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NOTE

Fatal *Diplostomum phoxini* infection in captive Atlantic puffin *Fratercula arctica* chicks following ingestion of infected European minnows *Phoxinus phoxinus*

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ABSTRACT: Conservation of endangered animal species is a major task of zoos. Husbandry and breeding of Atlantic puffins *Fratercula arctica* in captivity is challenging. In 2019, the entire chick population (n = 4 chicks) in Berne Animal Park's Atlantic puffin colony (Bern, Switzerland) died within 7 d. Due to supply constraints, the chicks had been fed with wild-caught European minnows *Phoxinus phoxinus*. At necropsy, the main pathological finding in all deceased puffin chicks was a multifocal, moderate to severe subacute heterophilic and granulomatous enteritis with intralesional adult trematodes and eggs. Metacercariae surrounded by few necrotic cells and scattered macrophages were found in the brain and spinal cord of the food fish. Additional microbiological analyses of both the puffin chicks and fish were unremarkable. *Diplostomum phoxini* DNA could be identified in formalin-fixed paraffin-embedded tissue from the small intestine of all puffin chicks and European minnows following PCR and sequencing of the 18S ribosomal RNA gene and the internal transcribed spacer (ITS1) region. This report illustrates the importance of intensive health checks of food fish for animal species kept in captivity.

KEY WORDS: Atlantic puffin · European minnow · *Diplostomum phoxini* · Conservation · Food fish

1. INTRODUCTION

Zoos play an important role in the conservation of protected and endangered animal species worldwide within the context of the ongoing global extinction crisis (Conde et al. 2011, Godinez & Fernandez 2019). Good husbandry practices are essential for success. However, several factors can severely impair conservation efforts, namely poor breeding success, exposure to pathogens in non-adapted species and lack of suitable habitats for reintroduction (IUCN 2013).

The Atlantic puffin *Fratercula arctica* is a marine bird species belonging to the auk family (Alcidae) that was classified as Endangered by the International Union for Conservation of Nature (IUCN & BirdLife International 2022) due to its rapid decline across Europe. Despite their attractiveness to the public, Atlantic puffins are difficult to keep and rear in captivity due to low reproduction rates and high chick mortality (Tocidlowski et al. 1997). Notably, among Species360 Zoological Information Management Software (ZIMS 2022)-affiliated institutions,

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Atlantic puffins are only kept in 7 European, 5 North American, and 3 Asian zoological institutions, numbering a total of 171 individuals alive and only 11 births in the past 12 mo (data accessed on the 1 June 2022). Another limitation that impairs the maintenance of these animals in captivity is the limited knowledge of threatening diseases. From the 888 Atlantic puffin deaths recorded in ZIMS, the cause of mortality was determined in only 14% (n = 122). In Switzerland, this species is kept and bred at Berne Animal Park (Rosset & Blatter 2019) (at present 13 birds), which is one of 5 zoos worldwide that breed this species at present (ZIMS 2022). The most frequent cause of death since 2009 in the adult animals (n = 32) from the Bernese flock is aspergillosis (47%), followed by avian malaria (25%; Rosset & Blatter 2019, Meister et al. 2021).

The *Diplostomum* genus represents a large group of freshwater parasitic digenean trematodes infecting fish and birds in all major freshwater environments with a complex life cycle (Hoffman & Hundley 1957, Landeryou et al. 2020). Cercariae released from lymnaeid snails (first intermediate host) infect freshwater fish (second intermediate host) and migrate to brain, eyes, or central nervous system and develop into metacercariae. Fish-eating birds (definitive host) are infected upon ingestion of infected fish, and adult flukes live in the intestine (Karvonen et al. 2006). In recent years, several molecular studies revealed a substantial genetic diversity and provided a framework for new insights in the vast taxonomic diversity and distribution of the species of this genus (Georgieva et al. 2013, Blasco-Costa et al. 2014, Landeryou et al. 2020). Although infection with digenean trematodes has been described in different auk species, *Diplostomum* spp. has only been diagnosed in the common murre *Uria aalge*, the thick-billed murre *U. lomvia*, and the razorbill *Alca torda* (Muzaffar & Jones 2004), without any significant pathogenicity.

In this manuscript, we describe the fatal infection of Atlantic puffin chicks with *D. phoxini* following ingestion of infected European minnows *Phoxinus phoxinus*, which led to the loss of the entire 2019 offspring from the Atlantic puffin flock at Berne Animal Park.

2. MATERIALS AND METHODS

2.1. Atlantic puffins and European minnows

The total Atlantic puffin offspring of 2019, consisting of 4 males at age 5 d (body weight 60 g), 14 d (98 g), 34 d (280 g) and 45 d (323 g), died within a single week. They were raised as previously described (Rosset & Blatter 2019). Diet consisted of pellets (Ibis-See-(Eis-) Ente-Spezial size 5, Lundi) and thawed sand smelts *Atherina boyeri* (Parlevliet & Van der Plas). Chicks from earlier successful breeding seasons were additionally fed fresh juvenile captive rainbow trout *Oncorhynchus mykiss* from a local fish farm starting from the second day following hatching. However, due to supply difficulties of the usual food fish, the 2019 puffin offspring were fed fresh wild minnows *Phoxinus phoxinus* (1 fish of approximately 5 g bird^{-1} d^{-1}) caught in a local stream (Giesse, 46° 53′ 49″ N, 7° 31′ 57″ E), beginning 3 d before the first death.

After onset of the first clinical symptoms, the puffin chicks were immediately treated symptomatically with meloxicam (Metacam, 1 mg kg^{-1} 1× daily) and enrofloxacine (Baytril, 10 mg kg^{-1} 1× daily) or metronidazole (Flagyl, 60 mg kg^{-1} 1× daily), including forced feeding. Clinical investigations performed on site included general examination, fecal examination for parasites (flotation and sodium acetate–acetic acid–formalin [SAF] method), fecal bacterial culture, hematology, and blood chemistry.

2.2. Pathological and microbiological analysis of Atlantic puffin chicks and food fish

All dead puffins were submitted for pathological and microbiological analyses. Tissue samples from the intestine, proventriculus, gizzard, crop, pancreas, lung, trachea, heart, eye, skeletal musculature, kidneys, brain, liver, spleen, bursa, and cloaca were collected for histopathological analysis. The intestinal content from 2 chicks was submitted for parasitological flotation analysis. Lung, liver, and kidney samples from the same 2 chicks were submitted for bacteriological analysis.

All Atlantic puffin chicks were tested for avian influenza using cloacal swabs (FASTest® AIV Ag Testkit; Megacor, Hörbranz); an RT-PCR for Usutu virus according to S. R. R. Pisano et al. (unpubl. data) from brain, liver, and spleen of 2 chicks was also performed. Diagnostic tests for avian influenza and Usutu virus were negative.

Food fish were euthanized using 3-aminobenzoic acid ethyl ester (MS 222®, Argent Chemical Laboratories) at a dosage of 150 mg l^{-1} water buffered with bicarbonate at pH 7. All specimens were tested for the presence of external and intestinal parasites. Parasite exams involved direct microscopic examination of wet mounts of skin, gills, and intestinal content. A standard necropsy was performed. Spleen, liver and

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kidney were cultured on blood agar plates (Biomerieux) for bacterial growth for 48 h at 22°C and plates examined daily. Skin, brain, liver, spleen and kidney from 5 animals were fixed in RNAlater (Sigma Life Science) prior to fixation and stored at 4°C.

The tissue samples from both the puffin chicks and whole specimens of the European minnows showing macroscopical changes were fixed in 10% neutral buffered formalin, paraffin embedded, cut at 4 µm, stained with haematoxylin and eosin (H&E), and examined under the light microscope.

2.3. Molecular identification of trematode species

Formalin-fixed paraffin-embedded (FFPE) tissue and RNAlater-fixed fish brain material were used to identify the metacercariae and adult trematodes detected in the fish and bird tissue, respectively. Two 20 µm sections of FFPE material were prepared. Each section was deparaffinised and lysed, using 200 µl ATL buffer (QIAGEN) and 30 µl Proteinase K at 56°C for 2 h. DNA was extracted from lysed tissue and RNAlater-fixed brain tissue directly using the DNeasy tissue Kit (QIAGEN), according to the manufacturer's protocol. Samples were incubated with Proteinase K at 56°C and shaken at 300 rpm overnight. DNA yield was determined spectrophotometrically (NanoDrop Technologies). Conventional PCR was performed using HotStarTaq DNA Polymerase (QIAGEN) according to the manufacturer's instructions. PCRs were performed with newly designed primers based on alignments of publicly available sequences (GenBank: AB693170, HM064962, KX931440, KX931442, KX931443, MF171002, MF171009) targeting a 130 bp region of the internal transcribed spacer region 1 (ITS1; POSTHO-F1 5′-TTG CAT CTC ATA AGT ACG GTC-3′, POSTHO-R1 5′-GCA CTG AGA TAG ATA CAG TAC T-3′) and a 289 bp region of the 18S ribosomal RNA gene (POSTHO-F1, POSTHO-R2 5′-TAC CAG TCA TAC AAG ACA ACC-3′) of the Diplostomidae family. The following PCR conditions were used: initial denaturation at 95°C for 15 min; 35 × (95°C for 30 s, 50°C for 45 s, 72°C for 45 s); and final extension at 72°C for 10 min. Primers DIPLO-F2 (5′-ATG GGC TTC CCG CAA GGG AC-3′) and DIPLO-R3 (5′-CCA TCA CCC TTG GCA TGA CAA GGC-3′) were designed based on alignments of *Diplostomum* spp. sequences (GenBank: AY386141, MT990746, MT322848) and used to amplify a 222 bp fragment of the ITS1 region to further differentiate closely related *Diplostomum* spp. Promega GoTaq polymerase (Promega) was used according to the manufacturer's instructions and the following PCR conditions: 95°C for 2 min; 35 × (95°C for 45 s, 62°C for 30 s, 72°C for 30 s); and 72°C for 5 min. DNAse-free water was used as control. PCR products were purified with WIZARD SV Gel and PCR Clean-Up System (Promega AG), resolved on a 1.5% agarose gel and sent for Sanger sequencing (Microsynth AG). Sequences were deposited in GenBank (www.ncbi.nlm.nih.gov) and analysed with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The amplified sequences were aligned with closely related and identical sequences available in NCBI using the MUSCLE algorithm in the Geneious software package. Trimmed 88 or 178 bp products amplified with F1/R1 or F1/R2, respectively, aligned and analysed with jModelTest 2.1.10 (Darriba et al. 2012). Maximum likelihood (ML) trees were constructed in IQ-TREE (Trifinopoulos et al. 2016) with the TPM3+F and K80+G model, respectively, and visualized in Archaeopteryx 0.9928 (Han & Zmasek 2009).

3. RESULTS

3.1. Clinical, pathological, and microbiological findings in Atlantic puffin chicks

The youngest chick died peracutely, while the remaining chicks displayed inappetence to anorexia, regurgitation, and weight loss dying within 1 or 2 d after onset of symptoms. Blood chemistry and hematology were within normal limits, microbiological analyses were inconclusive, and the animals did not respond to symptomatic treatments.

All chicks were emaciated. One chick had apparent pericardial visceral gout, 2 chicks had dilated gallbladder and 1 chick a crop dilated with fish and pellets. Histologically, cross and longitudinal sections of adult trematodes, measuring approximately 700 × 300 µm, occasionally attached to the intestinal mucosa with an oral sucker, were found in the lumen of the small intestine of every chick (Fig. 1a). Moderate to high numbers of heterophils and macrophages and occasional multinuclear giant cells, fibroblasts, and collagen deposition infiltrated and expanded the lamina propria (Fig. 1b,d). Additionally, there were varying numbers of non-embryonated and embryonated trematode eggs measuring 50–70 µm in diameter with a smooth, approximately 4 µm thick yellow-brown shell (Fig. 1c) present within the intestinal lumen, which were often intermingled with cellular debris. The morphological diagnosis was moderate to severe, multifocal, subacute heterophilic and

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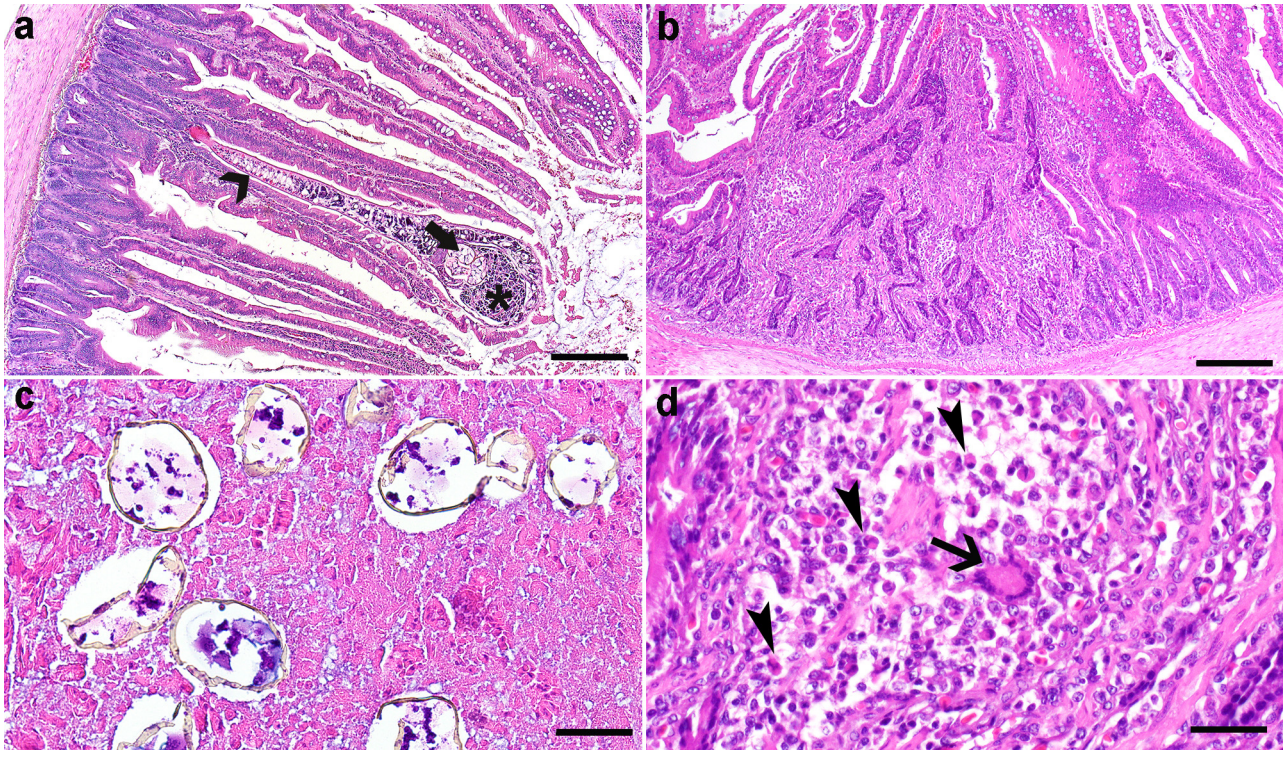

Fig. 1. Histopathological findings in the small intestine of the submitted Atlantic puffin chicks *Fratercula arctica*. (a) Longitudinal section of an adult trematode attached to the intestinal mucosa via an oral sucker. Yolk glands (arrowhead), testis (asterisk), and uterus containing eggs (thick arrow) can be clearly observed. H&E stain, scale bar = 200 µm. (b) Severe, focal thickening of the intestinal lamina propria due to severe infiltration with heterophils, macrophages and occasional multinuclear giant cells with incipient infiltrates of fibroblasts. H&E stain, scale bar = 200 µm. (c) Abundant nonembryonated and embryonated trematode eggs could be observed within the lumen measuring ca. 50–70 µm in diameter with a smooth, ca. 4 µm thick yellow-brown shell. H&E stain, scale bar = 50 µm. (d) Inflammatory infiltrate composed of abundant heterophils (arrowheads) and multinuclear giant cells (arrow) in the small intestine lamina propria. H&E stain, scale bar = 80 µm

granulomatous enteritis with intralesional adult trematodes and eggs and intraluminal cellular debris.

Visceral gout was present in 1 chick, and moderate to severe renal intraluminal minerali deposits with mild tubular degeneration were detected in 2 chicks compatible with dehydration/nutritional impairment secondary to the parasitic infection. Parasitology and bacteriology were negative.

3.2. Pathological and microbiological findings in European minnows

Several randomly distributed, brownish to black irregular-sized cutaneous discolorations were observed (Fig. 2a). Direct parasitological examination of the skin and gills revealed a severe infection with *Ichthyophthirius multifiliis*. Bacteriology showed low numbers of mixed bacteria deemed inconclusive.

Histopathologically, trematode metacercariae were found in the subcutis, muscle (measuring ca. 70 × 200 µm; Fig. 2b), and central nervous system (CNS) but not the eyes. Metacercariae were surrounded by basophilic granulated material, a fibrous capsule, macrophages, and melanocytes, responsible for the brownish cutaneous discolorations seen grossly. In the CNS, metacercariae measured 30 × 100 µm, with a clearly visible apical sucker (Figs. 2c,d). They were surrounded by cellular debris and scattered macrophages.

3.3. PCR and sequencing

Puffins' and minnows' FFPE material and directly fixed minnows were PCR positive. Sequences from intestines of the puffins and the brain of the minnows were 100% identical with *Diplostomum phoxini* (Figs. 3). For the fish skin, all amplicons were 100% identitical with *Posthodiplostomum cuticola* (Fig. 3a). All sequences from European minnows and puffins were deposited in GenBank under the following

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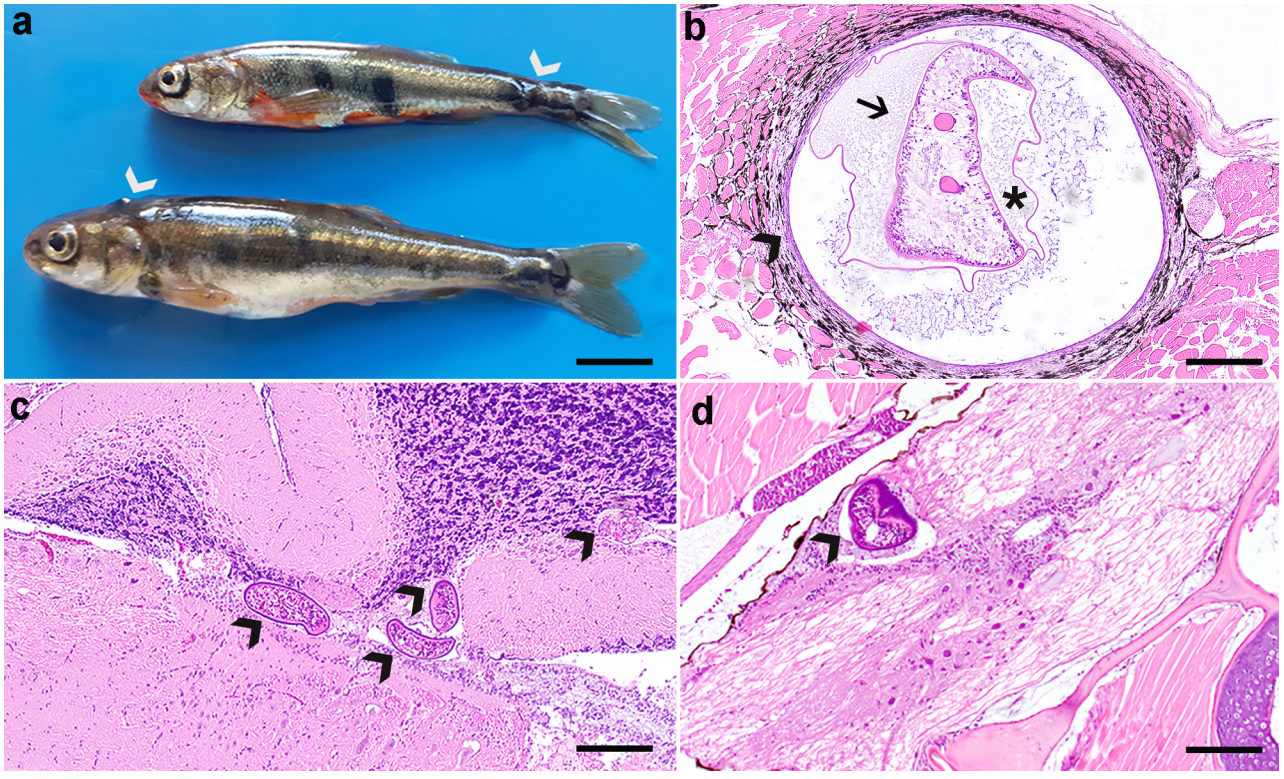

Fig. 2. Pathological findings in European minnows *Phoxinus phoxinus* used as food fish for puffin chicks. (a) Multiple randomly distributed, slightly elevated brownish to black discolorations of the skin (white arrowheads). Scale bar = 1 cm. (b) Trematode metacercaria in the subcutis (arrow) surrounded by basophilic granulated amorphous material (asterisk), surrounded by fibroblasts, macrophages, and melanocytes (arrowhead). H&E stain, scale bar = 50 µm. (c) Brain with multiple metacercariae in the ventricle (arrowheads). H&E stain, scale bar =100 µm. (d) Spinal cord with single metacercaria (arrowhead) with malacia in surrounding neuropil. H&E stain, scale bar = 50 µm

acession numbers: *D. phoxini* OM811655–OM811658 (ITS1 partial sequence obtained with POSTHO-F1 and POSTHO-R1 primers) and OM811659–OM811662 (ITS1 partial sequence obtained with DIPLO-F2 and DIPLO-R3 primers), and *P. cuticola* OM811653 and OM811654 (18S ribosomal RNA gene partial sequence obtained with POSTHO-F1 and POSTHO-R2 primers).

4. DISCUSSION

The infection with the digenean trematode *Diplostomum phoxini* of the entire 2019 Atlantic puffin offspring from Berne Animal Park colony probably led to nutrient and energy loss following damage of the intestinal barrier (Schmidt et al. 2006, McKay et al. 2017) and ultimately to peracute or acute death. This infection was possible because the strict Atlantic puffin rearing protocol was adapted by replacement of captive rainbow trout by wild-caught European minnows infested with *Posthodipostomum cuticola* and *D. phoxini* metacercariae.

D. phoxini is one of the few species of *Diplostomum* spp. with a strict secondary intermediate host as the European minnow, even though adult *D. phoxini* trematodes have been recovered from the small intestine of avian and mammalian hosts (Schwelm et al. 2021). Adult *D. phoxini* are not known to cause disease in the avian definitive host. However, digenean trematode infections can vary in pathogenicity, depending on infection burden, parasite, and affected avian species (Huffman 2008). As few as twenty *Sphaeridiotrema globulus* can lead to death in American coots *Fulica americana* and mute swans *Cygnus olor*, whereas in Muscovy ducks *Cairina moschata*, mallards *Anas platyrhynchos* and Canada geese *Branta canadensis*, the fatal worm burden may range from 100 to 3300 parasites (Trainer & Fischer 1963, Campbell & Jackson 1977, Roscoe & Huffman 1982). Experimental infection with only 7 *Cyathocotyle bushiensis* trematodes may be fatal in ducks (Hoeve & Scott 1988). Interestingly, both *Sphaeridiotrema* spp. and *C. bushiensis* are non-native in the midwestern North American states and Canada and were intro-

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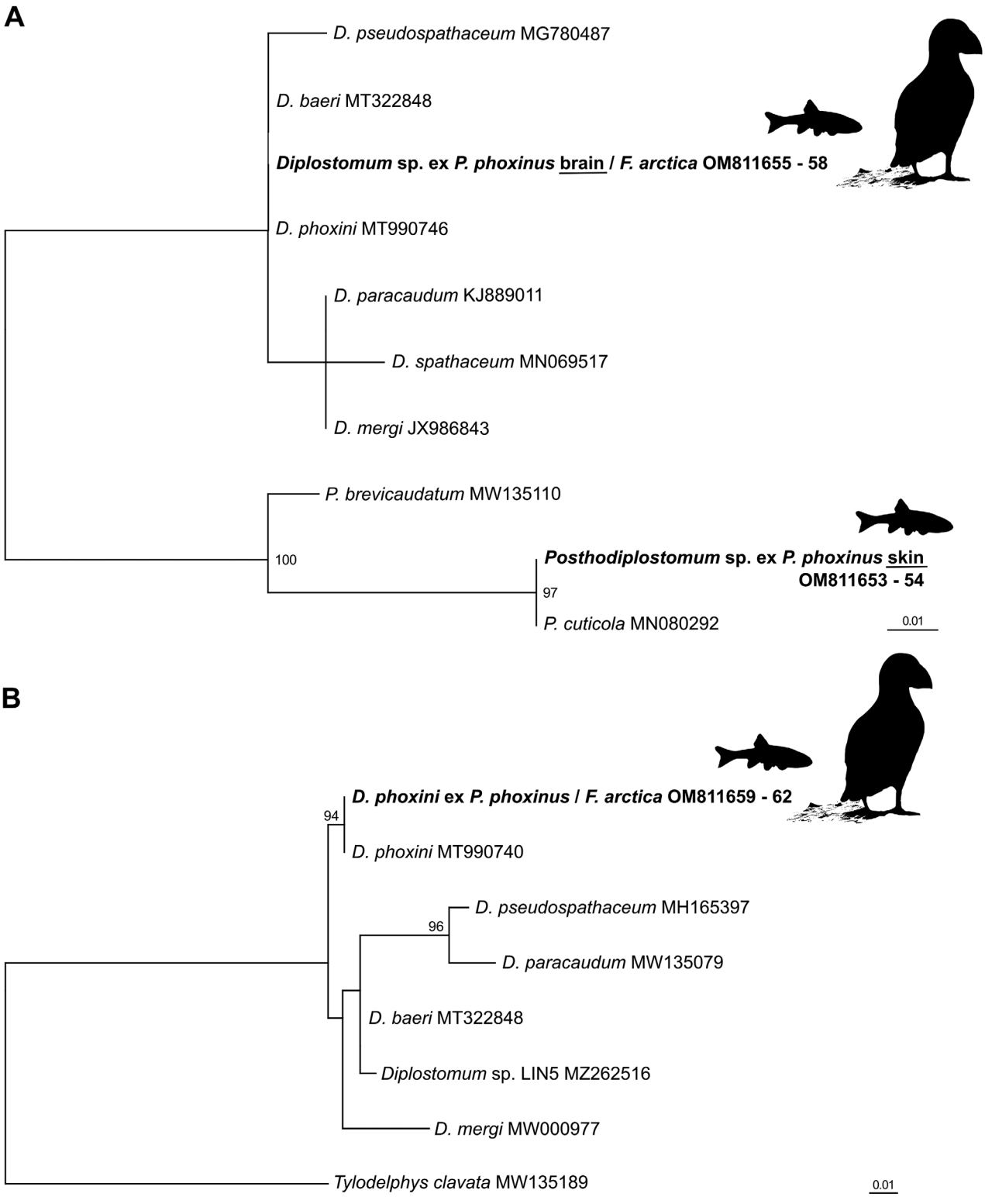

Fig. 3. (a) Unrooted phylogenetic tree revealed clustering of parasites in the brain of European minnows *Phoxinus phoxinus* and intestine of Atlantic puffins *Fratercula arctica* with *Diplostomum* spp. distant from the *Posthodiplostomum* parasite detected in the subcutis of the same European minnows. (b) Maximum likelihood (ML) tree depicts 100% nucleotide identity of the parasite species detected in the brain of the minnows and intestine of the puffins with *D. phoxini*. Bootstrap values above 70 are given, and *Tylodephys clavate* was used as an outgroup. Scale bars = number of substitutions

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duced in the continent via an invasive intermediate host, the Eurasian faucet snail *Bithnynia tentaculata* (Roy & St-Louis 2017). This explains the high susceptibility of some American bird species upon infection with these non-native trematode parasites. In parallel, free-living Atlantic puffins feed on marine fish species and never ingest freshwater European minnows. The living conditions for puffins in Switzerland are very different from those in the wild. It is also possible that the puffin chicks were more susceptible to infection due to their developing immune system.

In conclusion, we hypothesize that the ingestion of European minnows infected with *D. phoxini* led to the death of all Atlantic puffin offspring hatched in 2019 at Berne Animal Park. To prevent such incidents from recurring in the future, an intensive health check of fresh food fish and a closed supply chain with a known health history is essential, especially for protected, endangered, and vulnerable zoo animals that are particularly difficult to keep and breed in captivity, such as Atlantic puffins.

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