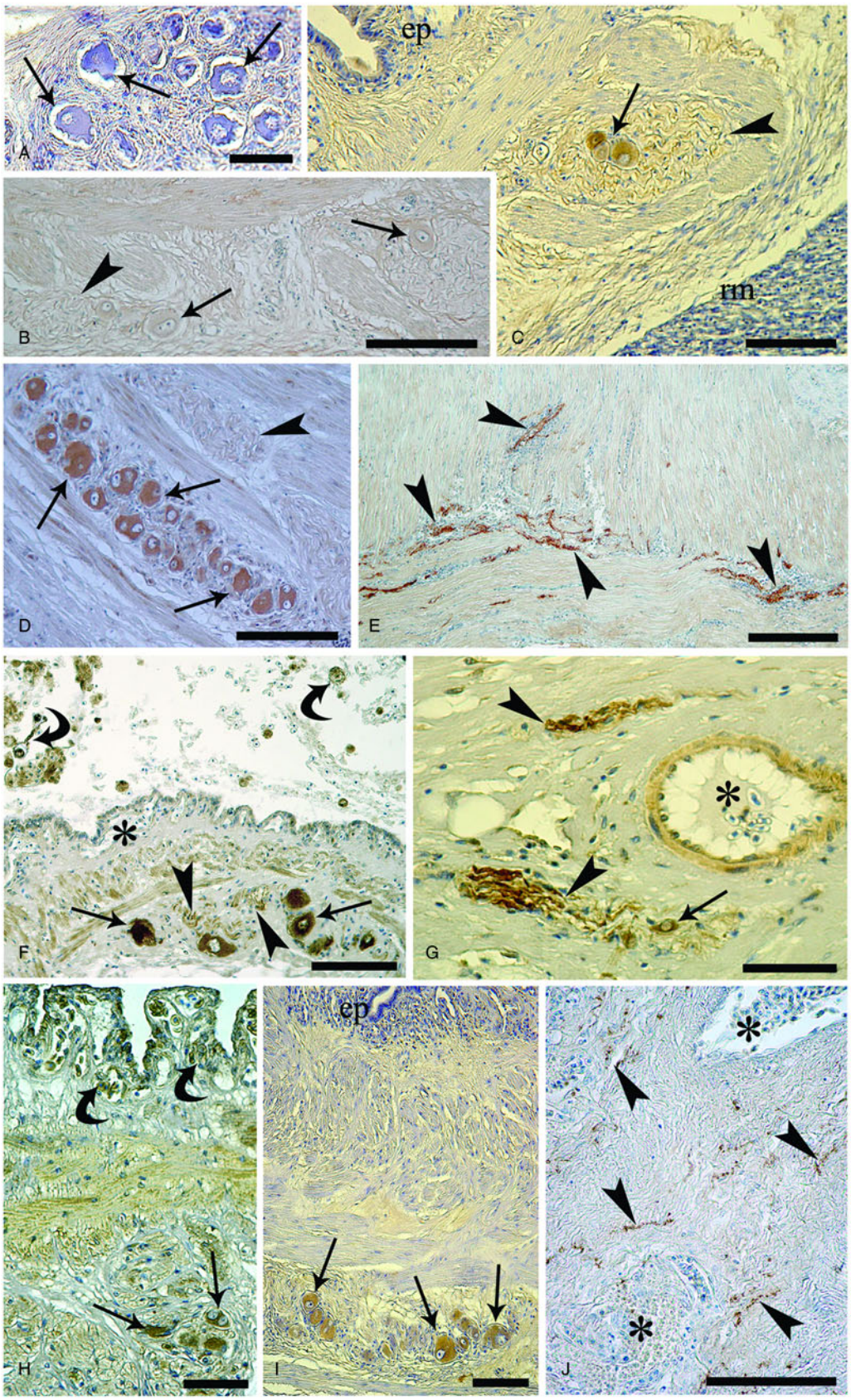

Fig. 6. Nervous components of the eel swimbladder uninfected (A–C) and infected with *Anguillicoloides crassus* (D–J). (A) A ganglion with no immunoreactive neurons (arrows) to the anti-5-HT. Scale bar = 50 μm. (B) Neurons (arrows) and nerve fibres (arrowhead) with negative immunoreactivity to the anti-NPY. Scale bar = 100 μm. (C) A little ganglion with three neurons (arrow) positive to the anti-VIP surrounded by unreactive nervous fibres (arrowhead); ep, epithelium; rm, *rete mirabile*. Scale bar = 100 μm. (D) A large ganglion with several neurons (arrows) immunoreactive to anti-CGRP; arrowhead shows a negative bundle of nervous fibres. Scale bar = 100 μm. (E) Region of the swimbladder wall with a high number of anti-CGRP immunoreactive nervous fibres (arrowheads). Scale bar = 100 μm. (F) A group of neurons (arrows) and nervous fibres (arrowhead) immunoreactive to anti-n-NOS; the oedematous epithelium shows numerous blood capillaries (asterisk); parasite larvae (curved arrows) are in the lumen with tissue debris and erythrocytes. Scale bar = 100 μm. (G) Two bundles of nerve fibres (arrowheads) and a little neuron (arrow) immunoeactive to anti-n-NOS close to a blood vessel (asterisk). Scale bar = 50 μm. (H) A ganglion with neurons (arrows) immunoreactive to anti-5-HT; curved arrows show blood capillaries in the epithelium. Scale bar = 50 μm. (I) Several neurons (arrows) immunoreactive to anti-VIP in a ganglion within the submucosal layer of the swimbladder wall, epithelium (ep). Scale bar = 100 μm. (J) Many nerve fibres immunoreactive to anti-VIP (arrowheads) close to the blood vessels (asterisks). Scale bar = 100 μm.

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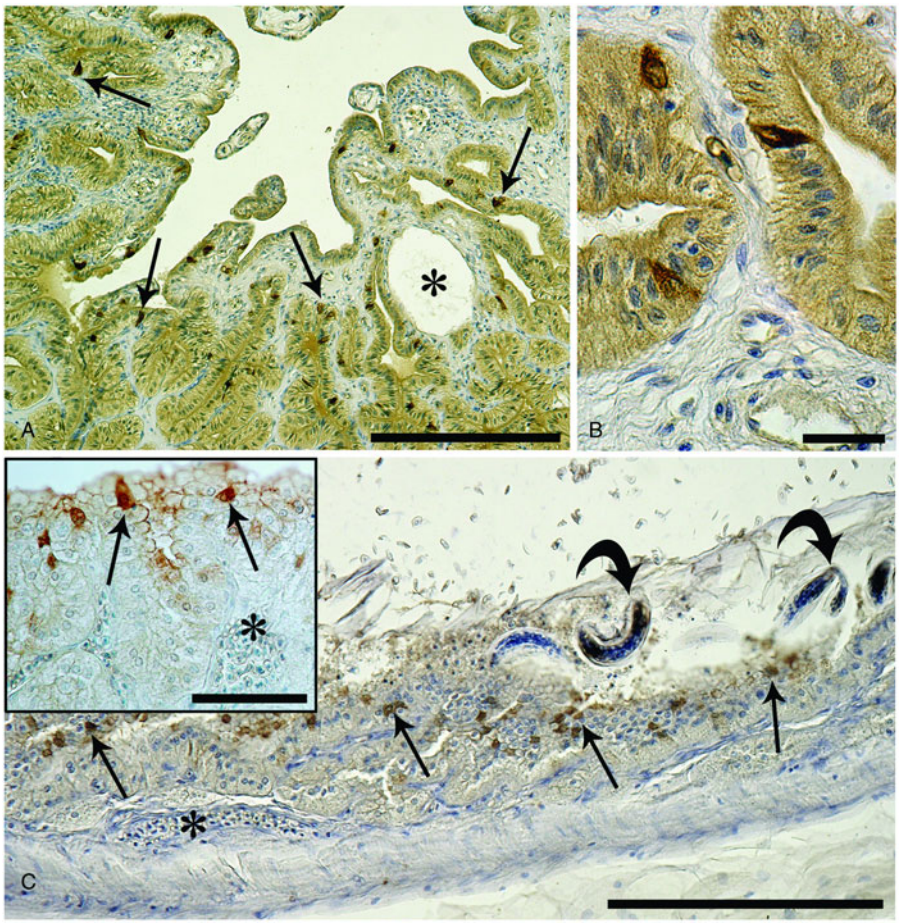

Fig. 7. Endocrine cells belonging to the diffuse endocrine system in the epithelium of the eel swimbladder infected with *Anguillicoloides crassus*. (A) Several endocrine cells immunoreactive to anti-5-HT (arrows); asterisk indicates a dilated blood vessel. Scale bar = 200 μM. (B) High magnification shows three endocrine cells positive to anti-5-HT. Scale bar = 20 μM. (C) Numerous endocrine cells (arrows) immunoreactive to anti-NPY; note erosion of *lamina mucosa* due to action of parasite larvae (curved arrows); asterisk indicates a blood vessel. Scale bar = 200 μM. The inset shows a high magnification of the immunoreactive endocrine cells. Scale bar = 50 μM.

cells to anti-NPY and -5-HT antibodies might suggest their involvement in inflammatory response against helminths, as they are active in immune cells recruitment and host local defence (Bosi *et al.*, 2005*a*; Gonzáles-Stegmaier *et al.*, 2017; Dezfuli *et al.*, 2018; Wang *et al.*, 2018).

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Conflict of interest. None.

Ethical standards. This study was conducted in accordance with the national law (D.L. 4 March 2014, n. 26) and was approved by the Ferrara University Ethical Committee (TLX n. 2/2018).

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