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# ***Anisakis* spp. induced granulomatous dermatitis in a harbour porpoise *Phocoena phocoena* and a bottlenose dolphin *Tursiops truncatus***

Steven J. van Beurden<sup>1,\*</sup>, Lonneke L. IJsseldijk<sup>1,\*</sup>, Herman J. W. M. Cremers<sup>1</sup>,  
Andrea Gröne<sup>1,2</sup>, M. H el ene Verheije<sup>1</sup>, Lineke Begeman<sup>1,\*\*</sup>

<sup>1</sup>Pathology Division, Department of Pathobiology, and <sup>2</sup>Dutch Wildlife Health Centre, Faculty of Veterinary Medicine, Utrecht University, PO Box 80.165, 3508 TD Utrecht, The Netherlands

**ABSTRACT:** Cetaceans are well known definitive hosts of parasitic nematodes of the genus *Anisakis* (Nematoda: Anisakidae). Anisakid nematodes are also a health hazard for humans, potentially causing gastrointestinal infections or allergic reactions following the consumption of infected fish. In marine mammals, the nematodes develop from third-stage larvae to adults in the stomachs. In the first (or fore-) stomach, these parasites are typically associated with mucosal ulceration; parasites have not been identified in other organs. Two small cetaceans, a bottlenose dolphin *Tursiops truncatus* and a harbour porpoise *Phocoena phocoena*, presented marked gastric *A. simplex* infection, as well as chronic granulomatous and ulcerative dermatitis with intralesional nematodes, bordered by epithelial hyperplasia. Nematodes in the skin of the bottlenose dolphin were morphologically similar to *Anisakis* spp. Morphology of the parasitic remnants in the skin lesion of the harbour porpoise was indistinct, but molecular identification confirmed the presence of *A. simplex*. This is the first report of *Anisakis* spp. infection in the skin of marine mammals.

**KEY WORDS:** Anisakid · Nematode · *Anisakis simplex* · Marine mammal · Cetacean · Skin lesion

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## INTRODUCTION

The life cycle of anisakid nematodes is indirect. In the stomachs of cetaceans, the main definitive hosts, the third-stage larvae (L3) develop to fourth-stage larvae (L4) and then to adults. Eggs pass in the faeces and embryonate in seawater to the second larval stage (L2). The ensheathed larvae are swallowed by euphausiid shrimps (krill), where the L3 larvae then develop in the shrimp haemocoel. These first intermediate hosts are eaten by fish or squid, which serve as transport hosts. In these hosts, the larvae migrate to the coelomic cavity from where they penetrate and encapsulate in

skeletal muscle. The L3 larvae that are present in muscle of commercial fish, such as herring, pose a threat to public health. If an infected fish is consumed raw or insufficiently cooked, the larvae may cause gastrointestinal infections in humans, known as anisakidosis. An allergic reaction may also occur in humans after ingestion or contact with infected fish, ranging from urticaria and contact dermatitis to life-threatening anaphylactic shock (Nieuwenhuizen & Lopata 2013).

*Anisakis simplex* (Nematoda, Ascaridida, Anisakidae) is a common parasite of many cetacean species, infecting toothed and baleen whales. The distribution of *A. simplex* is worldwide, particularly in colder

\*These authors contributed equally to the study

\*\*Corresponding author: l.begeman@uu.nl

and temperate seas. In harbour porpoises *Phocoena phocoena*, *A. simplex* is most commonly present in the first (or fore-) stomach with possible extension into the second, third and fourth compartments (Herreras et al. 1997). The parasites extending farther than the first stomach are mostly the larval stages (Herreras et al. 2004). *A. simplex* likely has little impact on the general health of harbour porpoises, but may cause mucosal ulceration of the first stomach in case of heavy infection (Smith 1989, Lehnert et al. 2005). Infection burden for harbour porpoises differs per region, possibly reflecting the share of certain fish species in the diet (Lehnert et al. 2005). Severity of infection does not differ in subadult or adult harbour porpoises. Less is known regarding *Anisakis* infection in bottlenose dolphins *Tursiops truncatus*, but prevalence and pathogenicity are very likely to be comparable between different cetacean species (Abollo et al. 1998, Motta et al. 2008).

Here we describe 2 cases in 2 small cetacean species in which *Anisakis* was identified intradermally. Definitive identification was based on either morphological features or molecular testing. To our knowledge, this is the first report describing the presence of anisakid nematodes in lesions outside the gastrointestinal tract in definitive hosts.

## MATERIALS AND METHODS

### Case histories

A juvenile male harbour porpoise (114 cm total length) was found in May 2013, stranded dead on the island of Texel, The Netherlands. The carcass was frozen until transport, and necropsy took place approximately 1 mo later. The carcass had freezing artifacts, but showed few signs of autolysis.

A juvenile female bottlenose dolphin (266 cm total length) was found in June 2013, shortly after it died, near Krabbendijke in Oosterschelde National Park, The Netherlands. A solitary bottlenose dolphin was sighted a week before in this estuary, and since this is very rare, it was presumably the same animal. The carcass was directly transported, showed very little evidence of autolysis, and the gastrointestinal parasites were still alive at the time of necropsy.

### Pathological examination

Both necropsies were carried out according to the protocol of Kuiken & Gacia Hartmann (1991). A stan-

dard set of organ samples, including grossly visible skin and stomach lesions of both the harbour porpoise and the bottlenose dolphin, were collected for histology in 10% formalin. Formalin-fixed tissues were then embedded in paraffin according to standard protocols, and 4 µm sections were stained with haematoxylin and eosin (H&E) for light microscopic examination. Periodic acid-Schiff (PAS) stains were additionally made from skin, stomach and lung lesions to enhance visibility of aetiological agents.

### Morphological identification of the nematode species

Nematodes found in the lumen of the stomach in both the bottlenose dolphin and harbour porpoise were collected during necropsy and stored in 70% ethanol until morphological determination by a veterinary parasitologist (H. J. W. M. Cremers). Light microscopic H&E and PAS stained sections of the bottlenose dolphin skin lesion with larval and egg stages of nematodes were also evaluated by the same veterinary parasitologist. Distinction from other dermal or subcutaneous helminths known in cetaceans, including the nematode *Crassicauda* sp. (Tetrameridae, Spirurida) and the cestode *Phyllobothrium delphini* (Tetraphyllidea), was based on the distinctive morphological features of *Anisakis* spp., including the Y-shaped lateral chords and the absence of lateral alae. Distinction from *Crassicauda* sp. eggs was based on shape and wall thickness, which are more oval and thicker in the latter species.

### Molecular identification of the nematode species

Molecular identification of the nematode species in the skin lesion of the harbour porpoise was conducted by determining the partial gene sequences of 2 species-specific regions of the rRNA. Genomic DNA was extracted from formalin-fixed tissue of the skin lesion using the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's protocol for formalin-fixed animal tissue. Two PCRs were performed using *Taq* DNA polymerase (Thermo Fisher Scientific). The first PCR targeted the nematodes' partial 18S gene sequence using the previously published primers Nem\_S\_F (forward) and Nem\_18S\_R (reverse) (Floyd et al. 2005). The second PCR targeted the 18S-28S region (including ITS1, 5.8S and ITS2), using the previously published primers nemspec

18SF (forward) and NC2 (reverse) (Umehara et al. 2008). The thermal profile of both PCRs consisted of a denaturation step of 94°C for 5 min, 45 cycles of 94°C for 30 s, 54 or 52°C, respectively, for 30 s and 72°C for 1 min, and a final extension step at 72°C for 10 min, and was carried out in a T100 Thermal Cycler (Bio-rad). PCR products were analysed by gel electrophoreses, and resulting fragments of expected sizes (926 and 1396 bp, respectively) were cut from the gel and extracted using the QIAquick gel extraction kit (Qiagen). PCR products were subsequently ligated into the pGEM T-easy vector (Promega), transformed into *Escherichia coli* HB101 and sequenced from both ends (Macrogen). The resulting sequences were subjected to BLAST searches (with the primers removed) in the NCBI nucleotide database using default settings.

## RESULTS

### Necropsy findings

The harbour porpoise died due to a severe fungal pneumonia, caused by *Aspergillus fumigatus*, as confirmed by culture. The gastric lumen contained approximately 60 ascarids identified as *Anisakis simplex* based on morphology. The first stomach had a single 1.5 cm diameter, round mucosal ulceration (Fig. 1). Ventrally, between the pectoral flippers, there was a 7 × 3.5 cm oval skin lesion characterized by villar to filariform epithelial hyperplasia alternated with small erosions and ulcerations (Fig. 2).

Bottlenose dolphins are rarely seen near The Netherlands, and this animal was observed towards the upstream end of an estuarine zone with little suitable prey, approximately 30 km away from the open sea. The carcass weighed 196.5 kg, had an average blubber thickness of 14 mm, and its nutritional state was poor. The lumen of the stomachs of the bottlenose dolphin contained hundreds of ascarids morphologically identified as *A. simplex*. Six nodules, approximately 2 cm in diameter, with central ulceration in the mucosa of the first stomach, affected 10% of the mucosal surface. Along the base of the keel and extending transversely along the ventral aspect of the right fluke, there was a 15 × 4 cm oval region of depigmentation, an irregular surface with multiple 2 to 4 mm diameter cutaneous ulcers, surrounded by irregular epithelial hyperplasia (Fig. 3). The animal presumably died as a consequence of live stranding. It is not clear to what extent *Anisakis* infection in the stomach lumen might have played a role in starvation.

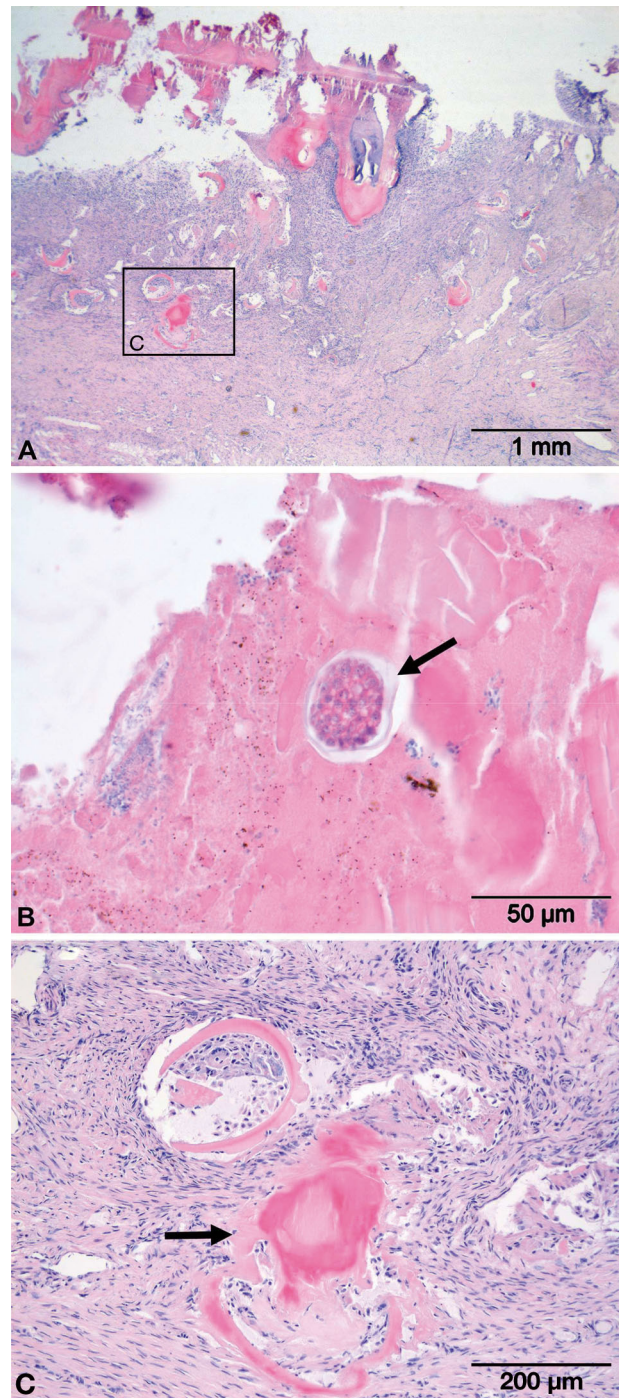


Fig. 1. Histology (with freezing artifacts) of the first stomach of a harbour porpoise *Phocoena phocoena*, showing severe gastric ulceration. (A) The submucosa is expanded by abundant mixed inflammatory cells admixed with parasitic remnants (box) and nematode eggs. Periodic acid-Schiff stain (PAS). (B) A nematode egg ~50 µm in diameter with a 2 to 3 µm capsule (arrow), the morphology of which is consistent with *Anisakis* eggs. The egg was present within the exudate on the surface of the ulceration in panel A. PAS. (C) Higher magnification of panel A, showing the crescent-shaped parasitic remnants (arrow). PAS

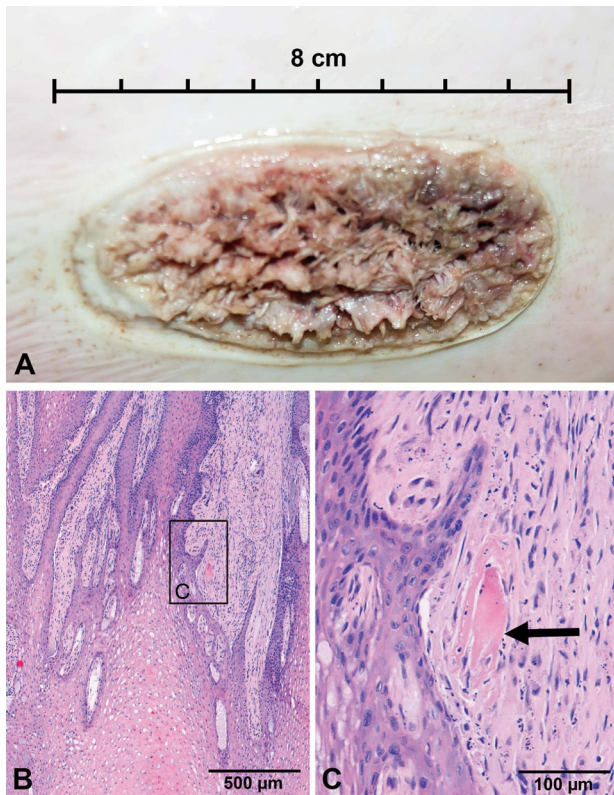


Fig. 2. Skin lesion of a harbour porpoise *Phocoena phocoena*. (A) Macroscopic picture showing an oval ulceration with irregular epidermal hyperplasia centrally. (B) Microscopic overview of the skin lesion shown in panel A, revealing marked epithelial hyperplasia. Within the dermis are occasional eosinophilic crescent-shaped structures surrounded by few macrophages and granulation tissue. Haematoxylin and eosin stain (H&E). (C) Higher magnification of an eosinophilic crescent-shaped structure in panel B (arrow). H&E

### Histopathological findings

The stomach of the harbour porpoise showed deep ulceration of the mucosa (Fig. 1A). The surface was covered with cellular debris admixed with fibrin and several 150 to 500 µm diameter cross-sections of coelomic nematodes with an undulating spiculated 40 µm cuticle, Y-shaped lateral cords, coelomyarian polymyarian musculature characteristic for *Anisakis* spp. Also present were loose nematode eggs of 45 to 49 µm in diameter and surrounded by a thin 2 to 3 µm capsule, consistent with *Anisakis* spp. (Fig. 1B). The underlying submucosa contained a mixed infiltrate, with variable numbers of eosinophils and giant cells with fibrosis; scattered throughout were crescent-shaped, brightly eosinophilic structures, interpreted as parasitic remnants (Fig. 1C).

Freeze artifacts hampered histopathology of the harbour porpoise's skin lesion. Erosion and ulceration of the epidermis, surrounded by hyperplastic epidermis with irregular broadened rete ridges were apparent (Fig. 2A). The underlying dermis with blubber exhibited focally extensive fibrosis with infiltration by moderate numbers of macrophages, lymphocytes and plasma cells (Fig. 2B). Eosinophilic crescentic structures were present in the superficial dermis (Fig. 2C). Further morphological characterization of these putative parasite remnants was impossible, as no intact nematodes were identified histologically.

Histologically, the skin lesion of the bottlenose dolphin (Fig. 3A) was ulcerated and surrounded by hyperplastic epidermis. On the surface and within the ulcerated area, several cross-sections of coelomic nematodes were present with similar morphology as described above for the harbour porpoise stomach, characteristic for *Anisakis* spp. (Fig. 3B). Some nematodes were partly degraded, leaving only eggs surrounded by neutrophils, cellular debris and fibrin (Fig. 3C,D). The dermis and underlying blubber were completely replaced by vascularized fibrous (granulation) tissue, in which several circular and crescent-shaped structures composed of glassy homogenous eosinophilic material (interpreted as remnants of nematodes) surrounded by multinucleated giant cells and macrophages were present (Fig. 3E). Adjacent dermis was infiltrated with moderate numbers of lymphocytes and plasma cells, indicative of chronic inflammation. In another cross section, *Anisakis* larvae were visible in between the dermis and the epidermis, surrounded by inflammatory infiltrates (Fig. 3F). Additional samples of the lesion were not available for molecular analysis and confirmation.

### Molecular identification of the nematode species

BLAST searches of the sequences generated by PCR on DNA extracted from the harbour porpoise's skin lesion revealed 100% sequence identity (885/885) to GenBank record U94365 for *Anisakis* (partial 18S gene) and 99.9% sequence identity (1352/1353) to GenBank record AB277822 for *A. simplex* (18S-28S region), identifying the nematode in the harbour porpoise skin as *A. simplex*.

### DISCUSSION

Post mortem examination and histopathology of a bottlenose dolphin and a harbour porpoise revealed

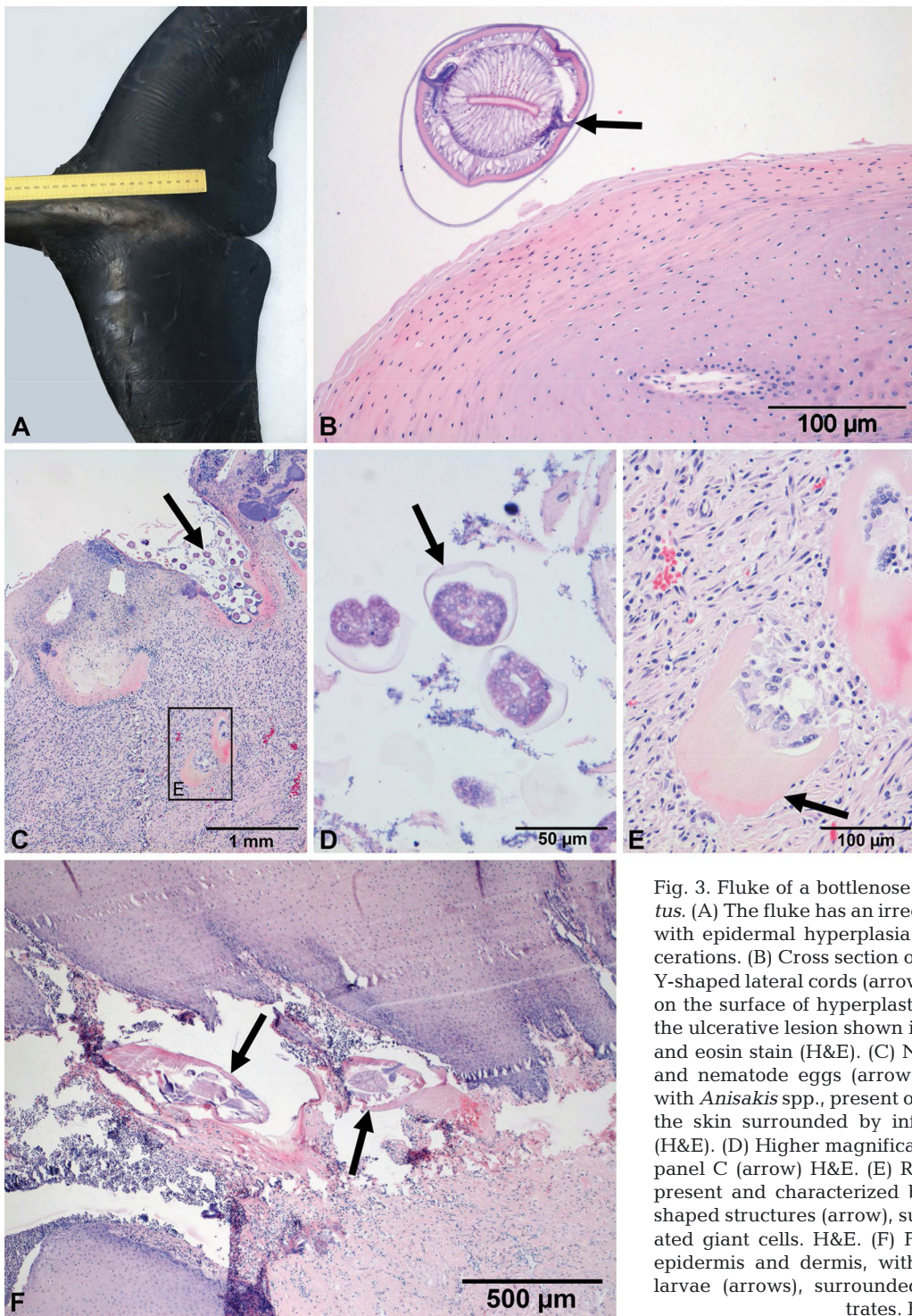


Fig. 3. Fluke of a bottlenose dolphin *Tursiops truncatus*. (A) The fluke has an irregular depigmented lesion with epidermal hyperplasia and small multifocal ulcerations. (B) Cross section of a nematode with visible Y-shaped lateral cords (arrow) typical of *Anisakis* spp. on the surface of hyperplastic epithelium adjacent to the ulcerative lesion shown in panel C. Haematoxylin and eosin stain (H&E). (C) Nematode remnants (box) and nematode eggs (arrow), morphology consistent with *Anisakis* spp., present on the ulcerated surface of the skin surrounded by inflammation and bacteria (H&E). (D) Higher magnification of nematode eggs in panel C (arrow) H&E. (E) Remnants of parasites are present and characterized by eosinophilic crescent-shaped structures (arrow), surrounded by multinucleated giant cells. H&E. (F) Focal disruption between epidermis and dermis, with intralesional nematode larvae (arrows), surrounded by inflammatory infiltrates. H&E

gastric and intradermal *Anisakis simplex* infection, resulting in ulcerative and granulomatous dermatitis. *Anisakis* larvae are not known to migrate in tissues of the definitive host, and in both cases described here, we observed no gross pathological

lesions in underlying tissues that would indicate migration from the digestive tract to the skin. Therefore, the route of infection of the skin in these 2 cases remains speculative. A potential route of infection is that L3 or L4 larvae left the stomachs,

passed through the intestines and were excreted with the faeces, or were regurgitated and excreted via the oral cavity. After excretion, they might have managed to adhere to and infiltrate the skin, as they normally would have done in the first stomach, likely through a skin abrasion already present. In both cases, the skin lesions were on the ventral side of the animal, with substantial distance (approximately 40–50 cm) from the anus. It is surprising that the larvae managed to attach to and invade the skin, and we hypothesize that social behaviour with congeners might have played a role here. The severe chronic inflammatory process (fungal pneumonia) and emaciation in the harbour porpoise and bottlenose dolphin, respectively, might have induced immunosuppression and made these animals more susceptible to *Anisakis* infection.

Histologically, the ulcerative skin lesions in these animals were similar to gastric (first compartment) lesions previously described in harbour porpoises (Smith 1989). Smith (1989) found that adult stages of *A. simplex* were mostly free living in the first stomach, while L3 and L4 larvae were most often clustering within stomach ulcers. It is not clear whether the *Anisakis*-associated stomach ulceration is induced by the larval stages of the nematode, or whether the larvae make use of a stomach ulcer already present due to another cause (Young & Lowe 1969).

The skin lesions in the bottlenose dolphin and the harbour porpoise were small and chronic, and probably did not significantly contribute to disease or death of these animals. The macroscopic changes of the skin (Figs. 2A & 3A) were different in size, location and aspect, which might be suggestive of a different primary cause for the lesions to have arisen. Histologically for both skin and stomach, the pattern of inflammation is typical for an inflammatory reaction against a parasite (Motta et al. 2008). The changes due to the inflammatory reaction against a parasite range from acute to chronic. In the bottlenose dolphin, it was an active lesion with clearly identifiable parasites and parasitic remnants, with the more superficial infiltrate surrounding the eggs representing an acute foreign-body-material response. In contrast, the lesion found in the harbour porpoise seemed to represent an older and almost completely resolved infection with few parasitic remnants remaining in the lesion. It is impossible to date the origin of the skin lesions in relation to the presence of the anisakid nematodes, but the amount of fibrosis and inflammatory infiltrates surrounding the parasitic remnants in the dermis indicates that inflammation was chronic.

In humans, most clinical cases are allergic responses to the ingestion of or contact with anisakid-parasitized fish. The contact dermatitis seen in people frequently processing fish is not associated with the actual presence of the parasite in human skin. When ingested alive, L3 larvae remain predominantly in the stomach and can develop to the L4 stage, but development to the adult stage does not occur in humans (Nieuwenhuizen & Lopata 2013). Lesions in the stomach and intestines of humans induced by the presence of the parasite (anisakidosis) are histopathologically classified into 5 types, progressing from a more acute to a chronic lesion. The acute phase is characterized by neutrophils, eosinophils, oedema and necrosis, whereas chronic lesions include lymphocytes, with foreign body type multinucleated giant cells surrounding degenerate larvae in cases of infections that have lasted over 6 mo. Sometimes the only lesion that remains is granulation tissue with few eosinophils, lymphocytes and total absence of the inciting nematode, categorized as type 5 (Sakanari & McKerrow 1989). The histopathological changes seen in the bottlenose dolphin skin show characteristics of both the acute and chronic forms, while the case in the harbour porpoise fits the description of the type 5 category in humans.

One human case of anisakiasis in the tonsil has been described, where the larva was suggested to have entered through the crypt of the tonsil (Bhargava et al. 1996). Interestingly, the crypt of the tonsil is also lined by stratified squamous epithelium, like the epidermis and the first stomach compartment in cetaceans. Hence, it seems that both in humans and in cetaceans, *Anisakis* may incidentally invade non-gastrointestinal tissues lined by stratified squamous epithelium, either with or without prior ulceration.

In future cases of acute or chronic skin ulceration, especially if there is a concurrent heavy gastric infection with *Anisakis* spp., clinicians and pathologists should be aware of *Anisakis* infection as a differential diagnosis. In case of inconclusive histology, e.g. when only parasitic remnants remain, frozen samples of the lesions should be subjected to molecular analysis in order to confirm diagnosis.

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