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Prevalence and description of *Ichthyophonus* sp. in yellowtail flounder (*Limanda ferruginea*) from a seasonal survey on Georges Bank

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ABSTRACT

Ichthyophonus sp. is a ubiquitous parasite infecting numerous fish species. *Ichthyophonus* sp. infection of yellowtail flounder (*Limanda ferruginea*) was first identified in Canadian waters in 1968. Between 2012 and 2014, fifteen seasonal survey cruises by Coonamessett Farm Foundation, randomly sampled a total of 1325 fish for at-sea examination at fixed stations on the U.S. Georges Bank sea scallop fishing grounds. A subsample of yellowtail flounders were examined internally on board the ship and animals with visible lesions were collected, processed, and examined histologically. Infected fish were concentrated in eastern Georges Bank with only two of the 32 infections collected from western Georges Bank. Both macroscopic and histological evaluation of tissues showed that 81.3% of the animals infected with *Ichthyophonus* sp. were severely infected.

1. Introduction

The yellowtail flounder (*Limanda ferruginea*) is a commercially important species in New England waters and is a limiting bycatch species in many fisheries including the Atlantic sea scallop (*Placopecten magellanicus*) fishery (O'Keefe and DeCelles, 2013). Yellowtail flounder are distributed from the Chesapeake Bay to the Gulf of St. Lawrence, with highest abundance occurring on Georges Bank (Bigelow and Schroeder, 1953). Yellowtail on Georges Bank is managed as a unique stock (Legault et al., 2014). The estimated abundance of Georges Bank's yellowtail flounder has substantially decreased in the fall survey trawls by the Northeast Fisheries Science Center from 17,000 mt in 1963 to 1000 mt in 2013 (Legault et al., 2014). While fishing pressure has lessened over the past decade, total population numbers have not increased (Legault et al., 2014).

Ichthyophonus sp., a protozoan parasite, has been identified as a cause of disease in over a hundred species of marine, fresh, and brackish teleost fish, as well as marine copepods and crustaceans, since its original classification in 1911 (Lauckner, 1984; Rahimian, 1998; Rand, 1992). Infection is known to be lethal or debilitating in many fish species including herring and various flounders (Rahimian, 1998; Vollenweider et al., 2011). *Ichthyophonus* sp. outbreaks have resulted in epizootics of disease in herring from the northwest Atlantic, with the disease remaining at enzootic levels between epizootic events (Kramer-Schadt et al., 2010; Lauckner, 1984; Patterson, 1996; Sindermann, 1958).

To demonstrate the quick progression of the disease, laboratory-reared Atlantic herring and mummichogs (*Fundulus heteroclitus*) were fed food contaminated with *Ichthyophonus* spores. The fish developed systematic infections, resulting in many infected organs within 18 days, with death occurring between 70 and 110 days (Sindermann and Scattergood, 1954). Other laboratory studies of herring and rainbow trout (*Oncorhynchus mykiss*) infected with *Ichthyophonus* sp. have shown weakened responses, swimming abnormalities, loss of pigment, or swelling of the abdomen (Kocan et al., 2009; Vollenweider et al., 2011).

Infections of *Ichthyophonus* sp in the yellowtail flounder were first described from samples near Western Sable Island, 300 km east of Nova Scotia (Powles et al., 1968). Powles *et al.* (1968) collected yellowtail flounder from the Canadian waters of the Gulf of St. Lawrence, Banquereau, Middle Ground, Western Bank, and Sable Island Bank. Prevalence of *Ichthyophonus* sp. infections ranged from 2.8% to 57.4% of sampled fish. However, high prevalences were relatively concentrated and limited to areas adjacent to Sable Island in the western Atlantic (Ruggieri et al., 1970). Rand (1994) reported that the yellowtail flounder on Brown's Bank, Nova Scotia in 1987 were infected with an unusual form of *Ichthyophonus* later classified as a new species, *Ichthyophonus irregularis* (Rand et al., 2000). To date, this species has only been identified in yellowtail flounder in that study. Of the 222 yellowtail flounder examined by Rand *et al.* in 1997, seven fish were infected with *I irregularis* and eleven fish were infected with *I. hoferi* (Rand et al., 2000).

In 2011 during the seasonal bycatch surveys conducted by

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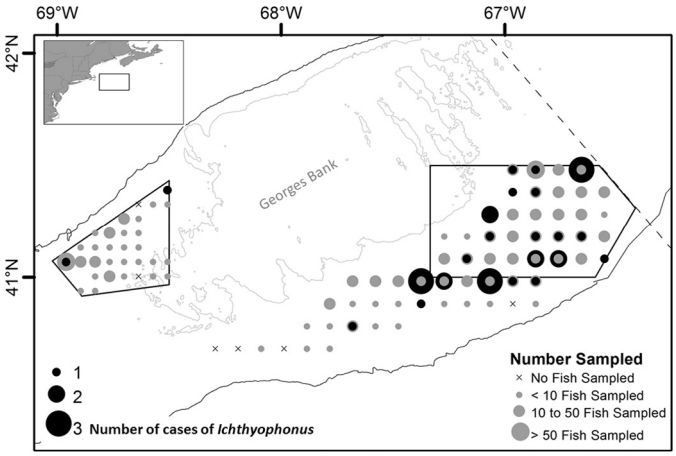

Fig. 1. Location of fixed fishing stations for the CFF Seasonal Bycatch survey. Positive cases of *Ichthyophonus* infection are shown as black circles with the size relative to the number of cases. The number of yellowtail flounder examined at each station are represented by the size of the gray circles. Bathymetry lines are shown as 50 m (gray) and 100 m (black).

Coonamessett Farm Foundation, *Ichthyophonus* sp. was identified in yellowtail flounder from Georges Bank. This report describes the protocol establish and results obtained during the remaining surveys to document prevalence and severity of *Ichthyophonus* sp. infections in yellowtail flounder caught on Georges Bank from June 2012 to March 2014.

2. Methods

2.1. Collection

Sea scallop fishing grounds on Georges Bank were routinely sampled on dedicated research cruises using commercially rigged scallop dredges between June 2012 and May 2014. Sampling locations were selected using a fixed station systematic grid in the scallop access areas of Closed Area I (CAI) and Closed Area II (CAII), as well as open fishing ground between the two closed areas (Fig. 1). Sampling protocols were standardized for all fifteen sampling cruises, and the entire catch was evaluated for abundance of sea scallops and ground fish. Associated environmental factors were collected at each station (Winton et al., 2017). During each of the fifteen cruises from which data is reported here, at least 70 yellowtail flounder were randomly selected for evaluation of *Ichthyophonus* sp. resulting in examination of a total of 1325 yellowtail flounder.

2.2. Macroscopic Identification of infected flounder

Length, weight, sex, reproductive stage, and catch location were recorded for each animal and the peritoneal and pericardial cavities of each fish were opened and examined. The fish were classified into four groups based on macroscopic appearance of lesions: no observable lesions, lesions not identified as *Ichthyophonus*, minor macroscopic signs of *Ichthyophonus* (+/− other lesions) and major macroscopic signs of *Ichthyophonus* (+/- other lesions). *Ichthyophonus* presented distinct macroscopic lesions characterized by frim, off-white cysts in the parenchyma or on the surface, and/or a thickened whitish deposit on the serosal surface of the peritoneal organs or heart. In fish identified with *Ichthyophonus* sp. the animal was fileted and muscles were examined from the spine to the base of the fin rays. Minor cases of *Ichthyophonus* sp. were classified as a fish with fewer than fifty nodules macroscopically observed on any tissue and no other macroscopic signs of inflammation or necrosis. Individuals with macroscopic signs of necrosis or any tissue containing more than fifty nodules were classified as major infections.

All macroscopic abnormalities were noted and photographed. Pieces of the infected tissue, regardless of parasite observed, were preserved in 10% neutral buffered formalin for histological evaluation to confirm identification (Humason, 1972). Tissues were not collected from fish if lesions were not seen. Based on previous work (Sindermann, 1966) and observations from authors of this paper early in the study, infection occurs through the gut. In order to detect a very early infection with no nodules grossly visible, numerous histological sections of intestine and stomach, which is approximately 20 cm in length, would need to be examined in order to determine where early invasion by single *Ichthyophonus* sp. organisms is occurring.

2.3. Histopathologic examination

Fixed tissues were trimmed and transferred into 70% ethanol solution, embedded in paraffin and 6 μm-sections were cut and stained with hematoxylin and eosin (Humanson, 1972). *Ichthyophonus* infections were identified and intensity of the microscopic parasitic infection was rated using a scale of 1–5, where:

- 1 = 1–2 parasites observed
- 2 = 3–10 parasites observed
- 3 = 11–20 parasites observed
- 4 = Over 20 parasites observed
- 5 = More parasites than tissue present

Other parasites were identified to the lowest possible taxonomic level.

3. Results

3.1. Ichthyophonus prevalence

Macroscopic examination of fish, confirmed by histology, indicated 2.0% of all the yellowtail sampled were infected with *Ichthyophonus* sp. Histological evaluation identified five early stages of infection for a total of 2.4% infected. Prevalence of infection was calculated for the following size classes and sexes using estimated values similar to market categories: sub-legal (< 30.5), "small" (30.5–37 cm), and "large" (> 38 cm) (Legault et al., 2014; Wildlife and Fisheries, 2013). Infection of *Ichthyophonus* sp. was most common in males representing the "small" size class (6.67%). No infected animals were identified from the "large" or sub-legal males sampled. The female infection levels increased with market category with a 1.10% infection in the "small" class and 1.94% in the "large" size class (Table 1). A chi square test showed a significant higher infection in males ($\chi^2 = 13.8, p = 0.0005$).

While no formal seasonal analysis was completed, the percent of fish with *Ichthyophonus* sp. was calculated for each season. Infection was lowest in the winter (December–February) at 0.84%, higher for spring (March–May) 2.42%, summer (June-August) 1.95%, and highest in the fall (September–November) at 4.44%.

Table 1
Percent of yellowtail flounder infected with *Ichthyophonus* by sex at size categories with the number of animals observed in each category (Legault et al., 2014; Wildlife and Fisheries, 2013).

	Sub-legal (< 30.5 cm)	"Small" (30.5–37 cm)	"Large" (> 38 cm)	All sizes
Males	0%	6.67%	0%	5.49%
	$n = 33$	$n = 225$	$n = 15$	$n = 273$
Females	0%	1.10%	1.94%	1.61%
	$n = 18$	$n = 365$	$n = 669$	$n = 1052$

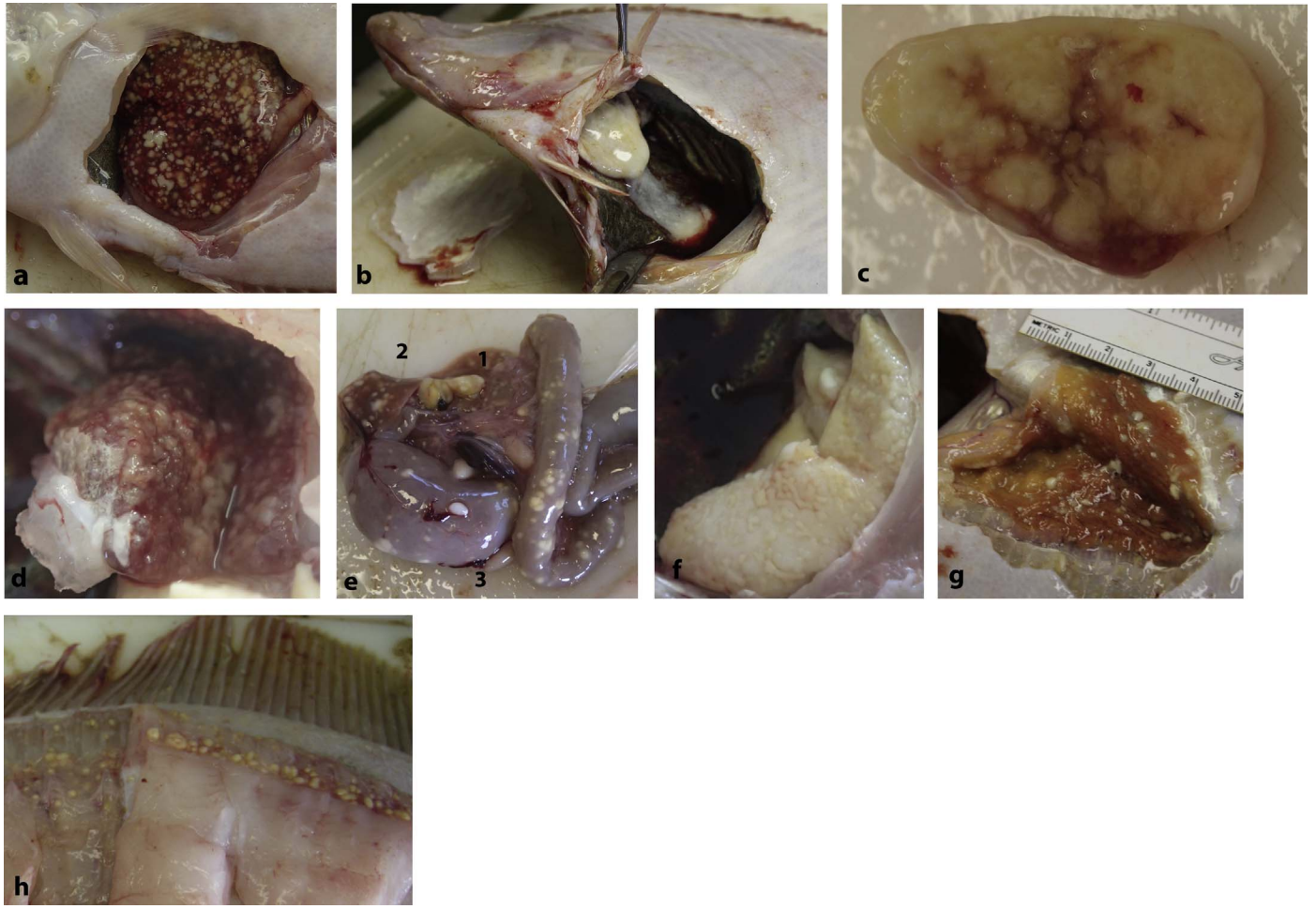

Fig. 2. Severe infection of Ichthyophonus in yellowtail flounders. (a) Numerous white/tan nodules are noted in the parenchyma and protruding from the serosal surface of the liver; (b) the pericardial sac and sinus venosus are filled with white/tan exudate (arrows); (c) severe granulomatous inflammation of the myocardium and epicardium; (d) extensive granulomatous nephritis; (e) multifocal Ichthyophonus granulomas in the intestinal wall (1) and liver (2), cestode cysts present on the serosa of the stomach (3); (f) multifocal Ichthyophonus granulomas in the parenchyma of the testis (arrows); (g) multifocal granulomas in the parenchyma of the spent ovary; (h) *Ichthyophonus* granulomas are concentrated along the radial cartilage at the base of the fin rays (arrows).

Table 2
Macroscopic intensity of *Ichthyophonus* characteristics by tissue for all fish positive for *Ichthyophonus*. Infection levels were classified as no macroscopic signs of *Ichthyophonus* (−/0), minor signs, less than 50 nodules on any tissue (+/1) or major infections by *Ichthyophonus* (++/2).

Sex	Size (cm)	Infection level	Liver	Heart	Stomach	Intestine	Gonad	Kidney	Myotomes	Mesentery
M	34	++	2	2	1	0	2	0	0	0
F	42	++	2	1	2	0	1	1	2	0
F	36	−	0	0	0	0	0	0	0	0
M	35	+	1	1	1	0	1	1	0	0
F	42	−	0	0	0	0	0	0	0	0
M	34	++	2	2	0	1	2	2	2	0
M	35	−	0	0	0	0	0	0	0	0
F	45	++	2	0	1	0	1	2	0	2
F	34	−	0	0	0	0	0	0	0	0
F	40	−	0	0	0	0	0	0	0	0
M	35	++	2	2	1	2	2	0	1	0
F	40	++	2	2	1	0	2	0	0	0
M	36	++	2	2	0	0	2	0	0	0
M	36	++	2	1	0	0	2	0	1	0
F	40	++	2	2	0	0	0	2	2	0
M	35	+	1	1	0	0	1	0	0	0
F	37	+	0	0	0	0	0	0	0	1
F	37	+	1	1	0	0	0	0	0	0
M	34	++	2	2	2	2	0	0	2	0
F	40	+	1	1	1	0	1	0	1	0
F	42	++	2	2	1	0	2	0	0	0
M	31	++	2	2	1	0	2	2	0	0
M	35	+	1	1	0	0	1	0	0	0
M	33	++	2	2	1	0	2	2	2	0
F	39	++	2	2	1	2	2	2	2	0
F	39	++	2	2	1	1	2	2	2	0
M	34	++	1	1	2	0	2	1	0	0
M	36	+	1	1	0	0	1	0	0	0
M	36	++	1	2	0	0	2	0	0	0
F	40	++	2	2	2	1	2	2	2	0
F	40	+	1	1	0	0	0	1	1	1
F	45	+	1	1	0	0	0	0	0	0

3.2. Macroscopic appearance

Lesions caused by *Ichthyophonus* infection were easily identified upon opening the peritoneal cavity. In many cases peritonitis was noted. Peritonitis was characterized by flocculent to fibrillar white material overlying and attached to the serosal surfaces and similar-appearing whitish exudate in the abdomen that had filled and expanded the abdominal cavity. *Ichthyophonus* sp. nodules both projected from the surface and were embedded in the parenchyma of the liver. Nodules in the liver and other organs were characterized by small (1 mm) firm nodules which were white, clear, or yellowish in color. Even in low to moderate infections, the individual foci were distinct in appearance and were identifiable as *Ichthyophonus* sp. infections, compared to other causes of cysts and nodules in the tissues (all infections were confirmed by histological examination). In severe infections, *Ichthyophonus* sp. resulted in diffuse confluent micro-nodular growths on the serosal surface and in the parenchyma leaving little intact hepatic tissue (Fig. 2a).

In cases where the liver was severely infected, the heart was also often severely infected macroscopically (Table 2). In some cases, the infection was so severe that lesions were noted on all surfaces of the heart (epi- and endocarditis), the inner surface of the pericardial sac (pericarditis) and the myocardium (myocarditis). In these cases the pericardial sac enclosing the heart was filled with flocculent to fibrillary white exudate (Fig. 2b and c).

In fish with severe systematic *Ichthyophonus* sp. infection, the kidneys were swollen either with many small, white, firm nodules, or with single nodules (Fig. 2d). *Ichthyophonus* sp. infections were also macroscopically identified in the stomach, intestine, and reproductive organs (Fig. 2e–g). *Ichthyophonus* sp. nodules were observed more often in testis (86%) than ovaries (44%) (Table 2; Fig. 2f and g). *Ichthyophonus* sp. nodules were present in the myotomes in 36% of the *Ichthyophonus* positive animals. The nodules were concentrated near the fins along the radial cartilage at the base of the rays (Fig. 2h).

3.3. Histopathology

Microscopic examination confirmed all macroscopic cases of *Ichthyophonus*, although there were five cases of minor infection in which the abnormality observed was not macroscopically identified as *Ichthyophonus* (Table 3).

Histopathological examination showed *Ichthyophonus* sp. organisms

Table 3
Number of flounder examined during each survey trip with percent infection by parasite type. *Ichthyophonus* infections were identified both macroscopically and microscopically, but other parasites were only identified microscopically. In many cases multiple species of parasites were identified in the same host.

		Number Sampled	*Ichthyophonus*		Microscopic ID	
			Macroscopic	Microscopic	Cestode	Ascarid
2012	June	83	3.61%	6.02%	62%	60%
	August	76	0.00%	0%	52%	51%
	September	91	1.10%	2.20%	50%	73%
	November	83	6.02%	8.43%	56%	80%
	December	78	0.00%	0.00%	37%	76%
2013	January	73	2.74%	2.74%	21%	74%
	March	78	1.28%	1.28%	18%	21%
	April	102	2.94%	2.94%	10%	27%
	June	105	0.00%	0.00%	12%	30%
	July	95	1.05%	1.05%	23%	33%
	September	101	4.95%	4.95%	27%	28%
	October	85	2.35%	2.35%	22%	54%
	December	97	0.00%	0.00%	26%	35%
2014	January	108	0.93%	0.93%	31%	31%
	March	70	2.86%	2.86%	37%	66%
	Average		2.0%	2.4	32.3%	49.3%

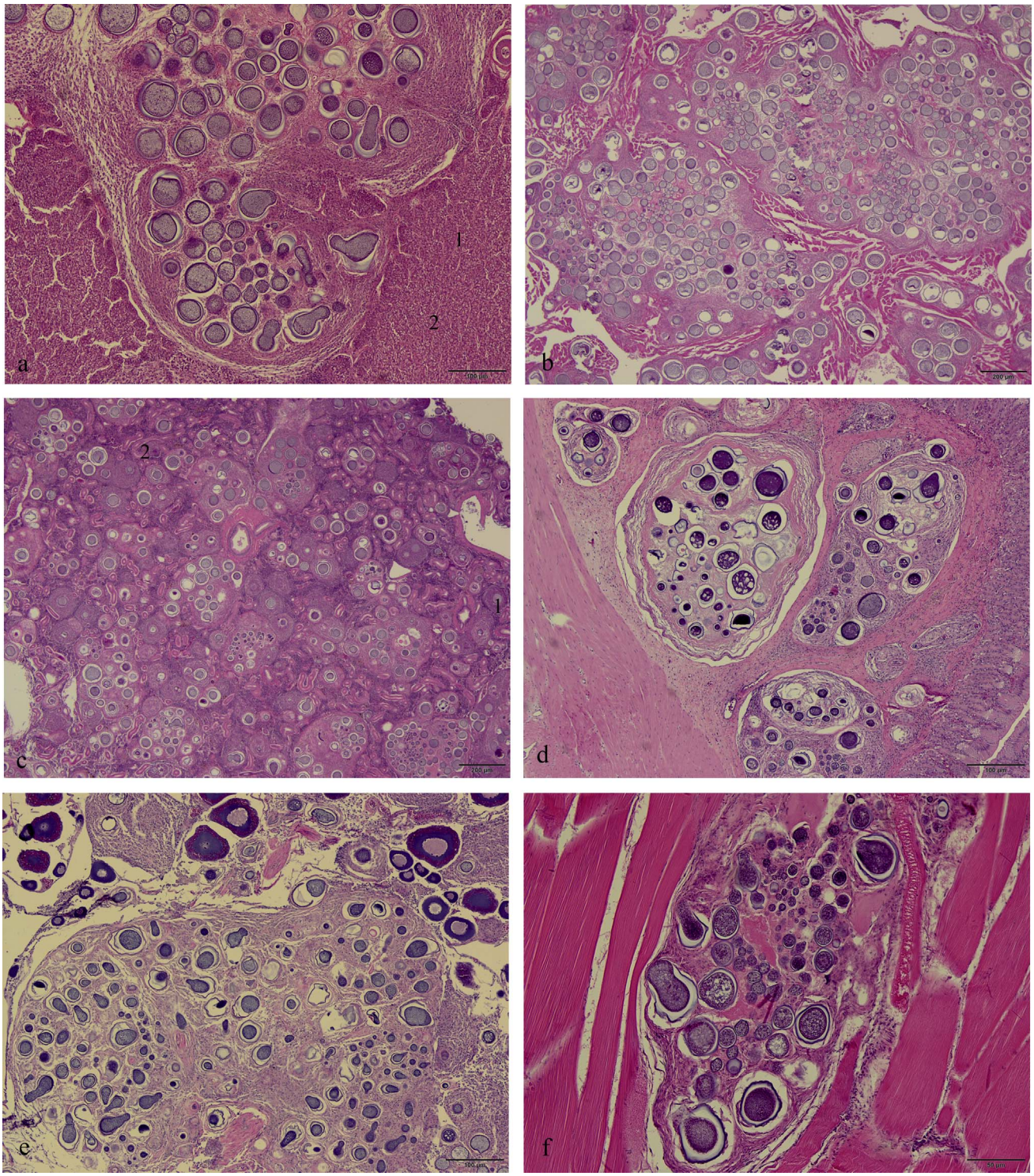

Fig. 3. (a) Granulomatous inflammation surrounds this focus of hepatic *Ichythyophonus* infection (grandulomatous inflammation (1), budding of the Ichthyophonus organism (2)); (b) severe destruction and loss of the myocardial fibers (arrow) by abundant *Ichythophonus* organisms and associated inflammation; (c) renal tissue is almost obliterated by the Ichthyophonus infection and the accompanying granulomatous inflammation (renal tubule, arrow); (d) foci of *Ichythyophonus* infection in the submucosa of the stomach (gastric epithelium, arrow); (e) severe Ichythophonus infection in the lamellae of the ovary; (f) a focus of *Ichythophonus* organisms in necrotic skeletal muscle (arrows); (6 μm sectioned, paraffin embedded, H & E stained histological sections).

throughout the liver parenchyma and extending to and through the serosa of the liver causing mild to severe, localized to diffuse granulomas and granulomatous inflammation associated with mild to extensive hepatic necrosis (Fig. 3a). Budding was commonly noted with hyphae extending through the ruptured cell wall into the surrounding inflamed and necrotic liver parenchyma.

Severe necrotizing granulomatous myocarditis was accompanied by severe diffuse granulomatous epicarditis and pericarditis (Fig. 3b). This condition was noted in almost all animals with significant liver infections and was consistently associated with abundant, *Ichthyophonus* sp. cells.

Ichthyophonus sp. was identified histologically in many of the tissues from severely affected animals (Fig. 3c–f) including the kidney, stomach, intestine, spleen, gonad, muscle, and the brain. In each of

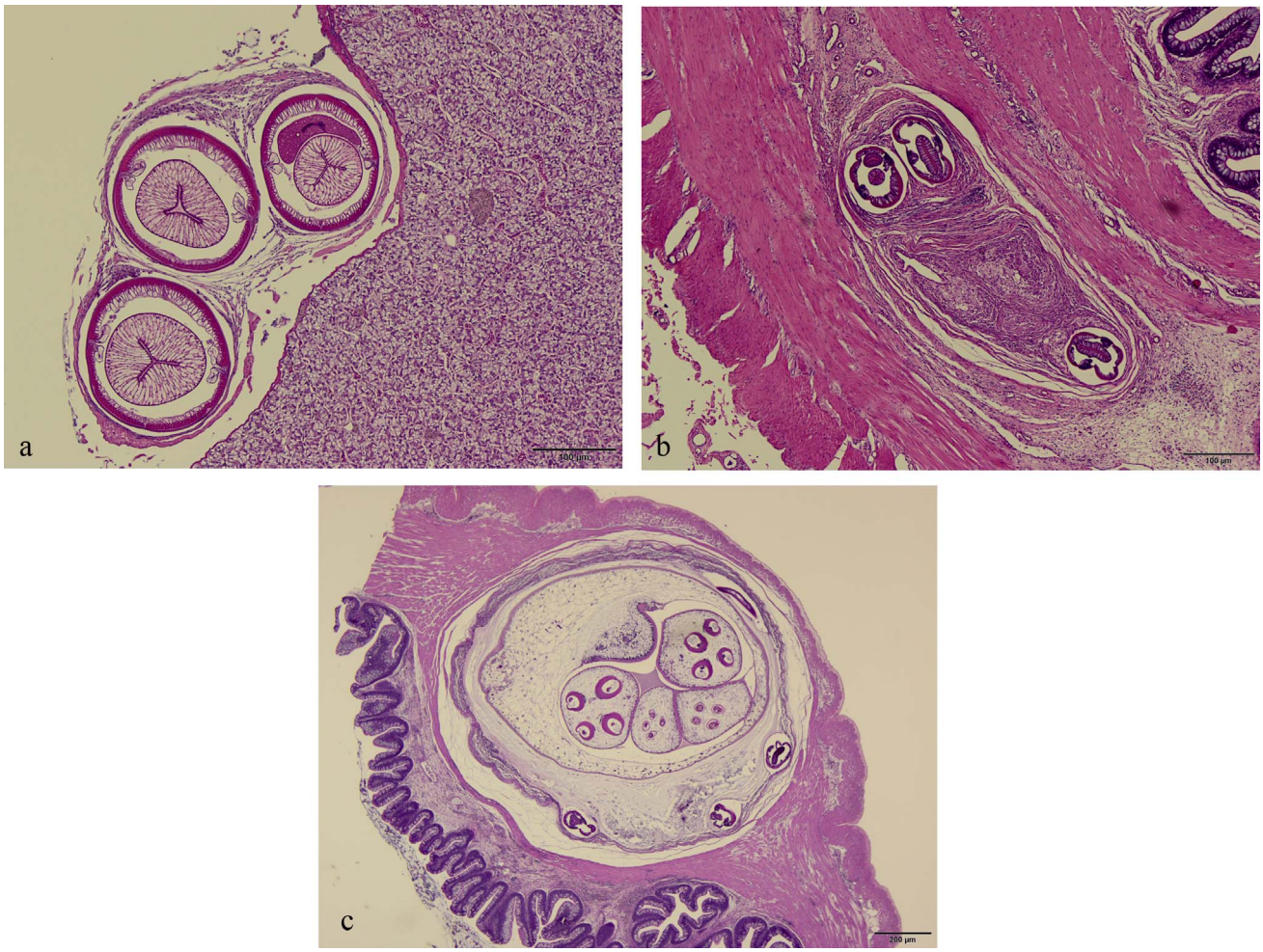

Fig. 4. (a) Mild chronic inflammation surrounds ascarid larvae (arrow) on the serosal surface of the liver; (b) ascarid larvae incite chronic inflammation forming granulomas between muscle layer of the intestine (intestinal epithelium, arrow); (c) A cestode larval is noted in the tunica muscularis of the stomach. Interestingly, four ascarid larvae are present within the larval cestodes capsule (arrows); (6 μm, paraffin embedded, H & E stained histological sections).

these locations multifocal areas of necrosis and granulomatous inflammation with few to multiple distinct granulomas were noted *Ichthyophonus* sp. organisms were noted in the vessels in the intestinal wall and in the attached mesentery and vessels, albeit rarely. *Ichthyophonus* sp. caused multifocal granulomatous inflammation and granulomas, in the lumen and leaflets of the gonads.

3.4. Other lesions

Other swelling and nodules were grossly apparent in the yellowtail. Upon histological examination, these were caused by various parasites including larval ascarids and cestodes. Cestodes and nematodes were the most commonly identified cause of lesions averaging 32.3% and 49.3% respectively (Table 3).

In almost all animals, larval nematode parasites in various sizes and possibly species were commonly visible. Larvae were macroscopically identified as small (1 mm) white or clear nodules attached to the serosal surface of the liver, intestine and throughout the mesentery. Histologically, mild chronic fibrosis of the adjacent mesenteries and serosa were usually noted (Fig. 4a) but occasionally moderate to severe inflammation of the tissues were noted, causing localized to extensive subacute peritonitis, gastritis, and enteritis. These nematodes are most likely Anasarca-like larvae. Larval ascarids of unknown species were also noted in stomach submucosa (Fig. 4b) and there resulted in mild to focally extensive, subacute to chronic granulomas or more extensive granulomatous enteritis.

In many cases, multiple large cysts containing larval cestodes (Fig. 4c) and characterized by round, white, 2–3 mm capsules were observed in the mesenteries, on the serosa of the liver and other abdominal organs, and embedded in the walls of the intestine and stomach. In the intestine and stomach, they were identified in the tunica muscularis or submucosa. When they occurred at the pyloric junction (between the stomach and intestine), they usually caused moderate, focally extensive, mixed cell inflammation and thinning and necrosis of the wall. Rarely, migration routes thorough the gastric wall were noted, resulting in localized necrosis and inflammation. Cestodes in these cysts are most likely the previously reported *Rhynchobothrium imparispine* (Linton, 1901).

4. Discussion

Ichthyophonus sp. was first described in yellowtail flounder in 1968 from Canadian waters, including Browns Bank and Sable Island Bank (Powles et al., 1968; Rand et al., 2000). Browns Bank is separated from Georges Bank by waters over 300 meters deep, and the yellowtail populations in these two areas are managed as separate stocks (Cadrin, 2010; Legault et al., 2014). There was one documented case of *Ichthyophonus* infection in yellowtail flounder in the Gulf of Maine, but this study documents the first case on Georges Bank (Hendricks, 1972).

Tagging studies have shown there is high residency for Georges Bank yellowtail flounder suggesting that the flounder were likely

becoming infected on the bank (Wood and Cadrin, 2013). Yellowtail flounder are also more abundant in the sandy bottom of CAII and open fishing ground to the south than in the rocky bottom of CAI and the eastern open area stations (Cadrin, 2010). In this study, we report that the infection appeared to be concentrated in yellowtail flounder on the eastern U.S. portion of Georges Bank (CAII), with only two positive animals identified on western Georges Bank in CAI. It is possible this finding is a function of the population of yellowtail interacting with potentially high prevalence of infected prey or substrate. *Ichthyophonus* sp. has been observed in many intermediate prey species including crustaceans and copepods, all which are part of the diet of yellowtail flounder (Rahimian and Thulin, 1996). Alternately, the higher number of yellowtail examined in CAII may have resulted in a more accurate detection level.

Past studies have demonstrated that the pathogenicity of *Ichthyophonus* sp. is highly variable depending on hosts and environmental conditions (Kocan et al., 2009; Mellergaard and Spanggaard, 1997). In one study, *Ichthyophonus* was microscopically identified penetrating the stomach epithelium of the goby (*Glossogobius giuris*) three days after infection. The parasite caused massive secondary infection in similarly treated rainbow trout (*Oncorhynchus mykiss*) after 8 days, resulting in visible nodules (McVicar, 1982). The reported time until death is rapid in other host species, with time-until-death ranging from 7 days to a few months (Kocan et al., 2009; Mellergaard and Spanggaard, 1997). The level of tissue damage observed in combination with no evidence of recovery from *Ichthyophonus* in any animal examined (scars, granulomas with no visible cause, etc.), leaves little doubt that this infection is lethal in yellowtail flounder. Data presented here shows the heart is severely affected, often showing more severe lesions than the livers of the same fish. Heart lesions result in an inability of the fish to pump blood appropriately to the body, thus making the fish lethargic and easy prey, as well as resulting in death due to a non-functioning vital organ.

Ichthyophonus sp. has been reported to have devastating effects on fish populations even when identified at a low prevalence. Rahimian and Thulin (1996) reported a prevalence of 2.4% in stocks of Atlantic herring (*Clupea harengus*) by *I. hoferi* which assuming 100 day survival resulted in an estimated a mortality of 10% of the population off the west coast of Sweden. Mellergaard and Spanggaard (1997) suggested that low prevalence can lead to high fish mortality due to the possible quick progression of the infection. Sindermann (1966) indicated that infected animals held in a laboratory setting would develop severe systemic disease and die within six months.

Identification of infected individuals using visual and histological identification could underestimate the number of samples with low infection levels (Kocan et al., 2011). In this study, the lack of macroscopic lesions was consistent with the lack of microscopic lesions due to *Ichthyophonus* sp. The potential reasons for this finding are several. If no tissue abnormalities were visible macroscopically, then identifying infection in tissues sections from these fish would be unlikely due to the small amount of tissue/organ that could be examined from each of these fish histologically. Additionally, during the very early stages of *Ichthyophonus* sp. infection, macroscopic lesions are probably cryptic and are associated with the gut, adjacent mesentery, and localized vessels only. If the disease progresses quickly, it would be hard to find very early infections in the gut and adjacent mesentery. Thus, in yellowtail flounder, mild infections identified both macroscopically and microscopically as a few *Ichthyophonus* sp. nodules in or on the liver are considered early infections, especially since the route of entry appears to be through the gut wall into vessels leading to the liver.

It is possible that many species previously identified as *I. hoferi* may be reclassified, which might explain the variability of *Ichthyophonus* pathogenicity reported between hosts species (Rand et al., 2000). Culture and genetic analysis is needed to determine if the species of *Ichthyophonus* in Georges Bank yellowtail flounder is the ubiquitous species, *I. hoferi*; the relatively recently described species, *I. irregularis*; or a new species yet to be classified.

Results from this project indicate a 2.4% infection level of *Ichthyophonus* sp. in yellowtail flounder within our sampling location. While this is a low percentage, based on previous research and data, there appears to be a rapid progression of this disease in each fish likely resulting in many more animals being lost to the disease than can be estimated in this study. We also observed no infections in the sub-legal size classes for either sex, which is not surprising given the low sample size. Percent of infection increased with age (using size as a proxy for age) in the females. Although no infected males were observed in the "large" size class, only a few animals were examined, and it is possible infected males were dying before they reached the "large" size class. Also, males do have a smaller maximum size and slower growth after age two than females (Dwyer et al., 2003). Further research is needed to identify the species of *Ichthyophonus* as well as to determine if there is a primary first infection location in the gut, to estimate progression rate of the infection, and most importantly the possible implications for Georges Bank yellowtail flounder population restoration.

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