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Parasitic infections in the East Asian finless porpoise *Neophocaena asiaeorientalis sunameri* living off the Chinese Yellow/Bohai Sea coast

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ABSTRACT: Parasitic disease is among the major causes of health problems in marine mammals. However, little information on parasitic species composition and infection levels in finless porpoises (*Neophocaena* spp.) is available. In this study, we report the first systematic survey on parasitic infections in the East Asian finless porpoise *N. asiaeorientalis sunameri* (EAFP) living off the Chinese Yellow/Bohai Sea coast. Using both morphological and molecular methods, 5 parasitic helminths were identified: *Campula oblonga* in the liver and pancreas; *Synthesium seymouri* in the pyloric stomach and duodenum ampulla; *Anisakis pegreffii* in the forestomach, main stomach, and esophagus; *Halocercus* sp. in the lungs; and *Crassicauda magna* in the mammary glands and muscle. Among these helminths, *C. oblonga* (80.7%), *S. seymouri* (80.7%), *A. pegreffii* (80.7%), and *Halocercus* sp. (77.4%) were the most prevalent, whereas *C. magna* (6.5%) were only observed in 2 EAFP individuals. All juvenile and adult EAFPs were parasitized by at least 3 parasites species (*C. oblonga*, *S. seymouri*, and *A. pegreffii*), whereas in neonates, only *Halocercus* sp. were detected. We observed no significant difference in parasite prevalence between males and females. In addition, *A. pegreffii* and *C. magna* represented new infection records in *Neophocaena*. A pathological examination associated with parasitic lesions in EAFPs showed damage or destruction of cells or tissues to some extent. This study represents the first systematic survey on parasitic infections in EAFPs, providing important and valuable parasitological information for the research and conservation of this coastal marine mammal.

KEY WORDS: Cetacean · Helminth · Trematoda · Nematoda

INTRODUCTION

One of the major causes of health problems in marine mammals are parasites. Cetaceans are infected with a wide variety of parasites in various tissues (Dailey 2001). Although some parasites are apparently not debilitating to their hosts, other species can cause important health problems, even death, and can be harmful enough to affect an entire population (Smith & Wootten 1978, Dailey 2001). The parasitic fauna of cetaceans has become an important cause of death, and data for further ecological studies are needed (Raga et al. 2002). However, parasites are almost unknown in the finless porpoises of the genus *Neophocaena*, which have been reclassified as the Indo-Pacific finless porpoise *N. phocaenoides* and

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the narrow-ridged finless porpoise *N. asiaeorientalis*, including its 2 subspecies (the freshwater Yangtze finless porpoise *N. a. asiaeorientalis* and the coastal East Asian finless porpoise *N. a. sunameri*, hereafter EAFP) (Jefferson & Wang 2011).

The EAFP is the marine subspecies of the narrow-ridged finless porpoise and has a wide distribution from the Taiwan Strait north to the Yellow/Bohai Sea in China and waters of Korea and Japan (Jefferson & Wang 2011). Because of a suspected past decline in population size, primarily reflecting a wide variety of human activities, such as bycatch, ship strikes, and other anthropogenic activities, narrow-ridged finless porpoises have been classified worldwide as 'Vulnerable' (VU) according to the IUCN Red List of Threatened Species (Wang & Reeves 2012). However, unlike its freshwater counterpart, the Critically Endangered Yangtze finless porpoise, insufficient efforts have been made to conserve EAFPs, and accordingly less conservation biology information is available about this subspecies. In recent parasitology studies, for example, although parasitic infection is common in stranded or bycatch EAFPs, this condition has not drawn enough attention from biologists. Indeed, in recent years, only sporadic parasitology studies have been conducted on finless porpoises living in Japanese waters (Nakayama et al. 2009, Isobe et al. 2011); this population was reclassified as *N. a. sunameri* in 2011 (Jefferson & Wang 2011). Among the studies of the parasites of EAFPs living off the Chinese Yellow/Bohai Sea coast, there are only 2 morphological reports describing 1 nematode (*Halocercus pingi* Wu 1929) and 2 trematodes (*Campula oblonga* and *Hadwanius seymouri*) in some finless porpoises *N. phocaenoides* stranded in 1983 and 1985 in Jinzhou City, Liaoning Province, China (Tao 1983, Zhang 1985); this population was also reclassified as *N. a. sunameri* in 2011 (Jefferson & Wang 2011). Recently, molecular approaches have been widely applied to identify parasites in cetaceans (Cavallero et al. 2011); however, these techniques have not been applied in parasitology studies of finless porpoises. Thus, parasitology studies focusing on a larger population of EAFPs living off the Chinese Yellow/Bohai Sea coast and a more accurate identification of parasites based on molecular methods are necessary. In addition, histopathological studies on parasite-infected organs are also important to better characterize parasite-associated pathology in EAFPs and provide insights into the evaluation of the health conditions of these animals.

The purpose of the present study was to survey the composition, prevalence, and distribution of parasitic helminths in the EAFP living off the Chinese Yellow/Bohai Sea coast. In addition, pathological findings associated with parasite-infected organs are also presented. These results might be used as a baseline for future studies estimating the impact of parasitic infections on EAFPs, thereby contributing to the protection of this poorly understood porpoise population.

MATERIALS AND METHODS

Sample collection

We collected 31 bycatch EAFPs (21 females and 10 males) from the coastal waters of Penglai City, Shandong Province, China (Fig. 1), between April 2015 and May 2016. Biological information on the sampled EAFPs is provided in Table 1. These animals were accidentally caught in gill nets and discovered within a few hours after death by local fishermen. Fresh carcasses were transported to a necropsy suite at the Penglai Sea World for necropsy. Each organ was examined. The collected data included biological features, e.g. sex, body length, body weight, developmental stage, sampling date, and gross pathology, including a macroscopic examination of the presence or absence of parasites-infected organs. The developmental stage of the sampled EAFPs was determined based on the estimated age and ultrasonic examination of the genital organs. The age of each porpoise was determined using the age–length curve established for this population (Gao & Zhou 1993). Developmental stage was classified as fetus, newborn, juvenile, and adult (Table 1). Newborns were identified as infants retaining rostral hairs, an unhealed umbilicus, and fetal folds; juveniles were identified as both females and males with body lengths >95.5 cm (based on weaning time at approximately 6 mo of age; Zhang 1992). Adults were identified as both females and males with body lengths >137.1 cm (based on sexual maturation at 5 yr of age; Gao & Zhou 1993). Accurate estimates of the infection intensity were generally impossible, reflecting the large number of parasites observed in most sampled porpoises. Thus, the numerical data for parasites was restricted to prevalence (Bush et al. 1997). A Fisher's exact test was used at a statistical significance level of $\alpha = 0.05$ (2-sided) to determine differences between parasite prevalence of male and female EAFPs.

For parasite species identification, different types of parasites were collected during the necropsy, washed in physiological saline, and preserved in

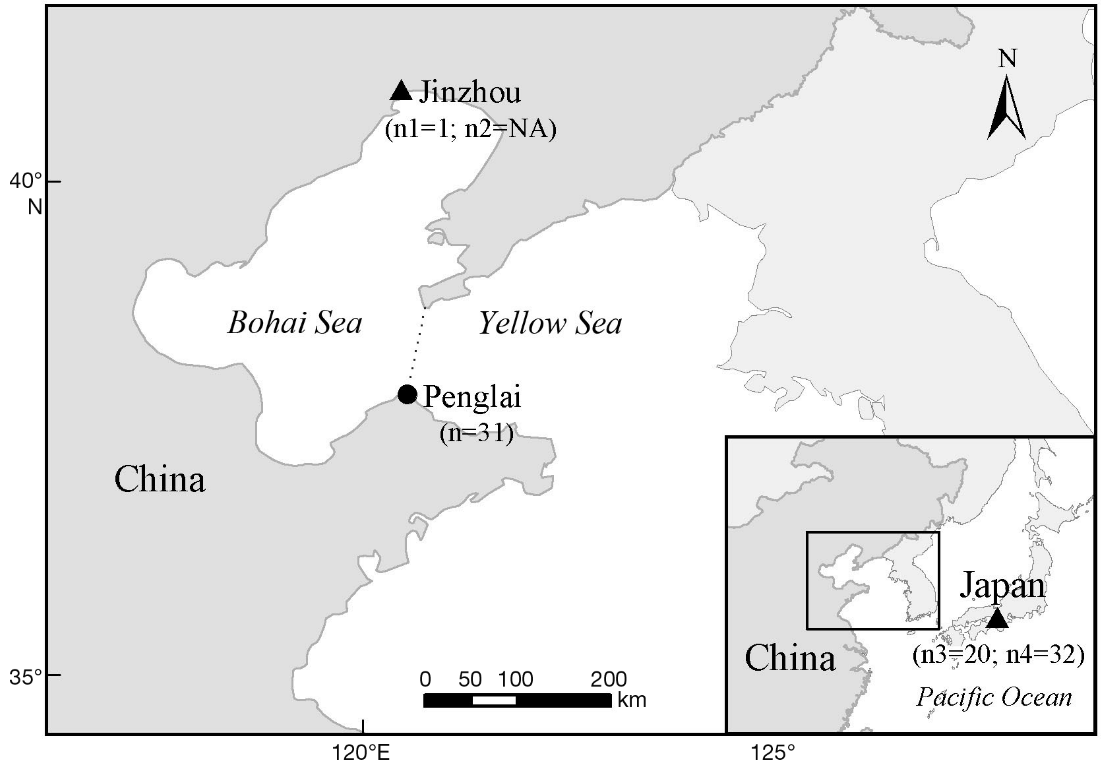

Fig. 1. Sampling location (black dot) and sites of previous studies (black triangles) on parasites of East Asian finless porpoises *Neophocaena asiaeorientalis sunameri*. n: sample number in our study; n1–4: sample numbers in previous studies (Zhang 1985, Tao 1983, Isobe et al. 2011, Nakayama et al. 2009, respectively). NA: not available. Dotted line shows the rough boundary of the Bohai and Yellow Seas

70% ethanol. In addition, typical pathological tissues associated with parasite infection were sampled and fixed in 10% neutral-buffered formalin for conventional histopathology examination.

Parasite identification

Microscopic examination was initially performed to distinguish nematodes and trematodes. Nematodes were identified according to Gibson et al. (1998). The internal structures of nematodes were visualized under an optical microscope, and morphological identification was performed based on available identification keys (Dougherty 1944, Davey 1971, Lambertsen 1985). Trematodes were stained using a previously described protocol (Gibson et al. 1998). Morphological identification of trematodes was conducted according to Marigo et al. (2008) and Gibson (2005).

For widely studied parasites with public genetic information, genomic DNA was extracted using a Tissue & Cell Genomic DNA Purification Kit (GeneMark) for further accurate identification based on molecular methods. In the present study, different primers were used to amplify the ribosomal RNA gene or mitochondrial cox2 gene of the sampled parasites (Table 2). The PCR products were subsequently purified using a DNA Gel Extraction Kit (Bioteke) following the manufacturer's instructions. The purified PCR products were sequenced using the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) on an ABI® 3730 automated DNA sequencer. The sequences were assembled using DNAMAN 8.0. For parasite identification, the sequences were compared with the ribosomal RNA gene and mitochondrial cox2 gene of existing organisms using the NCBI GenBank nucleotide database. Identification at the species level was defined as a sequence showing ≥97% similarity with that of the sequence in GenBank, and identification at the genus level was defined as a similarity of ≥95%.

All of the sequences obtained in the present study were deposited in the GenBank nucleotide database. The accession numbers and sequence similarities are given in Table 2.

Table 1. Basic information on the East Asian finless porpoises *Neophocaena asiaeorientalis sunameri* examined in the present study. F: female; M: male. Body length was measured as the straight length from snout to fluke notch. C: *Campula oblonga*; S: *Synthesium seymouri*; A: *Anisakis pegreffii*; H: *Halocercus* sp.; Cr: *Crassicauda magna*. +: presence; −: absence

Specimen ID	Developmental status	Sex	Body length (cm)	Weight (kg)	Presence of parasites C	S	A	H	Cr
PL01	Fetus	F	77.5	7.0	−	−	−	−	−
PL02	Fetus	F	80.5	6.6	−	−	−	−	−
PL03	Fetus	M	78.0	7.6	−	−	−	−	−
PL04	Newborn	F	79.0	9.0	−	−	−	+	−
PL05	Newborn	M	82.0	13.0	−	−	−	+	−
PL06	Newborn	M	84.0	8.5	−	−	−	−	−
PL07	Juvenile	F	104.0	20.0	+	+	+	+	−
PL08	Juvenile	F	105.6	21.0	+	+	+	+	−
PL09	Juvenile	M	107.0	22.0	+	+	+	−	−
PL10	Juvenile	M	109.0	21.2	+	+	+	+	−
PL11	Juvenile	F	110.0	22.3	+	+	+	+	−
PL12	Juvenile	M	110.0	26.4	+	+	+	+	−
PL13	Juvenile	F	114.0	23.4	+	+	+	+	−
PL14	Juvenile	M	120.0	30.2	+	+	+	+	−
PL15	Juvenile	F	120.0	26.4	+	+	+	+	−
PL16	Juvenile	M	128.0	21.2	+	+	+	+	−
PL17	Juvenile	F	131.0	33.4	+	+	+	+	−
PL18	Juvenile	F	135.0	34.2	+	+	+	+	−
PL19	Juvenile	F	137.0	37.0	+	+	+	+	−
PL20	Adult	F	138.0	35.2	+	+	+	+	−
PL21	Adult	F	140.0	28.5	+	+	+	+	−
PL22	Adult	F	148.0	47.2	+	+	+	+	−
PL23	Adult	F	150.0	60.6	+	+	+	+	−
PL24	Adult	F	153.0	52.0	+	+	+	−	−
PL25	Adult	M	156.0	51.8	+	+	+	+	−
PL26	Adult	F	156.0	50.0	+	+	+	−	−
PL27	Adult	F	162.8	41.4	+	+	+	+	−
PL28	Adult	F	163.0	64.6	+	+	+	+	−
PL29	Adult	F	169.2	60.0	+	+	+	+	+
PL30	Adult	F	171.0	61.0	+	+	+	+	+
PL31	Adult	M	191.6	75.0	+	+	+	+	−

Histopathological examination

The pathological tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin wax, cut into 5–7 µm sections, and stained with hematoxylin and eosin (H&E) for histological examination.

Table 2. Parasites, sources of the primers used in the present study, and GenBank accession numbers of the obtained sequences

Sequence ID	Accession no.	Closest species	Similarity (%)	Primer source
PL-01	KX354832	*Anisakis pegreffii*	99	Zhu et al. (1998)
PL-02	KX354833	*Anisakis pegreffii*	99	Nadler (2000)
PL-03	KX354834	*Campula oblonga*	98	Littlewood et al. (2000), Tkach et al. (1999)
PL-04	KX354835	*Crassicauda magna*	99	Nadler et al. (2007)

RESULTS

In all evaluated porpoises, using both morphological and molecular methods, 5 endoparasites belonging to 5 genera were identified: *Campula oblonga* (Trematoda), *Synthesium seymouri* (Trematoda; junior synonym: *Hadwenius seymouri* Price 1932), *Anisakis pegreffii* (Nematoda), *Halocercus* sp. (Nematoda), and *Crassicauda magna* (Nematoda). The data for the infected organs and the prevalence of each identified parasite are provided in Table 3.

The numbers of EAFPs at different developmental stages infected with different parasites are listed in Table 4. All juveniles and adults were par-

Table 3. Prevalence of parasites and infected organs in East Asian finless porpoises *Neophocaena asiaeorientalis sunameri* (EAFPs). Prevalence was calculated as (no. of infected porpoises/total sample number) × 100 %; percentage (%) was calculated as (no. of infected organs/no. of infected EAFPs) × 100 %

Parasites	No. of infected EAFPs (% total prevalence)	Infected organs	No. of infected organs (%)
Digeneans			
Campula oblonga	25 (80.7)	Liver	25 (100)
		Pancreas	2 (8)
Synthesium seymouri	25 (80.7)	Pyloric stomach	25 (100)
		Duodenum ampulla	25 (100)
Nematodes			
Anisakis pegreffii	25 (80.7)	Forestomach	25 (100)
		Esophagus and main stomach	2 (8)
Halocercus sp.	24 (77.4)	Lung	24 (100)
Crassicauda magna	2 (6.5)	Mammary gland	2 (100)
		Muscle	1 (50)

Table 4. Numbers of East Asian finless porpoises *Neophocaena asiaeorientalis sunameri* in different developmental stages infected with different parasites

Developmental stage (n)	Parasites				
	Campula oblonga	*Synthesium seymouri*	*Anisakis pegreffii*	*Halocercus* sp.	*Crassicauda magna*
Fetus (3)	0	0	0	0	0
Newborn (3)	0	0	0	2	0
Juvenile (13)	13	13	13	12	0
Adult (12)	12	12	12	10	2

asitized by at least 3 species (*C. oblonga*, *S. seymouri*, and *A. pegreffii*; Tables 1 & 4). No parasites were observed in the fetuses. Except for *Halocercus* sp. in 2 newborns, no other parasites were detected in other neonates. There was no significant difference in parasite prevalence between males and females (Table 5).

Table 5. Effect of sex on the prevalence of different parasites found in East Asian finless porpoises *Neophocaena asiaeorientalis sunameri*

Parasites	Prevalence (%)		p (1-sided)
	Female	Male	
Campula oblonga	85.7	70	0.284
Synthesium seymouri	85.7	70	0.284
Anisakis pegreffii	85.7	70	0.284
Halocercus sp.	81.0	70	0.401
Crassicauda magna	9.5	0	0.097

Digeneans

In the present study, 2 digeneans of the Brachycladiidae were identified: *Campula oblonga* and *S. seymouri*.

C. oblonga was one of the most prevalent parasites in EAFPs (80.7 %), where it occurred in the liver (100 %) and pancreas (8 %) (Table 3). Generally, the surface of infected livers showed several large white to grey solid nodules. Inside the liver, multifocal fibrotic nidi of distended bile ducts which contained numerous trematodes in a dark green liquid were observed (Fig. 2A). Histopathology revealed multiple necrotic foci in the infected livers with surrounding abundant fibrosis and necrotic debris (Fig. 2B). Macroscopic lesions with *C. oblonga* surrounded by yellow mucoid fluids were observed in the infected pancreas (Fig. 2C).

S. seymouri was another prevalent parasite in EAFPs (80.7 %). This species appeared with equal frequency (100 %) in the lumen of the pyloric stomach (Fig. 2D) and the duodenum ampulla (Table 3). No macroscopic lesions were observed in the infected organs.

Nematodes

In total, 3 genera of nematodes were identified: *Anisakis* (Anisakidae), *Halocercus* (Pseudaliidae), and *Crassicauda* (Tetrameridae).

A. pegreffii was the most prevalent nematode in EAFPs (80.7 %), where it was primarily collected from the forestomach (the nonglandular compartment, 100 %; Fig. 2E), main stomach (the glandular compartment, 8 %), and esophagus (8 %; Table 3). Additionally, 2 infected EAFPs, presenting gastric ulcers in the main stomach and esophagus, were associated with these nematodes. Histopathological analysis of the ulcer in the main stomach revealed that the nematodes had penetrated into the tunica submucosa of the infected area (Fig. 2F).

Halocercus sp. was the second most prevalent nematode in the EAFPs (77.4 %) and was generally detected in the bronchioles and bronchi (Fig. 2G) as well as in small light-colored encapsulations on the

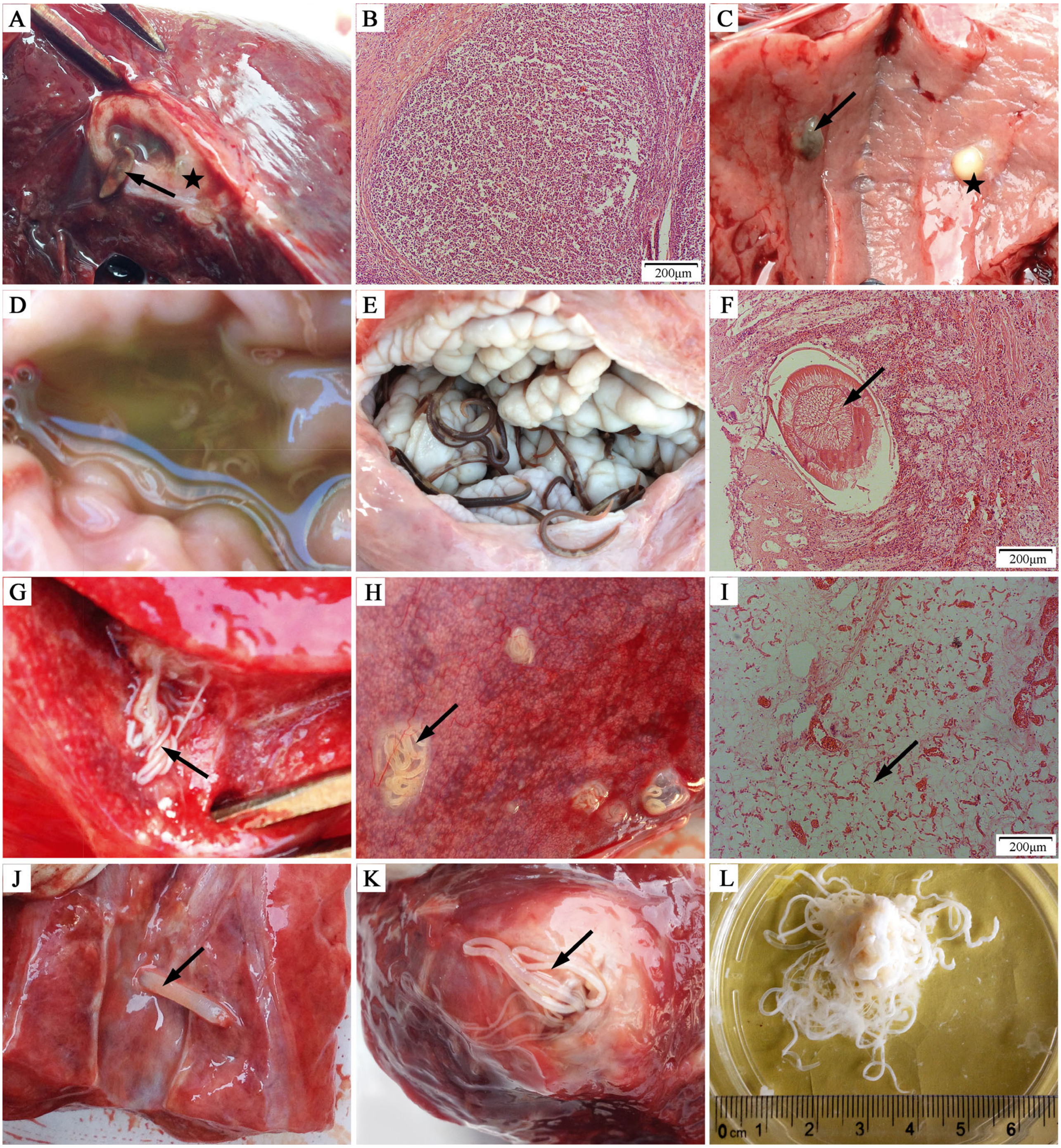

Fig. 2. Parasites and lesions in East Asian finless porpoises *Neophocaena asiaeorientalis sunameri*. (A) *Campula oblonga* (arrow) and the white fibrotic nidus (star) in the liver. (B) Histological section of the nidus infected by *C. oblonga* in the liver (H&E). (C) *C. oblonga* (arrow) in the pancreas and the yellow pyogenic fluid in the nidus (star). (D) White *Synthesium seymouri* within the lumen of the pyloric stomach. (E) *Anisakis pegreffii* in the forestomach. (F) Histopathological analysis of *A. pegreffii* (arrow) under the tunica submucosa of the infected main stomach (H&E). (G) *Halocercus* sp. (arrow) in the lungs. (H) Light-colored nodules and the encapsulation (arrow) of lungworms. (I) Histopathological analysis of the infected lung shows pulmonary alveoli fusion (arrow) in the nodule areas (H&E). (J) *Crassicauda magna* (arrow) in the mammary glands. (K) *C. magna* (arrow) in the muscles. (L) Free *C. magna* isolated from the muscles

surface of the lungs (Fig. 2H). Histopathological analysis showed pulmonary alveoli fibrosis in the nodule areas (Fig. 2I).

The large nematode *C. magna* was only detected in 2 adults (PL29 and PL30), and this worm was encysted in the mammary gland ducts (Fig. 2J) and muscle tissues (Fig. 2K). Necrosis and hemorrhage were observed in the infected muscle. The extraction of intact worms was nearly impossible, reflecting issues associated with the parasite itself, and the specimens recovered during this study were badly fragmented (Fig. 2L).

DISCUSSION

Parasites in marine mammals are relatively common, and some of these organisms have been implicated in disease processes and prominent causes of cetacean strandings (Gibson et al. 1998, Mignucci-Giannoni et al. 1998, Colón-Llavina et al. 2009). The present study represents the first systematic survey of parasitic infection in the EAFP population living off the Chinese Yellow/Bohai Sea coast. In 31 examined porpoises, 5 parasitic species were identified: *Campula oblonga*, *Synthesium seymouri*, *Anisakis pegreffii*, *Halocercus* sp. and *Crassicauda magna*. Among them, *C. oblonga*, *S. seymouri*, and *Halocercus* sp. were previously identified more than 30 yr ago from finless porpoises stranded in Jinzhou City, Liaoning Province, China (Tao 1983, Zhang 1985). However, *A. pegreffii* and *C. magna* represent new infection records in finless porpoises of the genus *Neophocaena*.

All of the parasites identified in our study have been commonly reported in other cetaceans (Lambertsen 1986, Dailey et al. 1991, Gibson et al. 1998, Siebert et al. 2001, Marigo et al. 2008, Carvalho et al. 2010, Oliveira et al. 2011). Previous studies have demonstrated that parasite infections can result in moderate to severe pathology in cetaceans. Liver parasite infections might damage liver function and lead to bacterial diseases in chronic cases or hepatic trauma (Dailey 2001). *S. seymouri* can be destructive when these parasites reside within the parenchyma of the organs (Geraci & Aubin 1987). The presence of *Anisakis* species in the stomach can lead to ulcers and bleeding in hosts (Dailey 2001). The formation of ulcers, gastritis, and granulomatous inflammation due to *Anisakis* typical infections was observed in cetaceans (Motta et al. 2008). The pulmonary nematode *Halocercus* sp. might be directly associated with stranding and death of cetaceans as the presence of this parasite induces parasitic pneumonia, predisposing the animal to secondary bacterial infections (Dailey et al. 1991, Jepson et al. 2000). It has been suggested that *Crassicauda* sp. might reduce the populations of certain baleen whales (Lambertsen 1986, 1992) and reduce the reproductive success of Atlantic white-sided dolphins *Lagenorhynchus acutus* by decreasing milk production (Geraci et al. 1978). EAFPs may be exposed to similar detrimental effects with heavy parasite loads. However, the effect of the parasites identified in the present study on EAFPs remains unknown, and further studies are needed.

There is evidence of prenatal *H. lagenorhynchi* infections in Atlantic bottlenose dolphins *Tursiops truncatus* (Dailey et al. 1991), and *H. pingi* in finless porpoises (*Neophocaena*) (Parsons & Jefferson 2000), indicating that a direct life cycle of *Halocercus* sp. is likely. Similarly, in the present study, lungworms were observed in the lungs of 2 of 3 newborns, although the lungs of all 3 fetal EAFPs were free of such parasites, suggesting that EAFPs are infected with these parasites before, or immediately after, birth.

Cetaceans are the definite host for *Anisakis* spp. (Motta et al. 2008). The life cycle of *C. oblonga* is uncertain, but might include fish as intermediate hosts and cetaceans as definitive hosts (Fernandez et al. 1998). In the present study, the absence of *A. pegreffii* and *C. oblonga* in fetuses and newborns and the 100% prevalence of these 2 parasites in juveniles and adults (Table 4) indicate that juveniles and adults might become infected with these parasites from prey fish. Notably, the youngest juvenile EAFP (PL07, 10.8 mo old with a body length of 104 cm; Table 1) was infected with these 2 parasites, which likely occurred just after the general weaning time of EAFPs (6 mo old) (Zhang 1992). However, future studies on the feeding behavior and diet composition of EAFPs are urgently needed to elucidate the sources and life cycles of these parasites.

Our study represents the first systematic survey on parasitic infections in EAFPs living in the Chinese Yellow/Bohai Sea, and these results could provide some important and valuable parasitological information for future research and the conservation of this coastal marine mammal. However, additional studies are needed to understand the sources and life cycles of these parasites, host–parasite relationships, and the use of these parasites as biological indicators in marine mammal conservation.

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