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# Gastric lesions in dolphins stranded along the Eastern Adriatic coast

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ABSTRACT: Stranded cetaceans are often found with gastric lesions associated with the presence of parasites; most frequently, nematodes of the genus *Anisakis* and the heterophyd digenean trematode *Pholeter gastrophilus*. In this study, we present histopathology mainly (but not exclusively) related to these 2 parasite species. Macroscopically, lesions associated with the presence of *Anisakis* spp. were characterised by the presence of ulcers within the gastric mucosa, while the digenean *P. gastrophilus* was found within large submucosal fibrotic nodules in the gastric wall. *Anisakis*-induced alterations included severe ulcerative gastritis with mixed inflammatory infiltrate often associated with colonies of bacteria, and mild to moderate granulomatous gastritis with eosinophilic infiltrate. *P. gastrophilus*-associated lesions were characterised by fibrogranulomatous gastritis with mixed inflammatory infiltrate. Additionally, immunohistochemical (IHC) analysis of *P. gastrophilus* lesions was consistent with the histopathologic findings, revealing inflammation-mediated stimulation. IHC-positive localisation of CD3+, iNOS+ and caspase-3+ cells suggests intensive accumulation of cytotoxic T-cells, proinflammatory cytokines and execution-phase of cell apoptosis at the parasitized area. In contrast, mechanical damage, rather than visible inflammatory response could be observed at the site of attachment of *Braunina cordiformis* recorded in 4 animals. Lesions not associated with the presence of parasites were mostly characterised by focal loss of superficial epithelial cells and accumulation of brown hemosiderin-like pigment or fibrous gastritis with lymphoplasmacytic infiltrate. In light of these results, we argue that observed 'tolerant' host–parasite interactions that led toward gastric lesions do not represent the cause of death and stranding of cetaceans included in this study.

KEY WORDS: Gastric lesions · Cetaceans · *Anisakis* spp. · *Pholeter gastrophilus* · *Braunina cordiformis* · Adriatic Sea



## INTRODUCTION

Toothed whales (Odontoceti) represent a suborder within the order of whales (Cetacea) that comprises 10 families and 75 recognized species, with the majority belonging to Delphinidae and Ziphiidae families (Rice 1998). Approximately 20 different cetacean species inhabit the Mediterranean Sea (UNEP/IUCN 1994), of which 8 species of toothed whales have been recorded in the Adriatic Sea (Gomerčić & Huber 1989). Among those, only the bottlenose dolphin *Tursiops truncatus* (Montagu, 1821) is a resident Adriatic species (Bearzi & Notarbartolo di Sciara 1995). Other observed species, such as striped dolphin *Stenella coeruleoalba* (Meyen, 1833), Risso's dolphin *Grampus griseus* (G. Cuvier, 1812), common dolphin *Delphinus delphis* Linnaeus, 1758 and Cuvier's beaked whale *Ziphius cavirostris* Cuvier, 1823,

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migrate from the Mediterranean Sea. Approximately 3% of total Mediterranean strandings of Cuvier's beaked whale occur in the southern Adriatic Sea, suggesting a potentially relevant habitat for this species (Holcer et al. 2007).

The biological diversity in feeding habits and digestive system structure of the suborder Odontoceti could contribute to specific gastrointestinal parasitic composition (Poulin et al. 2011, Benavides et al. 2012, Lafferty 2012). Firstly, their diet comprises a variety of fish and squid species, and secondly, they show compartmentalization of the stomach into a non-glandular forestomach, glandular main or fundic stomach, connecting chamber or channel that communicates between the main and pyloric chamber, and a pyloric stomach that differs among the Odontoceti families (Smith 1972, Mead 2002). Consequently, each gastric chamber represents a particular microhabitat and, combined with a diverse diet, can account for the diversity of parasites found in the gastrointestinal tract of odontocetes. Therefore, frequent histopathological findings in the gastric wall of toothed whales associated with parasite infection include mild to severe ulcerations and inflammatory infiltrate within one or more stomach compartments, mostly affecting the submucosal layer (Abollo et al. 1998, Lehnert et al. 2005, Jaber et al. 2006, Motta et al. 2008, van Beurden et al. 2015). Infection with at least 2 species of parasites results in these lesions in Odontoceti; the most abundant are nematodes of the genus *Anisakis* Dujardin, 1845 (Anisakidae, Nematoda) composed of 9 species (Mattiucci & Nascetti 2006, 2008), which use marine mammals as definitive hosts (Marcogliese 1995). In stranded toothed whales along the east coast of the Adriatic Sea, fourth-stage larvae and adults of *A. simplex*, *A. pegreffii* and *A. physeteris* have been isolated and molecularly characterised (Blažeković et al. 2015). The second most frequent gastric parasite is *Pholeter gastrophilus* (Heterophydae, Digenea), a heterophyd cosmopolitan and generalist digenean found in many cetacean species (Aznar et al. 1994, 2003, Gibson et al. 1998, Cornaglia et al. 2000, Berón-Vera et al. 2001). The life cycle of these parasites most likely includes molluscs and fish as intermediate hosts (Gibson et al. 1998). Adult parasites are typically encapsulated and located within the gastric submucosa of infected hosts (Woodard et al. 1969, Migaki et al. 1971). In addition, the digenean species *Braunina cordiformis* (Braunindae, Digenea) has been reported from the fundic stomach, pyloric stomach or ampulla duodenalis of Indo-Pacific bottlenose dolphin *T. aduncus* (Ehrenberg, 1832) (Schryver et al. 1967, Santos et al. 1996, Romero et al. 2014), short-beaked common dolphin *D. delphis* (Berón-Vera et al. 2007), Commerson's dolphin *Cepahlorhynchus commersonii* Lacépède, 1804 (Berón-Vera et al. 2001), Chilean dolphin *Cephalorhynchus eutropia* Gray, 1846 (Torres et al. 1992), dusky dolphin *Lagenorhynchus obscurus* Gray, 1828 (Dans et al. 1999), tucuxi *Sotalia fluviatilis* (Gervais & Deville, 1853) (Santos et al. 1996, Melo et al. 2006), Guiana dolphin *S. guianensis* (van Bénéden, 1864) (Marigo et al. 2010) and rough-toothed dolphin *Steno bredanensis* (G. Cuvier in Lesson, 1828) (Ott & Danilewicz 1996, Santos et al. 1996). The features of this digenean include a velum-like structure that encapsulates the body of the adult leaving its ventral part open, a large holdfast organ overlaid by a ventral fold, and the absence of both oral and ventral suckers (Niewiadomska 2002). It is doubtful whether this species causes significant lesions; however, localised regions of chronic gastritis at the site of attachment have been reported (Schryver et al. 1967, Sweeney & Ridgway 1975).

The primary aim of this study was to evaluate the histopathology associated with these parasite species, observed in the gastrointestinal tract of toothed whales stranded along the eastern coast of the Adriatic Sea during a 26 yr period. This represents the largest collection of cetacean samples evaluated over time for this area. Because a serious impediment in assessment of histopathological lesions in stranded cetaceans is the freshness of the collected tissue, in the majority of cases ultrastructural details of the extent of pathology associated with the parasites was impeded. In this study, we describe ultrastructural changes induced by the digenean *P. gastrophilus* inferred by transmission electron microscopy (TEM) and immunohistochemistry (IHC) to screen for CD3, inducible nitric oxide synthase (iNOS) and caspase-3.

## MATERIALS AND METHODS

### Gastrointestinal lesion sampling

As part of a long-term project to investigate marine mammal strandings along the Croatian coast of the Adriatic Sea between 1990 and 2016, gastrointestinal lesions in toothed whales were sampled during necropsies. Date and stranding site location, species determination, age, sex, body mass and external body measurements were recorded for each carcass as previously reported (see Table 1) (Gomerčić et al. 2009), and mean total body length (TBL) was measured. Necropsies were performed following the standard protocol (Kuiken & García Hartmann 1991).

Gross lesions in the wall of gastric chambers, duodenum and jejunum were sampled from fresh and moderately decomposed carcasses. Tissue sample size varied, depending on the extent of each lesion, but always included the whole lesion and surrounding tissue, later dissected into smaller pieces. All tissue samples were fixed and stored in cold buffered 4% formaldehyde until processing.

### Tissue preparation and histopathological analysis

Tissue samples were processed for histologic slide preparation in a Shandon Citadel 2000 (Thermo Scientific) tissue processor using standard techniques. After processing, samples were paraffin-embedded and cut to 5 µm sections. Sections were stained with haematoxylin-eosin (HE) in MICROM Autostainer HMS 70 (Thermo Scientific), coverslip mounted by Neomount (Merck) and evaluated for pathohistological changes using an Olympus CX40 microscope equipped with an Olympus Camedia C-4040 camera.

### IHC

Immunohistochemical staining was performed on lesions caused by *Pholeter gastrophilus* and sampled from fresh carcasses only, previously fixed in 4% paraformaldehyde (tissue to preservative volume, 1:10). Prior to staining, tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed by incubating of sections for 20 min at 95°C in Dako Target Retrieval Solution, pH = 6 (Dako). After cooling at room temperature, sections were incubated in 3% bovine serum albumin (BSA) solution in phosphate-buffered saline (PBS) (Sigma Aldrich) for 1 h at room temperature to prevent non-specific antibody binding. Following washing in Dako Wash Buffer (Dako) (TBS Tween-20), sections were incubated with rabbit anti-CD3 epsilon antibody (1:200, ab49943; Abcam), rabbit anti-active caspase-3 antibody [E83-77] (1:200, ab32042; Abcam) and rabbit anti-iNOS antibody (1:100, ab15323; Abcam) for 1 h at room temperature. After washing in Dako Wash Buffer, sections were incubated with donkey anti-rabbit IgG H&L (AlexaFluor®594) secondary antibody (2 µg $ml^{-1}$, ab150068; Abcam) for 1 h, washed in TBS and mounted in FluoroShield with DAPI (Sigma Aldrich). For a negative control, tissue sections were incubated with secondary antibody only (data not shown). Sections were inspected under an Olympus BX51 microscope equipped with a DP71 camera. Photographs were captured with CellA software and assembled with Photoshop CS4 (Adobe).

### TEM

For TEM, small tissue pieces of *P. gastrophilus* lesions sampled from fresh carcasses only were fixed in 4% paraformaldehyde on ice (tissue to preservative volume, 1:10). Samples were post-fixed in 1% osmium tetroxide, contrasted with 2% uranyl acetate, dehydrated in ascending series of acetone at room temperature and embedded in Durcupan resin (Fluka) at 64°C. Semi-thin sections (0.5 µm thick) were cut, stained with 1% toluidine blue, and inspected under light microscope for orientation. Ultrathin sections (0.07 µm) were cut based on semi-thin sections, contrasted with uranyl acetate and lead citrate and inspected under TEM (Jeol JEM-1400).

## RESULTS

During the 26 yr period, 387 marine mammals stranded along the Eastern Adriatic coast. There were 269 of 377 stranded toothed whales necropsied with sampled gastric lesions, including 38 dolphins (14.12%) ranging in age from 1 to 30 yr. These included bottlenose dolphins *Tursiops truncatus* (n = 23, mean TBL = 267.52 ± 29.021 cm), striped dolphins *Stenella coeruleoalba* (n = 12, mean TBL = 203.66 ± 6.81 cm) and Risso's dolphins *Grampus griseus* (n = 3, mean TBL = 294.33 ± 8.02). Data on species, age, sex and body measures of sampled animals are summarised in Table A1 in the Appendix.

For the dolphins, 4 of 38 (10.5%) animals presented with advanced autolysis, and thus were not assessed by histopathology and were excluded from the analysis. Four animals were diagnosed with intralumenal trematodiasis caused by *Braunina cordiformis* (Brauninidae, Digenea). In 23 dolphins (60.5%), histopathological changes associated with the presence of either adult parasites or their eggs were present. Prevalence of parasite species with respect to host species and sex in animals histopathologically evaluated is represented in Fig. 1. Most of the lesions affected deep mucosal layers, lamina propria and muscularis mucosae or submucosis. Additionally, 6 (15.8%) of the dolphins had various pathological changes that were not associated with presence of either nematode or trematode parasites. All findings are summarised in Table 1.

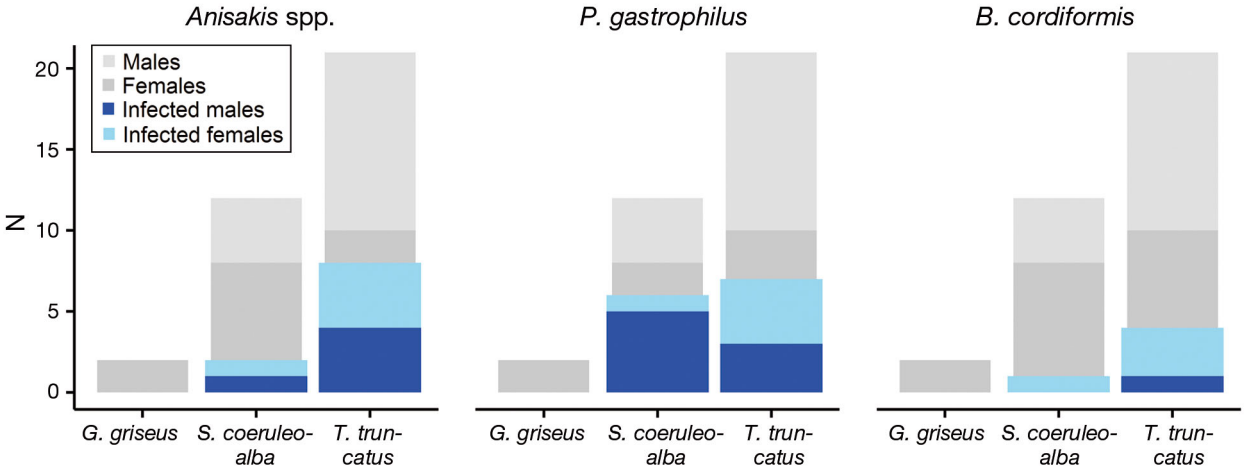


Fig. 1. Prevalence of parasite species in histopathologically examined dolphins with respect to host species and sex

### *Anisakis*-associated lesions

In 11 (28.9%) sampled dolphins, lesions associated with the presence of *Anisakis* spp. were observed (Fig. 2a). Various developmental stages of the nematode, or its remnants that included parts of worm body or only cuticule, were present. The most prevalent alteration induced by parasitation by *Anisakis* was ulcerative gastritis with abundant lymphoplasmacytic and eosinophilic infiltrate in underlying tissue, accompanied by colonies of coccobacillary bacteria (Fig. 2b,c). In other cases, changes included mild to moderate, multifocal to confluent, chronic granulomatous gastritis in the forestomach or forestomach and fundic stomach respectively, focal moderate oedema of muscularis mucosae in the forestomach, or mild focal fibrosis in forestomach and moderate, multifocal granulomatous jejunitis (Table 1). Lesions induced by the nematode were in a state of freshness that did not permit adequate TEM or IHC analysis.

### *Pholeter gastrophilus*-associated lesions

The intralesional trematode *Pholeter gastrophilus* (Heterophydae, Digenea) or trematode eggs embedded in fibrotic nodules (Fig. 2d, left) were observed in 13 (34.2%) of 38 sampled animals. Nodules were filled with thick, dark detritus (Fig. 2d, right), composed of cellular debris and surrounded by macrophages, neutrophils and eosinophils, and located in either the fundic or pyloric stomach or both compartments. Occasionally, paired or single adult individuals, or only their eggs, were observed inside the nodule (Fig. 2e). A total of 8 animals with intralesional trematodiasis showed focal or multifocal chronic fibrous gastritis, and one animal also had areas of chronic active suppurative inflammation (additionally to fibrous gastritis). Surrounding tissue was mostly infiltrated with mixed lymphoplasmacytic and eosinophilic or lyphoplasmacytic and hystiocytic infiltrate (Fig. 2e, insert). Four animals with intralesional trematodes had severe multifocal to confluent or focal granulomatous gastritis, 2 of which with lymphoplasmacytic and eosinophilic infiltrate. Finally, one animal with intralesional trematode eggs showed multifocal chronic fibrous duodenitis. Additionally, in one of the animals with fibrous gastritis, hyperplastic lymphoid follicles were found in the pyloric stomach and duodenum, respectively (Fig. 2f).

### Immunohistochemical analysis of *P. gastrophilus* lesions

Immunolabeling of *P. gastrophilus* lesions showed positive staining for all 3 antibodies applied, i.e. anti-CD3, anti-iNOS and anti-active caspase-3. Staining with CD3 antibody produced a moderate signal, but showed characteristic ring-like cytoplasmic staining consistent with thin cytoplasm of lymphocytes (Fig. 3a–c). CD3+ cells were scarce and often localised within blood vessels. A low number of CD3+ cells infiltrating the fibrous connective tissue was observed at the periphery of granulomas. Immunolabeling with anti-iNOS antibody yielded strong, diffuse cytoplasmic staining of irregularly shaped cells (Fig. 3d–f). In contrast to CD3+ cells, iNOS+ cells were more abundant and were also observed through-

out the whole lesion, but tended to be more concentrated around the center of the lesion where trematodes were located. Immunolabeling with anti-caspase-3 antibody also yielded a strong signal in the form of granular cytoplasmic staining (Fig. 3g–i). Caspase3+ cells were the most abundant of all 3 cell populations that were positively stained and were found throughout the whole lesion. Although various cells showed positive staining with anti-active caspase-3 antibody, based on the cell shape and nuclear morphology eosinophils were the predominant cell type to express active caspase-3.

Table 1. Gastrointestinal lesions and parasites recorded in sampled dolphins stranded along the East Adriatic coast. *Tt*: *Tursiops truncatus*; *Sc*: *Stenella coeruleoalba*; *Ggr*: *Grampus girseus*; Ug: ulcerative gastritis; Fb: fibrous gastritis; Gg: granulomatous gastritis; Eg: erosive gastritis; Lp: lymphoplasmacytic; Eo: eosinophilic; Hc: hystiocytic

| Designation | Species | Organ | Type of lesion | Other remarks |
|---|---|---|---|---|
| ***Anisakis*-associated** | | | | |
| D25 | *Tt* | Forestomach | Ug | Focal, severe |
| D40 | *Tt* | Fundic stomach | Fg | Severe, moderate to abundant Lp, Hc and Eo infiltrate |
| D71 | *Sc* | Forestomach | Fibrosis | Focal, mild |
| D99 | *Tt* | Forestomach | Ug | Lp and Eo infiltrate, bacteria |
| D143 | *Tt* | Fundic stomach | Ug | Lp and Eo infiltrate, bacteria, oedema of *muscularis mucosae* |
| | | Forestomach | Ug | |
| D177 | *Tt* | Forestomach | Ug | Lp and Eo infiltrate, bacteria |
| D206 | *Tt* | Forestomach | Gg | Multifocal, chronic, moderate, focally moderate oedema of *muscularis mucosae* Eo infiltrate, multifocal to confluent, moderate, chronic |
| | | Fundic stomach | Gg | |
| D211 | *Tt* | Fundic stomach | Gg | Chronic, mild |
| D212 | *Tt* | Forestomach | Oedema | Diffuse, moderate |
| | | Fundic stomach | Fg | Scant Lp and Hc infiltrate |
| D232 | *Tt* | Forestomach | Ug | Multifocal, chronic with focal ulceration of mucosa, Lp and Eo infiltrate, bacteria |
| D259 | *Sc* | Jejunum | Jejunitis | Granulomatous, multifocal, chronic, moderate |
| D27 | *Sc* | Fundic stomach | Fg | Severe, moderate Lp and Hc infiltrate |
| ***Pholeter gastrophilus*-associated** | | | | |
| D41 | *Tt* | Pyloric stomach | Fg | Chronic, Lp and Eo infiltrate, moderate fibrosis |
| D79 | *Sc* | Pyloric stomach | Gg | Multifocal to confluent, chronic, severe |
| D121 | *Sc* | Pyloric stomach | / | Trematodiasis |
| D124 | *Tt* | Fundic stomach | Fg | Multifocal, chronic, moderate |
| D225 | *Tt* | Pyloric stomach | Fg | Focal, chronic |
| D235 | *Tt* | Duodenum | Duodenitis | Fibrous, multifocal, chronic |
| D244 | *Sc* | Pyloric stomach | Gg | Lp and Eo infiltrate, multifocal to confluent, chronic, severe |
| D258 | *Sc* | Fundic stomach | Gg | Lp and Eo infiltrate, multifocal to confluent, chronic, severe |
| D263 | *Sc* | Fundic stomach | Gg | Eo infiltrate, focal, chronic, moderate to severe |
| D265 | *Sc* | Fundic stomach | Fg | Moderate Lp and Eo infiltrate |
| D371 | *Tt* | Fundic stomach | Fg | Eo infiltrate, chronic, severe |
| D373 | *Tt* | Fundic stomach | Fg | Eo and Hc infiltrate, chronic |
| | | Pyloric stomach | Gg | Focaly extensive, Eo infiltrate, partially suppurative, subacute; hyperplastic lymphoid folicles |
| | | Duodenum | Duodenitis | Multifocal, hyperplastic lymphoid folicles |
| ***Braunina cordiformis*-associated** | | | | |
| D102 | *Tt* | Pyloric stomach | / | Intraluminal trematodiasis |
| D200 | *Tt* | Pyloric stomach | / | Intraluminal trematodiasis |
| D276 | *Tt* | Pyloric stomach | / | Intraluminal trematodiasis |
| D288 | *Tt* | Pyloric stomach | / | Intraluminal trematodiasis |
| D70 | *Ggr* | Fundic stomach | Eg | Multifocal, mild |
| D84 | *Ggr* | Fundic stomach | Eg | Mild, multifocal, submucosal lymphatic dilatation |
| D89 | *Sc* | Forestomach | / | Muscular hypertrophy, submucosal oedema, vascular wall hypertrophy, perivascular Lp infiltrate |
| **Non-parasitic** | | | | |
| D115 | *Sc* | Duodenum | Duodenitis | Erosive, multifocal, moderate |
| D179 | *Sc* | Fundic stomach | Eg | Multifocal, moderate |
| D198 | *Tt* | Fundic stomach | Fg | Mineralisation |

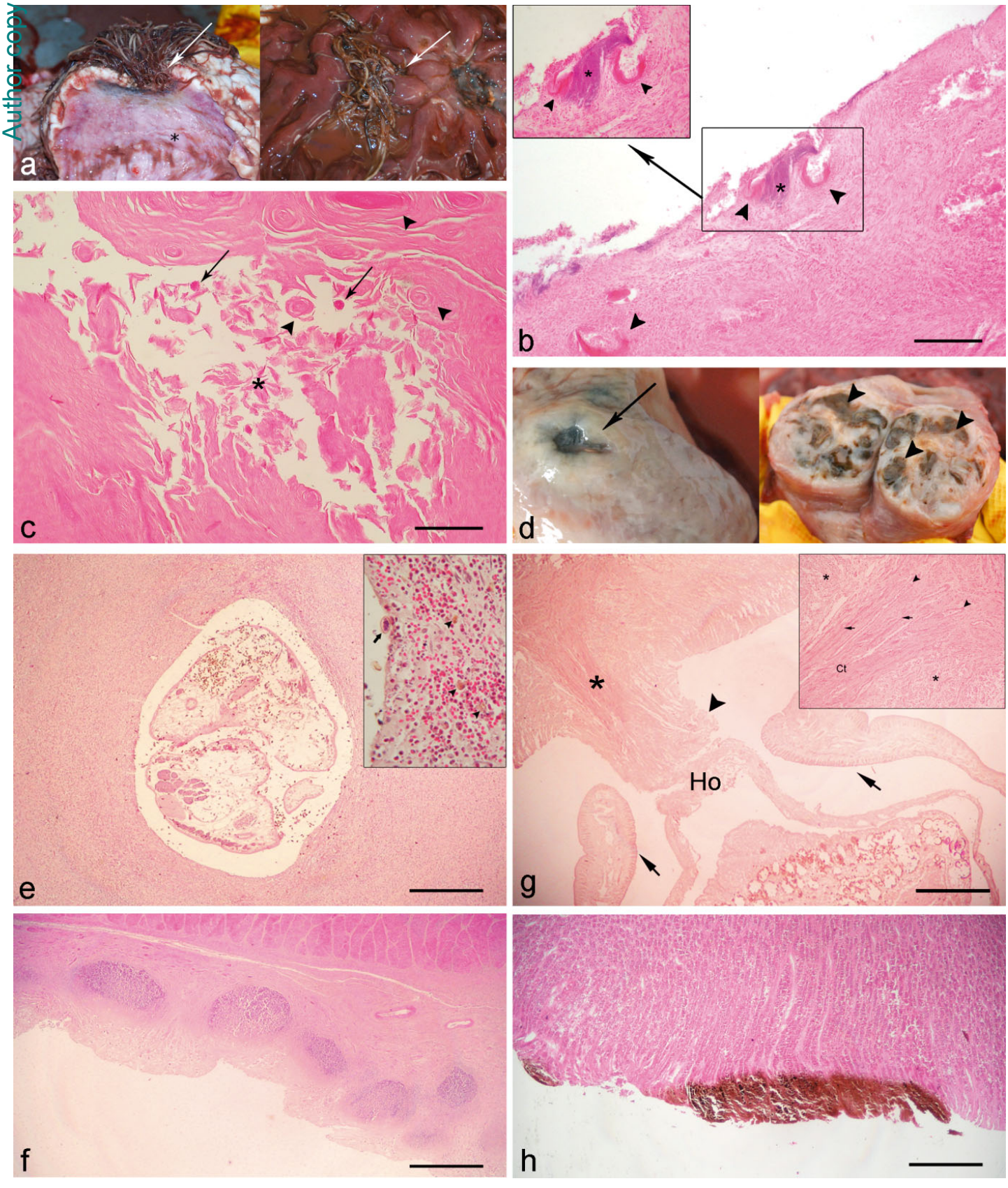

Fig. 2. Gross pathological and histopathological findings of gastric lesions in dolphins stranded along Eastern Adriatic coast. (a) Large ulcers from bottlenose dolphin forestomach (left) and fundic stomach (right) with numerous nematodes embedded inside the gastric mucosa (arrows) and extensive thickening of forestomach submucosal layer (asterisk). (b) Ulcerative gastritis in pyloric stomach of bottlenose dolphin. Present in submucosa are parasite remnants (arrow heads) surrounded by bacterial colonies (asterisk) and proliferating fibrous tissue. Haematoxylin-eosin (HE) staining, scale bar = 200 µm. (c) Deep ulceration with cellular desquamation (asterisk) in forestomach of bottlenose dolphin with multiple nematode eggs (arrows) and granulomas (arrow heads). HE staining, scale bar = 200 µm. (d) *Pholeter gastrophilus* fibrotic nodule, with apical opening, from striped dolphin pyloric stomach (left); inside of a nodule filled with thick, dark detritus (arrow heads) (right). (e) Transversal section of trematodes within fundic submucosa of bottlenose dolphin, surrounded by mixed inflammatory infiltrate composed of eosinophils, macrophages, few lymphocytes and plasma cells and activated fibroblasts. At the periphery of inflammatory node, formation of connective tissue is visible. HE staining, scale bar = 1 mm. Insert: high-power magnification of mixed inflammatory infiltrate from *P. gastrophilus* fibrotic nodule dominated by eosinophils and macrophages with intracytoplasmic hemosiderin-like brown pigment (arrow heads) together with extensive hemorrhage; at the periphery of lesion, an embryonated trematode egg is visible (arrow). (f) Hyperplastic lymphoid follicles composed of lymphocytes and plasma cells in lamina propria of bottlenose duodenum. HE staining, scale bar = 1 mm. (g) Transversal section of *Braunina cordiformis* attached to gastric wall inside pyloric stomach of bottlenose dolphin with its holdfast organ (Ho) attached to a connective bridge composed of host tissue (arrow head) with protruding submucosal connective tissue (asterisk). The trematode is surrounded by a vellum-like structure that connects ventrally to its body (not shown). HE staining, scale bar = 500 µm. Insert: detail of connective bridge showing protruding submucosal connective tissue (Ct) with bundles of collagen fibers (arrows), distended blood vessels (arrow heads) and surrounded by glandular cells of mucosal layer (asterisk). (h) Erosive gastritis in fundic stomach from striped dolphin. Focal erosion with loss of superficial epithelial cells and accumulation of brown hemosiderin-like pigment. HE staining, scale bar = 1 mm

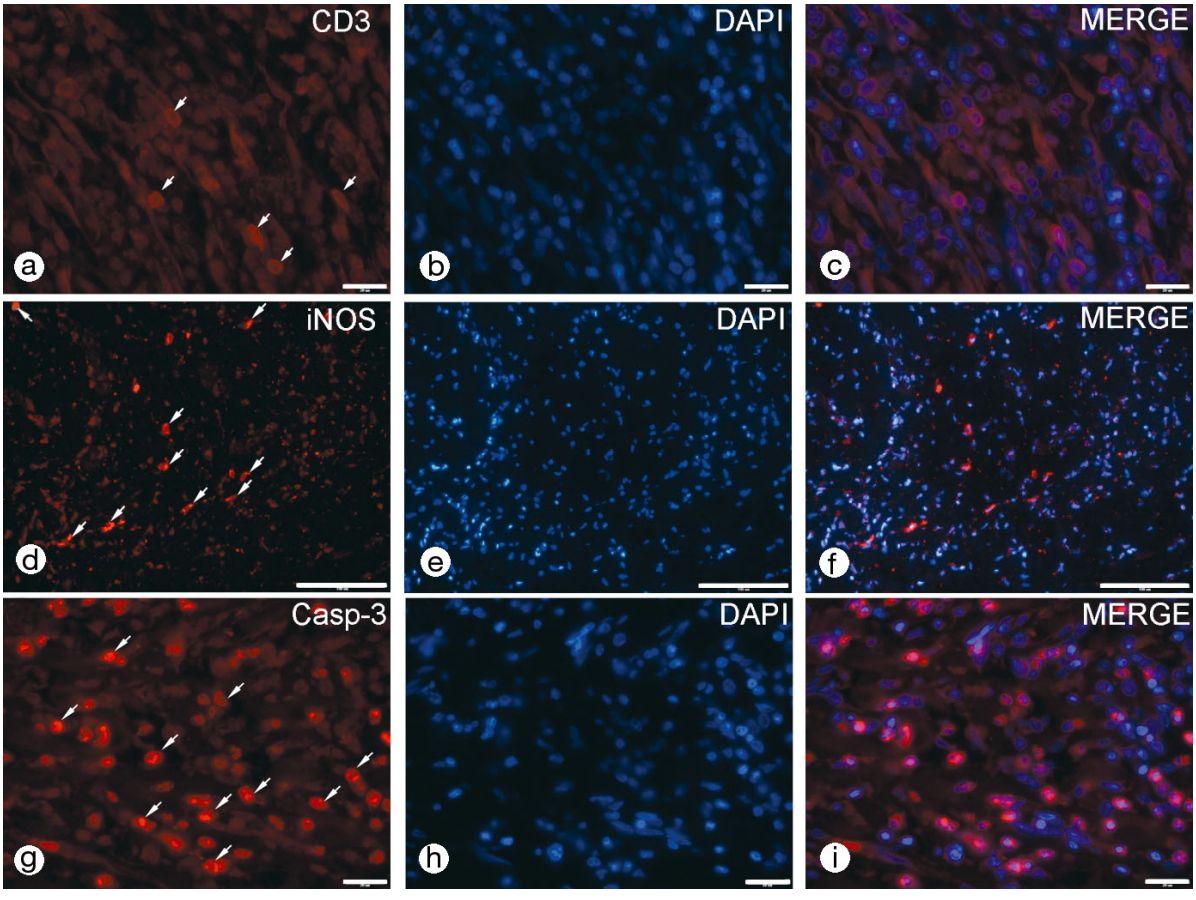


Fig. 3. Representative microphotographs of CD3, iNOS and caspase-3 immunolabelling in *Pholeter gastrophilus*-caused lesion. (a) Moderate expression of CD3 on T-lymphocytes (arrows) in peripheral part of the lesion. Cells show characteristic ring-like cytoplasmic staining consistent with thin cytoplasm of lymphocytes. (b) DAPI stains cell nuclei. (c) Merging of (a) + (b). (d) iNOS positive cells (arrows) in intermediate part of lesion, showing strong, diffuse cytoplasmic staining. (e) DAPI stains cell nuclei. (f) Merging of (d) + (e). (g) Caspase-3 positive cells (arrows) in central part of lesion (adjacent to trematodes), showing strong, granular cytoplasmic staining. (h) DAPI stains cell nuclei. (i) Merging of (g) + (h). Scale bars (a–c, g–i) = 20 µm, (d–f) 100 µm

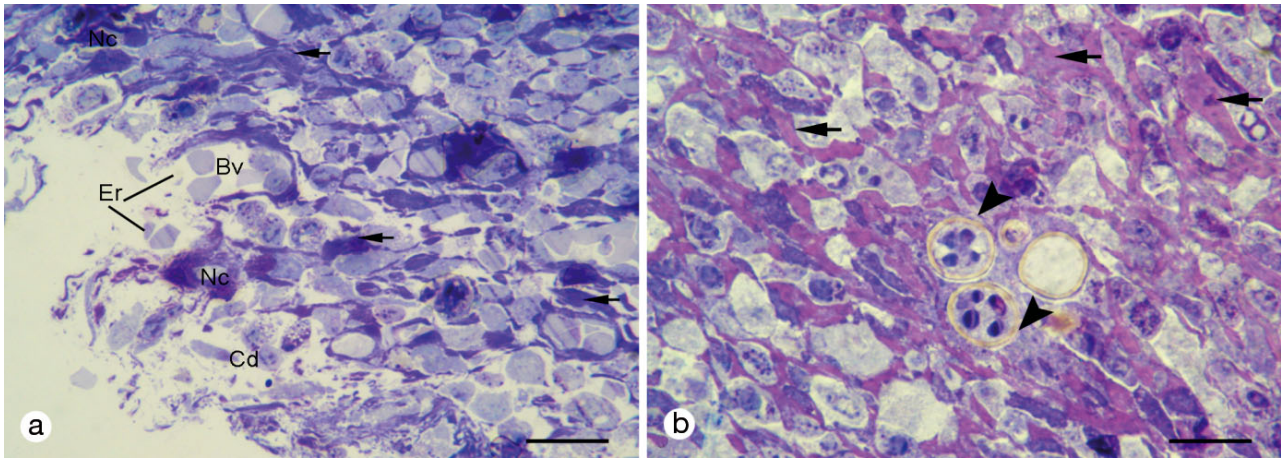


Fig. 4. Semithin sections of *Pholeter gastrophilus* lesion from the fundic stomach of a bottlenose dolphin, toluidine blue staining. (a) Bottom of lesion showing disruption of tissue structure, cellular debris (Cd) with necrotic cells (Nc) and ruptured blood vessel (Bv) with erythrocytes (Er). Proliferation of connective tissue is observed in the submucosa (arrows). (b) Embryonated parasite eggs (arrow heads) surrounded by thick bundles of proliferating connective tissue (arrows). Scale bars = 20 µm

### TEM of *P. gastrophilus* lesions

Out of 38 sampled carcasses, only 2 with *P. gastrophilus* lesions were appropriate for TEM analysis. Due to the size of the lesions, 3 regions, i.e. central, intermediate and distant part, were dissected.

In the central part of the lesions adjacent to a parasite, disruption of connective tissue and its cellular structures with accumulation of detritus could be observed (Figs. 4a & 5a). Acini of fundic glands were disrupted, and cells were observed in different stages of necrosis or apoptosis, recognizable within tissue detritus by remnants of dark, condensed and irregular nuclei and light and vacuolated cytoplasm. Such areas were usually colonised by bacteria (Fig. 5b). In one section interspersed with tissue debris, oval to elongated dark cells of unidentified fungi were observed, showing an electron-dense thick outer and electron-lucent inner layer, and vacuolated cytoplasm (Fig. 5c). Parasite embryonated eggs with thick electron-dense capsules were present throughout parasitised area, sometimes in close contact with neutrophil-like granulocytes (Fig. 5d). The latter were present within all lesion layers, showing abundant electron-lucent and few electron-dense granules. Few basophil-like cells with large dark granules of different size were also observed (Fig. 5e). In the intermediate part of the lesion, extensive proliferation of connective tissue was visible, with neutrophil- and basophil-like granulocytes and *Pholeter* eggs trapped within collagen fibers. Adjacent fundic glands were loosely situated within connective tissue, showing lack of visible intercellular connections, small basally situated nuclei, and electron-lucent vacuoles in the apical part. Feeble glandular activity was recognisable by secretion or absorption of small electron-dense vacuoles, visible above the apical cell membrane. Small blood vessels were filled with accumulated leukocytes; neutrophil-like and lymphocytes (Figs. 4 & 5f). In the outmost area, dense connective tissue encompassed thick bundles of collagen-like fibers and fibroblast with dark, elongated nuclei, apparently unaffected by *Pholeter* parasitation (Fig. 5a insert).

### *B. cordiformis* lesions

*B. cordiformis* was found in 4 (10.5%) of 38 sampled animals. Flukes could be found suspended in the luminal space attached to gastric walls (Fig. 2g) and all were found exclusively in the pyloric stomach. In histological sections, they appeared heart-shaped, surrounded by a velum-like structure and containing large oval eggs — most of which were empty and broken, most likely due to tissue processing. Although several flukes could be found in close proximity suspended from a singe site in gastric wall, no significant pathology could be observed. Mechanical damage, rather than visible immune response, could be observed at the site of attachment. Although lacking both suckers, flukes could be observed 'dragging' gastric wall tissue with their ventrally located holdfast organs, causing protrusion of submucosal

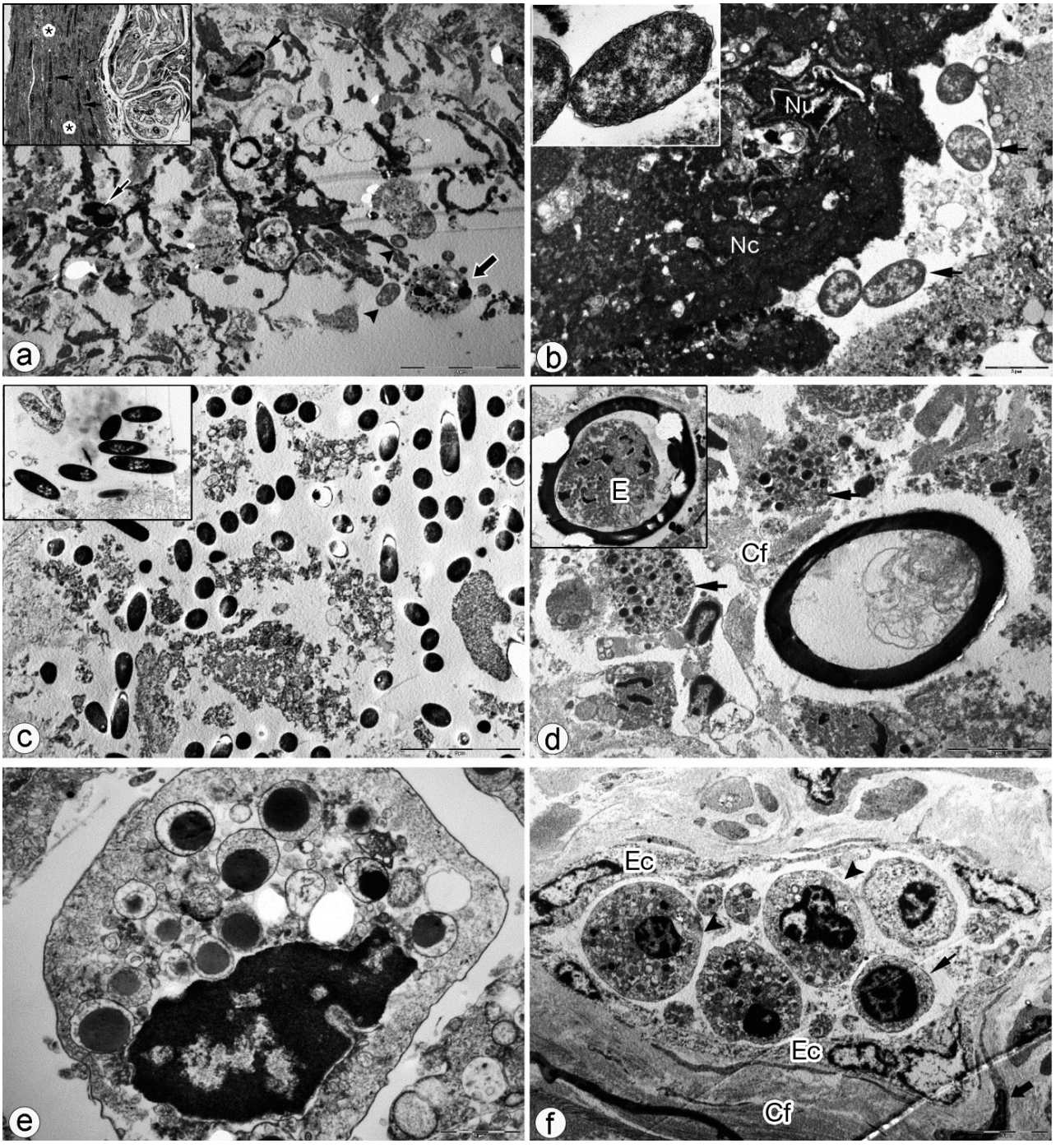


Fig. 5. Transmission electron micrographs of *Pholeter gastrophilus* lesion from the fundic stomach of a bottlenose dolphin. (a) Central part of lesion with accumulation of cellular detritus, 1 late apoptotic (thick arrow) and 2 early apoptotic cells (arrows) and occasional intralesional bacteria (arrow heads) (scale bar = 10 µm); insert: outmost part of lesion with thick bundles of collagen-like fibers and fibroblasts with dark, elongated nuclei apparently unaffected by *P. gastrophilus* parasitation. (b) Large areas of necrotic cells (Nc) and remnants of nucleus (Nu) surrounded by cellular detritus (asterisk) and numerous intralesional bacteria (arrows) (scale bar = 10 µm); insert: intralesional bacterium with undulating cell wall. (c) Colony of unidentified fungus admixed with cellular debris. Note electron-dense thick outer layer and electron-lucent inner layer, and vacuolated cytoplasm (scale bar = 5 µm). (d) Embryonated (E) parasite egg with thick electron-dense capsule surrounded by collagen fibers (Cf) and few neutrophil-like granulocytes (arrows) (scale bar = 5 µm). (e) Basophil-like cell, with large dark granules of different size (scale bar = 1 µm). (f) Small blood vessel (Ec: endothelial cells) inside lesion with accumulated lymphocyte (arrow) and neutrophil-like granulocytes (arrow heads). Adjacent to a blood vessel a fibrocyte is observed (thick arrow), while surrounding tissue shows extensive proliferation of collagen fibers (Cf) (scale bar = 5 µm)

connective tissue and destruction of superficial epithelium (Fig. 2g insert). This protrusion of connective tissue also caused mechanical damage to the mucosal layer in the form of destruction of glandular cells. No hemorrhages or inflammatory infiltrate could be observed.

### Lesions of unknown etiology

Out of 6 dolphins with various pathological changes not associated with the presence of either nematode or trematode parasites, 3 animals showed multifocal, mild to moderate erosive gastritis, one of which had signs of submucosal lymphatic dilatation. Histologically, erosions were characterised by focal loss of superficial epithelial cells and underlying glands and accumulation of brown hemosiderin-like pigment (Fig. 2h). One of the dolphins had muscular hypertrophy with submucosal oedema and vascular wall hypertrophy and scant perivascular lymphoplasmacytic infiltrate. Finally, the remaining 2 animals exhibited fibrous gastritis with mineralisation and moderate, multifocal erosive duodenitis, respectively. Although no parasite remnants or their eggs were found in the aforementioned animals, pathological changes suggest that some of them are most likely a consequence of intra vitam parasite migration.

## DISCUSSION

In total, 31 out of 38 sampled dolphins with gastric lesions were included in the analysis. In 23 animals, lesions were associated with the presence of either adult parasites or their eggs. According to the morphology of the parasites and lesions they caused, the parasites were identified as *Anisakis* spp. and the digenean trematode *Pholeter gastrophilus*. Additionally, in 4 animals the digenean trematode *Braunina cordiformis* was found, causing no significant pathology at the site of attachment.

Gastric ulcers associated with the presence of *Anisakis* spp. are often reported in stranded cetaceans (Abollo et al. 1998, Lehnert et al. 2005, Motta et al. 2008), although nematodes can also be found free in stomach chambers in larval stages causing no obvious damage to the gastric wall (Berón-Vera et al. 2001, Romero et al. 2014). Ten dolphins from the present study had lesions associated with *Anisakis* spp., 8 of which were bottlenose dolphins and 2 were striped dolphins. In contrast to this study, a previous study reported a significantly higher prevalence of *Anisakis* spp. and associated gastric lesions in striped dolphins compared to bottlenose dolphins (Blažeković et al. 2015). The reason for this bias could be in that we included only fresh samples appropriate for histopathological analysis, while in the former parasitological study this was not a prerequisite. Gastric lesions were most frequently found in the forestomach and occasionally in main (fundic) stomach. Surprisingly, in one dolphin a lesion associated with *Anisakis* spp. was found in the jejunum, while none of the dolphins had lesions in the pyloric stomach. This is in accordance with other studies that showed concentrations of lesions in the first 2 stomach compartments (Abollo et al. 1998, Lehnert et al. 2005, Motta et al. 2008). Moreover, it seems that adult *Anisakis simplex* showed a preference for the forestomach and main stomach, while larval stages had a wider distribution among the stomach compartments (Herreras et al. 2004, Aznar et al. 2006). It has been suggested that such distribution could be regarded as a mating enhancement strategy, or alternatively, a result of higher requirements of adult worms for the resources provided by the first chamber (Aznar et al. 2006).

Most of the ulcers appeared fresh indicating an acute process, although some of them had dark surfaces indicative of previous haemorrhages and the beginning of healing. In others, mild to moderate granulomatous or fibrous gastritis and jejunitis could be observed as a chronic process in various stages of healing. Similar findings were reported in cetaceans stranded along the Brazilian coast where *Anisakis typica* was the predominant species (Motta et al. 2008). Based on their phylogenetic distance and the fact that species from the *A. simplex* complex, i.e. *A. pegreffii* and *A. simplex* sensu stricto, and *A. typica* are generalists with regards to host specificity, a more general effect on the host is therefore likely. Recently, *Anisakis*-induced granulomatous dermatitis was reported in a harbour porpoise *Phocoena phocoena* and bottlenose dolphin *Tursiops truncatus* (van Beurden et al. 2015). In both animals, skin ulcerations surrounded by hyperplastic epidermis were visible with underlying dermis and blubber exhibiting focally extensive fibrosis and granulation with mixed cellular infiltrate. Similar findings were observed in mice experimentally infected with *A. simplex* (Jones et al. 1990) and in a case of human gastric anisakiasis that resulted in a bleeding ulcer (Kang et al. 2014). These findings all suggest an acute and localised but intense host response to *Anisakis* infection that progresses in a chronic process, regardless of the host species.

However, when comparing marine mammals with mice or human anisakiasis, it must be taken into account that the former is a definitive host that has developed a non-detrimental interaction with the parasite, while the later are accidental and naïve hosts that react to the unknown pathogen more intensively, as shown in murine models of few known human helminth parasites (summarised in Behm & Ovington 2000, Karasuyama et al. 2011, Gazzinelli-Guimarães et al. 2013)

Fibrotic nodules found in the gastric wall were occasionally filled with dark, thick detritus resembling that observed in *Fasciola* sp. infections causing chronic cholangitis in cattle (McGavin & Zachary 2008). Such nodules containing the trematode *P. gastrophilus* have been previously reported (Woodard et al. 1969, Migaki et al. 1971, Cornaglia et al. 2000, Berón-Vera et al. 2001, Fernández et al. 2003, Romero et al. 2014). Both bottlenose and striped dolphins from this study were almost equally infected with *P. gastrophilus* (6 bottlenose dolphins vs. 7 striped dolphins), and nodules were similarly distributed among main and pyloric stomachs (4 infected fundic stomachs vs. 3 infected pyloric stomachs in both species), as reported earlier by Aznar et al. (2006). Those authors suggested that *P. gastrophilus* behaves as a generalist parasite in regard to chamber selection, with no obvious preference for a specific part of the stomach. This is contrary to a study of parasites of the dusky dolphin *Lagenorhynchus obscurus* from Peru, where a majority of nodules was present in the pyloric stomach, only rarely in the main stomach or both stomach compartments (Van Waerbeek et al. 1993). Host diet and digestive physiology are thought to be involved in the stomach distribution of *P. gastrophilus*, which could explain different parasite localisation in hosts from different geographic areas. One of the dolphins from this study had chronic fibrous duodenitis, while all the other dolphins had chronic, moderate to severe fibrous or granulomatous gastritis. In few cases, mixed lymphoplasmacytic and eosinophilic or hystiocytic infiltrate was observed similar to that reported in trematode-associated gastric and hepatic lesions in cetaceans stranded in the Canary Islands (Jaber et al. 2004, 2006).

IHC analysis of *P. gastrophilus* lesions was congruent to histopathological findings, revealing inflammation-mediated stimulation. IHC-positive localisation of tCD3+, iNOS+ and caspase-3+ cells suggests accumulation of cytotoxic T-cells, pro-inflammatory cytokines and execution-phase of cell apoptosis at the parasitized area. Likewise, immunolocalisation of T-cells with pan T-cell marker CD3 showed infiltration of CD3+ cells in the peripheral parts of lesions, as observed by Jaber et al. (2006) in parasite-induced granulomas in stranded cetaceans. A number of cells strongly expressing iNOS has also been evidenced by immunolabelling corresponding to the observed histiocytic infiltrate. iNOS is 1 of 3 NO-synthases (the other 2 being neuronal [nNOS] and endothelial [eNOS]) that is not constitutively expressed and is strongly upregulated in response to various pathogens. Cells expressing iNOS include macrophages, dendritic cells, natural killer cells as (Bogdan 2001). The effects of NO vary greatly and among others include antiparasitic as well as cytotoxic effects (Bogdan 2001, Colasanti et al. 2002).

Most abundant inflammatory cells in the affected tissue were eosinophils. Eosinophilia is a known phenomenon associated with helminth infestations, where eosinophils attach directly to parasite's surface in the presence of antibodies or complement. Attachment causes degranulation of intracytoplasmic granules and release of various enzymes that can damage or kill parasite larvae (Meeusen & Balic 2000, Huang & Appleton 2016). Immunolocalisation of active caspase-3 as a marker for apoptotic cells showed numerous cells expressing this protein. Based on cellular and nuclear morphology, most cells expressing this protein appeared to be eosinophils. It has been previously shown that nitric oxide can trigger eosinophil apoptosis in a caspase-dependent mechanism (Zhang et al. 2003). Although caspase-3+ cells were much more abundant than those expressing iNOS, NO is not restricted to the site of its generation and is highly diffusible, thus potentially affecting distant cells (Bogdan 2001). However, a more plausible explanation for such a high number of eosinophils expressing caspase-3 could be the excretory–secretory products (ESP) released by the parasite. *In vitro* studies with ESP of 2 species of trematode parasites (*Fasciola hepatica* and *Paragonimus westermani*) showed that these products induced eosinophil apoptosis in both a time- and dose-dependent manner (Shin 2000, Serradell et al. 2007). Furthermore, a high number of apoptotic eosinophils were also evidenced in sheep experimentally infected with *F. hepatica*, suggesting that induction of apoptosis in host immune cells could be an immunosuppressive effect parasites have on their hosts (Escamilla et al. 2016). TEM revealed the presence of basophil-like cells within the lesions. Basophil granules are a known source of

interleukin-4 and interleukin-13, both of which stimulate a $T_H2$ response which is activated during helminth infections. Basophils also express IgE receptors on their cell surface, and elevated serum IgE is a feature of parasitic infestation. Moreover, basophils have the potential to process protein antigens, especially IgE-antigen complexes, and present antigens with MHC class II proteins, thus acting as antigen presenting cells (APC) (Min 2008, Voehringer 2009, Nakanishi 2010). TEM analysis also showed few neutrophil-like cells to be associated with the presence of parasite eggs in tissue. Neutrophils are the first cellular component to react to tissue damage and induce an inflammatory response. These cells can react to a wide array of pathogen- or damage-associated molecular patterns and subsequently recruit other granulocytes and monocytes that promote inflammation and immune response to pathogens (Jones et al. 2016). Although neutrophils are the most abundant leukocyte fraction, they mostly react to small pathogens which can be ingested and intracellularly degraded (Geering et al. 2013). However, in a murine model of *Schistosoma japonica* infection, egg-induced granulomas were heavily infiltrated with neutrophils and macrophages (Chuah et al. 2016). Despite their abundance and obvious role in parasite-induced immune response, we observed a fairly small number of neutrophil-like cells in *P. gastrophilus* lesions, while other components of cellular immunity (eosinophils, histiocytes, lymphocytes and plasma cells) were present in much larger numbers, indicating a later course of disease. Additionally, this discrepancy between the number of neutrophils and other immune cells could be due to the different regions of the lesions being sampled for the 3 analyses performed.

In the case of *B. cordiformis*, little is known about its pathology. Schryver et al. (1967) reported a localised region of chronic gastritis at the site of attachment of the fluke to host tissue. Although it has been reported in many cetacean species, as mentioned earlier, it is considered not to cause severe lesions in its hosts (Sweeney & Ridgway 1975). Our observations indicate that this species causes a 'reorganisation' of host tissue by utilising submucosal connective tissue as a means of attachment with only minor disruption of the surrounding mucosal glands. Furthermore, no inflammatory cells were observed at the site of attachment. Nonetheless, these results are inconclusive and should be interpreted with care due to the unknown ecology of the parasite and level of necrosis/apoptosis in pulled tissues. It is generally thought that the primary site of digenean parasitation in vertebrate hosts is the small intestine, where parasites can feed on chyme. Finding of parasites in more anterior parts of the digestive system, such as the pyloric or fundic stomach, is a result of radiation to other body parts and a strategy for occupation of new niches inside the host body (Galaktionov & Dobrovolskij 2010a). In light of this, the velum-like structure that surrounds the *B. cordiformis* body could serve as a protective layer against the acidic environment in stomach compartments. Although different from that in *B. cordiformis*, structural adaptation to acidic environment exists among the Hemiuroidea that parasitise fish stomachs. Members of this superfamily have eversible ecsoma that has a physiological function and is completely retracted during periods of low pH, while the tegument of the soma is thickened and has a protective function (Matthews & Matthews 1988, Galaktionov & Dobrovolskij 2010b)

Non-parasitic ulcers have been described in several cetacean species with *Helicobacter* spp. infections (Harper et al. 2000, 2002). It is unlikely that postmortem migration of 3 parasite species occurred, especially in the case of *P. gastrophilus*, which resides in firm fibrotic nodules. Alternatively, ulcers might have been left after the parasite's death, but since fibrosis (as indication of a healing process) was rarely observed, a non-parasitic etiology is feasible. Finally, such findings could be the result of mechanical damage caused by ingested objects or even prey in some cases (Kastelein & Lavaleije 1992, Byard et al. 2010, Krzyszczyk et al. 2013), rather than being caused by parasites.

In light of our results, we argue that the extent and quality of observed inflammatory processes, both of parasitic or non-parasitic origin, do not represent the cause of death and stranding of the cetaceans included in this study. Although severe infections can lead to gastric wall perforations, consequently causing peritonitis and death (Jaber et al. 2006), we did not observe such grave infection levels in this study. For a better understanding of the pathology of the cetacean gastrointestinal tract and the potential impact on the health status of dolphins, further studies using molecular approaches will help to elucidate mechanisms of host–parasite interactions leading to the observed alterations.

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## Appendix

Table A1. Species, age, sex and body measurements of 36 dolphins stranded along the East Adriatic coast between 1990 and 2014 bearing gastrointestinal lesions. *Tt*: *Tursiops truncatus*; *Sc*: *Stenella coeruleoalba*; *Ggr*: *Grampus griseus*

| Designation | Species | Age | Sex (yr) | Total body length (cm) | Body mass (kg) | Date |
|---|---|---|---|---|---|---|
| D17 | *Tt* | 13 | F | 274 | / | 30.7.1997 |
| D25 | *Tt* | 23 | F | 278 | 228 | 27.2.1999 |
| D27 | *Sc* | 11 | M | 198 | 99 | 23.6.1999 |
| D40 | *Tt* | 13 | M | 288 | 288 | 17.3.2000 |
| D41 | *Tt* | 12 | F | 261 | 224 | 27.4.2000 |
| D62 | *Tt* | 14 | M | 290 | 155 | 19.7.2001 |
| D69 | *Ggr* | / | M | 302 | 268 | 10.1.2002 |
| D70 | *Ggr* | / | M | 295 | 288 | 18.1.2002 |
| D71 | *Sc* | 13 | M | 208 | 99 | 19.1.2002 |
| D79 | *Sc* | 22 | F | 198 | 91 | 25.2.2002 |
| D84 | *Ggr* | / | M | 286 | 185 | 30.4.2002 |
| D89 | *Sc* | 23 | M | 209 | 98 | 21.6.2002 |
| D99 | *Tt* | 12 | M | 256 | 249 | 8.10.2002 |
| D102 | *Tt* | 20 | F | 262 | 216 | 24.12.2002 |
| D115 | *Sc* | 22 | F | 197 | 96 | 16.4.2004 |
| D121 | *Sc* | 12 | F | 203 | 80 | 4.7.2004 |
| D124 | *Tt* | 17 | M | 301 | 205 | 1.9.2004 |
| D143 | *Tt* | 20 | M | 263 | 163,5 | 25.10.2005 |
| D177 | *Tt* | 19 | M | 322 | 234 | 6.12.2007 |
| D179 | *Sc* | 11 | M | 192 | 59 | 11.1.2008 |
| D198 | *Tt* | / | F | 226 | 117 | 6.11.2008 |
| D200 | *Tt* | 28 | M | 298 | 277 | 26.11.2008 |
| D206 | *Tt* | / | F | 210 | 119 | 8.5.2009 |
| D211 | *Tt* | / | M | 302 | 232 | 21.10.2009 |
| D212 | *Tt* | 20 | M | 299 | 245 | 9.1.2010 |
| D225 | *Tt* | 11 | F | 264 | 163 | 22.9.2010 |
| D232 | *Tt* | 19 | F | 273 | 247 | 4.12.2010 |
| D235 | *Tt* | 5 | F | 220 | 117 | 9.12.2010 |
| D244 | *Sc* | 30 | M | 213 | 89 | 24.1.2011 |
| D258 | *Sc* | 18 | M | 208 | 83 | 25.1.2012 |
| D259 | *Sc* | 17 | F | 210 | 73 | 27.1.2012 |
| D263 | *Sc* | 17 | M | 210 | 78 | 16.2.2012 |
| D265 | *Sc* | 17 | M | 198 | 89 | 1.4.2012 |
| D276 | *Tt* | / | M | 275 | 227 | 12.9.2012 |
| D288 | *Tt* | / | F | 272 | 266 | 12.2.2013 |
| D289 | *Tt* | / | M | 226 | 174 | 24.3.2013 |
| D371 | *Tt* | / | M | 249 | | 2.12.2015 |
| D373 | *Tt* | / | F | 244 | | 5.2.2016 |

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