PREVALENCE AND CHARACTERIZATION OF CARDIAC PATHOLOGY INDUCED BY THE PARASITIC NEMATODE *PHILOMETRA SALTATRIX* IN JUVENILE BLUEFISH OF THE HUDSON RIVER ESTUARY, NEW YORK

A Final Report of the Tibor T. Polgar Fellowship Program

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ABSTRACT

Philometra saltatrix is a nematode parasite of bluefish that infects the ovaries of adult bluefish as well as the pericardial cavity of juveniles. Ovarian infection has been linked to decreased fecundity, and pericardial infection has been described as fatal via constrictive pericarditis. The life cycle of this parasite is unknown.

Eighty juvenile bluefish sampled from the Hudson River Estuary (HRE) between July and October 2010 were examined systematically for the parasite. Histopathology revealed pericardial infections were associated with chronic granulomatous inflammation, pericardial thickening and fibrosis, and pericardial adhesions over a range of severities. Mild pathology was associated with viable female worms, while the most severe pathology was associated with dead gravid females with or without the release of larvae in the pericardial cavity. Overall prevalence of infection in the pericardium was 67.5% (54/80). Overall prevalence of fatal severity infection was 25% (20/80). No difference in overall prevalence, severity of infection, or prevalence of fatal infection was observed between the spring and summer-spawned bluefish cohorts. No difference in condition factor was observed between parasitized and non-parasitized juveniles.

Pericardial infection with *Philometra saltatrix* should be considered an important source of mortality in juvenile bluefish of the HRE. In severely affected fish, constrictive pericarditis has the potential to impact ability to hunt and forage, pursue normal migratory behavior, and could result in increased susceptibility to predation. Future studies using full calendar year sampling periods, as well as experimental infections, are needed in order to fully evaluate the effects of this parasite on juvenile bluefish.

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INTRODUCTION

The mechanisms of recruitment in marine fishes have been a topic of much interest in the last 20 years (Rothschild 1986; Beyer 1989; Beamish and McFarlane 1989). Hjort (1914) proposed that the year-class strength of marine fish populations is determined in the very early life stages when mortality rates are high. Small variations in juvenile mortality rates can affect recruitment processes resulting in large fluctuations in abundance and survival to the adult population (Houde 1987). Factors that influence variable mortality rates in juveniles include egg size, larval and juvenile growth rates, predation, parasitism, and food availability (Juanes and Conover 1995; Chambers and Trippel 1997).

One species that is vulnerable to recruitment variability is the bluefish, *Pomatomus saltatrix*. Bluefish is a globally important, and highly migratory pelagic species found worldwide in subtropical and temperate waters (Juanes et al. 1996). Adults can reach one meter in length, and are important to the recreational game fishing industry and as a major marine predator. Along the United States coast, bluefish occur seasonally in the western Atlantic Ocean from Maine to Florida (Kendall and Walford 1979) migrating in loosely aggregated schools of similarly sized individuals (Olla and Studholme 1971). Bluefish reproduce multiple times along the eastern coast of the United States during annual spawning migrations. Although the exact temporal and spatial patterns of bluefish spawning remain uncertain, at least two cohorts (spring and summer) of juveniles are evident as a result of spawning over the continental shelf (Hare and Cowen 1996). The spring-spawned cohort results from spawning between Cape Hatteras, NC and Cape Canaveral, FL (March – May). The summer-spawned cohort

originates from spawning between Cape Hatteras and Cape Cod, Massachusetts (June – August).

The Hudson River estuary is an important juvenile bluefish nursery area, and is used by the spring and summer cohorts of juvenile bluefish throughout the summer. Juvenile bluefish typically migrate into the Hudson River estuary (HRE) in June and emigrate by November. Otolith microstructure analysis has revealed that in early summer, juvenile bluefish abundance is dominated by the spring cohort, while late summer and early fall catches are comprised mostly of summer-spawned juveniles (Stormer and Juanes 2008).

The relative contribution of the spring and summer cohort to the western Atlantic population varies and is the current topic of some debate (Juanes et al. 1996; Hare and Cowen 1996; Munch and Conover 2000; Conover et al. 2003). In the 1950s, the relative abundance of spring and summer-spawned cohorts was nearly equal (Lassiter 1962). From 1973-1995, spring-spawned bluefish dominated the cohort structure of juvenile bluefish inhabiting the Mid-Atlantic Bight (Munch and Conover 2000). However, for reasons unknown, an apparent shift in recruitment has favored the summer-spawned cohort since the mid 1990s (Conover et al. 2003). Unfortunately, the summer-spawned cohort does not appear to be contributing proportionally beyond the juvenile stage (Conover et al. 2003). The apparent recruitment failure of a large portion of the summer cohort coupled with disproportionate abundance of spring-spawned juveniles has been implicated in the recent decline in adult stock size (Klein-MacPhee 2002).

The host-parasite interaction

Parasitic infections by nematodes in marine fishes may make wild populations vulnerable to other biotic and abiotic stressors such as predation and water contamination. Conversely, environmental stressors such as coastal pollution, and climate change may make fish more vulnerable to infections through immunosuppression (Sures and Knopf 2004b). Infection may increase mortality, affect growth and reproduction, or compromise the condition of a fish.

Philometra saltatrix is a nematode parasite of the superfamily Dracunculoidea, family Philometridae, that appears to be specific to bluefish (Moravec et al. 2008). Dracunculoids, in general, are mostly parasites of various tissues and body cavities, and depending on species, can be found in skin, subcutaneous tissue, skeletal muscle, eyes, orbits, gills, swim bladder, kidneys, gonads, and circulatory system (Moravec 2004). *Philometra saltatrix* has been found in the adult female bluefish ovary (Ramachandran 1973; Clarke et al. 2006), as well as the pericardial cavity of juvenile bluefish (Cheung et al. 1984).

Gravid female worms reach a maximum of 300mm in length (Koske and Pinkerton 2010, unpublished research) and 300μm in width (Clarke et al. 2006). Subgravid females range from 36-75mm in length and 231-462μm in width. Mature male worms are 2-3mm long (Moravec et al. 2008) while juvenile male and female worms average 2mm in length (Clarke et al. 2006). The presence of *Philometra saltatrix* in the pericardial sac of juvenile bluefish and the ovary of adult females has led to the hypothesis that gravid females migrate to the ovary in adult female bluefish near the time of spawning, most likely under hormonal cues (Clarke et al. 2006). However, the life

cycle of this parasite remains unclear. Infections by Philometrid nematodes have been associated with ovarian damage in adults of several marine species (Ramachandran 1975; Hine and Anderson 1981; Oliva et al. 1992). In bluefish, Clarke et al. (2006) and Burak (2007) documented a variety of ovarian disorders associated with *Philometra saltatrix* infection including hemorrhage, inflammation, edema, fibrosis, and follicular atresia.

In addition, Koske and Pinkerton (2010) reported that severe infections with *Philometra saltatrix* in the pericardial cavity of juvenile bluefish cause severe chronic granulomatous inflammation and pericardial fibrosis, especially after the death of the gravid female parasite, with or without release of larvae into the pericardial cavity. Severe chronic inflammation leads to diffuse pericardial and epicardial thickening and fibrosis, a condition called constrictive pericarditis, where the fused, thickened pericardium restricts proper filling of the ventricle (Koske and Pinkerton 2010). This condition is capable of leading to eventual heart failure in affected individuals (Asher and Klein 2002). In mammals, symptoms of this condition include exercise intolerance, heart failure with liver congestion and ascites, and sudden collapse. By extension, we also expect the same physiological changes to occur in fish.

Clarke et al. (2006) observed significantly greater *Philometra saltatrix* prevalence in the ovaries of summer-spawning than in spring-spawning adult bluefish. It is possible that infection of bluefish offspring is associated with proximity to infected adults, possibly leading to increased vulnerability of summer-spawned juveniles to infection. Moreover, if both ovarian and pericardial forms of infection cause severe pathologies, the interaction between summer-spawned juvenile bluefish and *Philometra saltatrix* may be a causal mechanism for the recent recruitment failure exhibited by the summer cohort.

Characterization of the nature and severity of pathology induced by infection with

Philometra saltatrix in the pericardial cavity of juveniles, as well as estimation of the

prevalence of fatal infection, will help reveal the true impact of this parasite in bluefish.

METHODS

Sampling locations

Juvenile age-0 bluefish were sampled bi-weekly from late July 2010 to October

2010 from stations along a 65km stretch of the lower Hudson River (Figure 1) by the

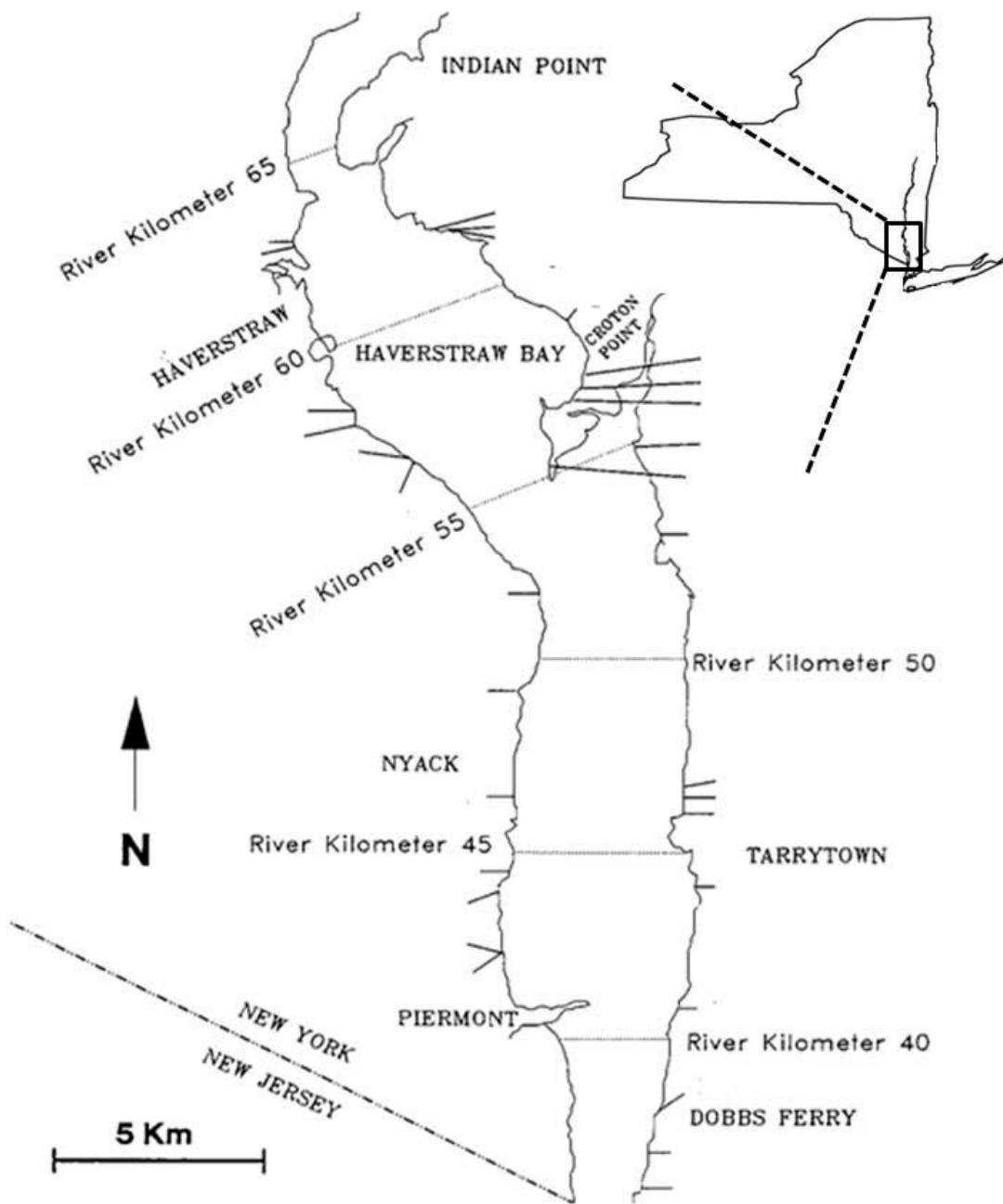New York State Department of Environmental Conservation.

Figure 1. Section of the lower Hudson River illustrating bluefish sampling locations. Straight lines indicate sampling stations.

Fish classified as juveniles were those spawned during the year 2010 of both spring and summer-spawned cohorts, and ranged in length between 53-190 mm fork length. Bluefish were collected with a 61m x 3m beach seine with 13 mm stretched mesh wings and a 6 mm stretched mesh bag. Seine hauls were set from a boat and parallel to shore. Catches were processed on shore, with juvenile bluefish preserved frozen for later dissection and analysis.

Sample Processing and Pathology Scoring

Frozen bluefish were thawed in cold water in groups of three to six fish at a time to minimize autolysis during room temperature conditions in the laboratory. The sampling date, cruise identification number, station number along the river, total length (mm), fork length (mm), standard length (mm), and wet weight (g) were recorded and the fish assigned a fish identification number. The external surfaces of the fish, the oral cavity, and the gills were examined for evidence of gross pathology such as traumatic lesions, external parasites, or signs of systemic disease. Any findings were noted for consideration in conjunction with later results if necessary.

The ventrum of the fish was incised with mayo scissors from the vent to the base of the opercula, carefully avoiding internal organs, especially the heart and pericardium. The surfaces of the internal organs, including intestine, stomach, pyloric caeca, liver, kidney, swim bladder, and coelomic cavity were examined closely for gross pathology, degree of autolysis, and evidence of nematode or other parasitic infections both with the naked eye and under a dissecting microscope. Any observed pathology was noted, as was

the location of any parasites found, including *Philometra saltatrix*. If *Philometra saltatrix* adults or larvae were found in the coelomic cavity, including on the mesenteries of organs or on the body wall itself, the reproductive stage of the parasite was determined using visualization of the uterine contents under a dissecting and compound microscope. The three stages included: immature (I), as described by a small, immature uterus containing small, undeveloped eggs; subgravid (SG), described by a mature worm with uterus containing well-developed eggs; and gravid (G), described by a uterus containing a mix of well-developed eggs and hatched larvae, or solely hatched larvae (after Moravec and de Buron 2009).

The intact pericardium was examined closely for presence of *Philometra saltatrix* adults and larvae. Digital photographs were taken to document the gross in situ appearance of the heart and nematode position, physical condition of the parasites, relative numbers, and any associated pathology.

Gross pathological findings were recorded and the heart assigned a gross pathology severity score of 0-5 (Figure 2) based on the severity of pathology in six specific areas outlined in Table 1. A score of 0 indicated no infection/pathology and a score of 5 indicated severe infection and pathology.

The heart and pericardium were carefully excised intact and as a whole and transferred to a jar containing 10% neutral buffered formalin (Globe Scientific, NJ, USA) for fixation. If the pericardium broke during transfer, the nematodes were individually reproductively staged as described above.

	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Inflammatory cell matrix	-	Focal	Multi-focal	Multi-focal to diffuse	Diffuse
Pericardium color	Clear/transparent	Slightly opaque	Slightly opaque to opaque	Multi-focally to diffusely opaque	Diffusely opaque
Pericardium texture	Smooth and thin	Smooth	Thickened, appears tacky	Thickened, rough	Thickened, rough
Adhesions between pericardium and body wall	-	-	+/-	+/++	++
Adhesions between epicardium and pericardium	-	-	+	++	+++
Condition of worms	None present	Fresh or degenerate	Degenerate or embedded in matrix	Worms embedded in matrix	Worms embedded in matrix

Table 1. Pathological characteristics evaluated on gross dissection. *+ = 25% of surface area affected, ++ = 50% surface area affected, +++ = 75% surface area affected.* Fresh worms are likely viable at time of collection and are not coated in inflammatory cell matrix. Degenerate worms are those that had died before collection and whose organs and cuticle are in various stages of physical deterioration. Worms embedded in inflammatory cells matrix are grossly visibly surrounded in a mixture of degenerate inflammatory cells, granulomatous inflammation, and cellular debris on histopathology.

Samples of liver and immature gonad were collected if the organs were not grossly severely autolyzed. Carcasses were then eviscerated and reweighed. Viscera were then re-examined for parasite adults and larvae. The body cavity was then flushed with 0.9% buffered saline solution and the flush solution caught in a Petri dish and examined under the dissecting microscope for the presence of *Philometra saltatrix* larvae and males. Any males or larvae found were quantified, photographed, and their location recorded. Representative samples of males and larvae were saved in 95% ethanol or 10% neutral buffered formalin for fixation.

Finally, otoliths were collected for future microstructure analysis and aging.
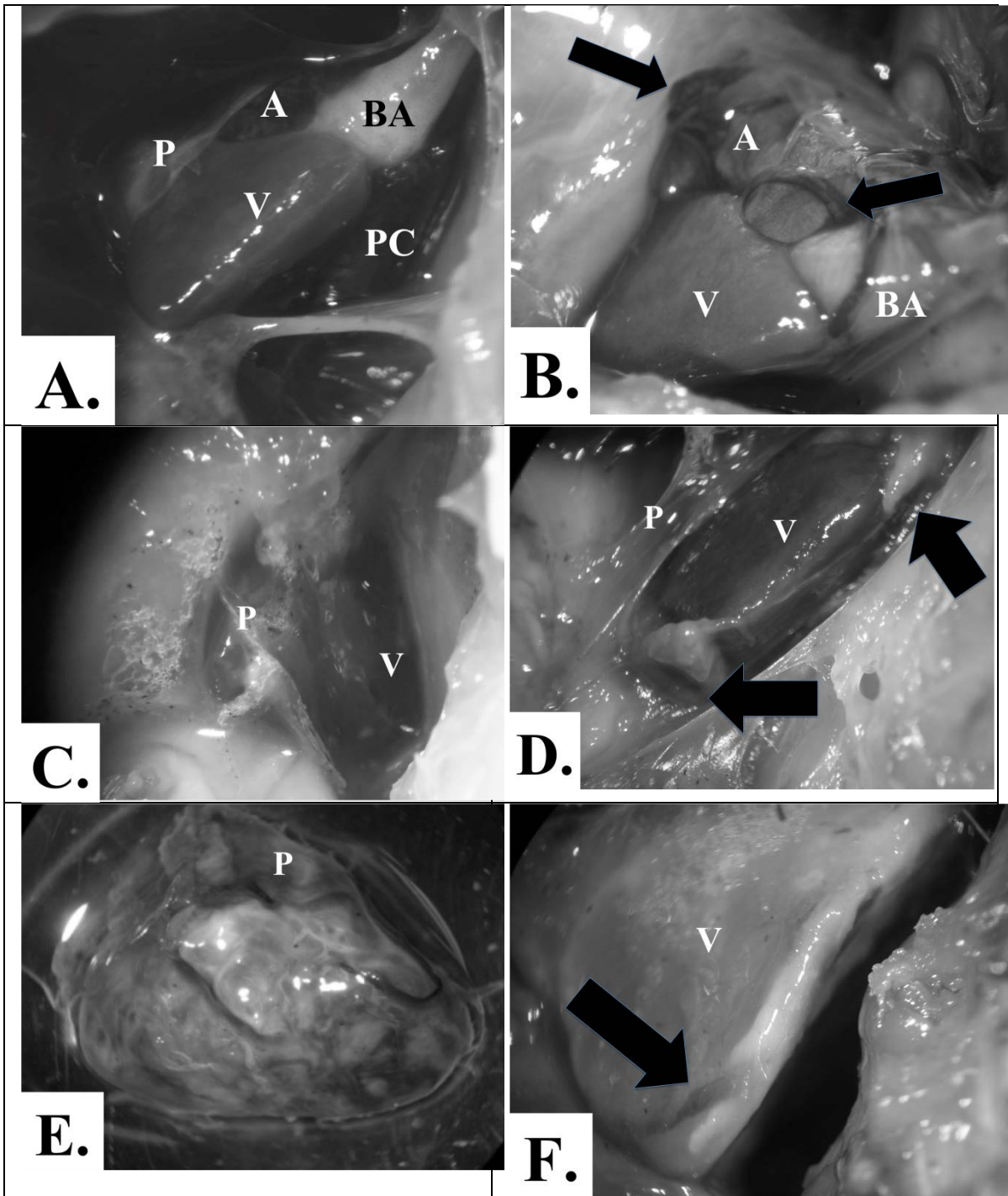
Figure 2. Example photographs of gross pathology scores. P = pericardium, V = ventricle, A = atrium, PC = pericardial cavity, BA = bulbous arteriosus. A. Normal juvenile bluefish heart. B. Grade 1 with *Philometra saltatrix* in pericardial cavity (arrows); C. Grade 2; D. Grade 3, arrows indicate inflammatory matrix and adhesions; E. Grade 4, locally extensive to diffuse fibrosis; F. Grade 5, ventricle encased in fibrotic pericardium. Parasites embedded in multi-focal inflammatory matrix within pericardial cavity (arrow).

Histopathology

Formalin-fixed hearts were sectioned using a sectioning blade and included a uniform section through the bulbous arteriosus, ventricle, and atrium. The sections were placed in fine mesh tissue cassettes in 10% buffered formalin and submitted for histological processing according to standard techniques and staining with hematoxylin and eosin. Hearts were examined via high-power light field microscopy and pathological findings and changes described for each sample. Slides with profound autolysis that precluded accurate description of the cellular characteristics as well as those with incomplete sectioning of all three heart divisions were omitted from histologic assessment.

Slides were evaluated to assess severity and extent of inflammation, degree and extent of pericardial fibrosis, physical condition of worms present in the pericardial cavity, reproductive stages of worms present, and cellular character and extent of inflammatory cell matrix present.

Histopathologic examination was used to confirm or amend the gross pathology score assigned at initial dissection, based on the criteria above. The score that resulted after any amendments was termed the Final Pathology Score (FPS). Final pathology scores of grade four or five were determined to be of fatal severity based on the severity and extent of pericardial fibrosis.

Slides of liver from both normal and pericardial cavity-infected fish were submitted for processing as well as immature gonad samples.

Data Analysis

Cohort assignment was accomplished by creating a length-frequency distribution for each month sampled using R statistical software, and the distribution analyzed to separate the fish into spring and summer-spawned cohorts by size.

Condition factor was calculated using the established formula:

$$K = \frac{\text{weight in grams} * 100}{(\text{fork length in cm})^3}$$

Infection in the pericardium was defined as the presence of any sex or stage nematode in the pericardial cavity. Overall prevalence of pericardial infection was defined as the number of fish from both cohorts, combined, with any sex or stage worm present in the pericardial cavity divided by the total number of fish examined from both cohorts combined. Prevalence of infection in the spring and summer-spawned cohorts individually was defined by the number of fish infected in the pericardial cavity in the cohort of interest divided by the total number of fish examined from the cohort of interest.

The prevalence of pericardial cavity infection over time in a cohort was calculated as the number of fish infected in the pericardium from the cohort of interest sampled during a specific week period divided by the number of fish from the cohort of interest sampled during that week period.

R statistical software was used for graphical representation of data and statistical analysis. Fish fork length, condition factor, and mass were tested for normality using the

Shapiro-Wilk normality test. Percent prevalence of infection, Final Pathology Scores (FPS), and numbers of infected individuals between cohorts were compared using Pearson's Chi-squared test with Yates' continuity correction. Means of non-normally distributed data were analyzed non-parametrically using a Wilcoxon rank sum test with continuity correction. A Welch Two Sample t-test was used to compare means among normally distributed data. Spearman's rank correlation rho was used to assess correlation between non-normally distributed numeric variables. A p value ≤ 0.05 was considered significant in all tests.

Data was analyzed with both cohorts combined, and with cohorts individually subsetted into spring and summer.

RESULTS

Cohort assignment

A total of 80 juvenile bluefish were processed. Fork lengths for all fish were not normally distributed (Shapiro-Wilk normality test, W = 0.9672, p-value = 0.03742). Length-frequency distribution analysis by month resulted in bimodal distributions with two distinct length populations of juveniles representing the two cohorts. The mean fork length (FL) for the spring cohort was 149.48mm (n=45) and 105.51mm (n=35) for the summer cohort. Spring cohort fork length was normally distributed, while summer cohort FL was not. A Wilcoxon rank sum test with continuity correction confirmed the difference between the mean fork lengths of the cohorts was significant (W = 1427.5, p-value = 5.531e-10).

Prevalence

The overall prevalence of infection in the pericardium was 67.5% (54/80). While the spring-spawned cohort prevalence of pericardial infection was 68.89% (31/45) and the summer-spawned 65.71% (23/35), the difference in infection prevalence between cohorts was not significant (Pearson's Chi-squared test with Yates' continuity correction, X-squared = 0.0036, df = 1, p-value = 0.952). When looking at only infected fish, 35.48% (11/31) of the spring cohort and 39.13% (9/23) of the summer cohort had potentially fatal infections. No significant difference in the number or percent prevalence of fatal grade infections in infected fish existed between cohorts (Pearson's Chi-squared test with Yates' continuity correction, p-value = 0.9916, p-value = 0.6984, respectively).

The percent prevalence of pericardial infection over time indicated that prevalence increased in the spring cohort over mid-summer and declined to zero as the spring cohort migrated out of the estuary (Figure 3). The spring cohort was no longer part of samples collected after 9/27/2010. The summer cohort was first found in samples beginning 8/2/2010. Infection prevalence was at 100% for the summer cohort when the sampling period for the year ended on 10/25/2010.
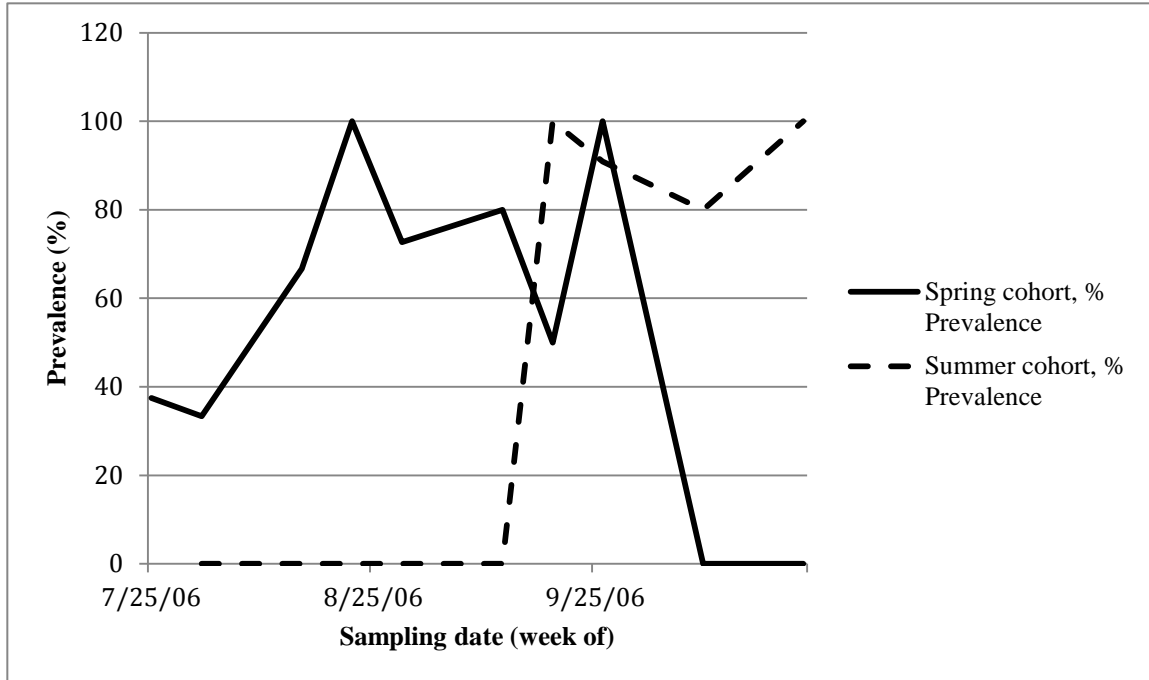
Figure 3. Weekly pericardial infection prevalence by cohort over sampling period.

A significant difference was observed in the mean fork length of fish infected in the pericardium versus those not infected in the pericardium (Wilcoxon rank sum test with continuity correction, p-value = 0.0004). Larger fish were more likely to be infected in the pericardium, with a mean value of 140.65mm, versus those not infected (mean=108.7mm FL). Only fish 97mm FL or larger were found infected in the pericardium.

Fish infected in the pericardium also had a larger mean mass than those not infected in the pericardium (Wilcoxon rank sum test with continuity correction, p-value = 0.0002).

Pathology

Histopathologic examination supported the assigned Gross Pathology Score in

100% of cases and increased the pathology score in 12.12% (4/33) of cases. The revised

score resulting from the combination of Gross Pathology Score (GPS) with

histopathology, the Final Pathology Score (FPS) was used in further pathology analyses.

Prevalence of individual Final Pathology Scores are shown in Table 2. There was

no significant difference in either the percent prevalence or frequency of an FPS between

cohorts (Pearson's Chi-squared test with Yates' continuity correction, p-values = 0.1-

0.99).

Prevalence of Final Heart Pathology Scores in Juvenile Bluefish of the HRE						
Final Path Score	Spring cohort	Prevalence (%)	Summer cohort	Prevalence (%)	Both cohorts	Prevalence (%)
0	14	31.11	12	34.29	26	32.5
1	8	17.78	8	22.86	16	20
2	9	20	4	11.43	13	16.25
3	3	6.67	2	5.71	5	6.25
4	5	11.11	4	11.43	9	11.25
5	6	13.33	5	14.29	11	13.75
n	45	100	35	100	80	100

Table 2. Prevalence of Final Heart Pathology scores in juvenile bluefish of the Hudson River Estuary. *0 = no infection present, 1 = mild pathology, 5 = most severe pathology.*

With both cohorts combined, a significant positive correlation existed between

increasing fork length and increasing FPS (Spearman's rank correlation rho, p-value =

0.0010).

When the cohorts were examined individually, however, there was a strong

positive but barely non-significant correlation between increasing FL and FPS in the

spring-spawned cohort (p-value = 0.0898), but a strongly significant positive correlation

in the summer-spawned cohort (p-value = 3.957e-07). The positive correlation between

increasing fork length and increasing Gross Pathology Score (GPS) was significant for both cohorts combined and for the summer cohort, but barely non-significant for the spring cohort alone (Spearman's rank correlation rho, both p-value = 0.0011; summer p-value = 4.254e-07; spring p-value = 0.0565).

Overall prevalence of fatal infection (grades 4 and 5) was 25% (20/80) in juvenile bluefish of the HRE during the 2010 sampling period. The average FPS for the spring and summer cohorts were 1.89 and 1.80, respectively. No significant difference in the number of fatal (grade 4 and 5) versus low-grade infections (grade 1 and 2) was observed between cohorts (Pearson's Chi-squared test with Yates' continuity correction, X-squared = 0.0018, df = 1, p-value = 0.9665). When looking at only infected fish, 35.48% (11/31) of the spring cohort and 39.13% (9/23) of the summer cohort had potentially fatal infections. The difference was not statistically significant (Pearson's Chi-squared test with Yates' continuity correction, X-squared = 1e-04, df = 1, p-value = 0.9916).

A significant positive correlation existed between increasing FPS and collection date later in the year for both cohorts combined and for the summer cohort alone (Spearman's rank correlation rho, both p-value = 0.0080; summer p-value = 0.0012) but not for the spring cohort (p-value = 0.1837). The FPS peaked for the spring cohort between days 230-240 (mid to late August) and at day 270 (9/27/2010) for the summer cohort. The peak refers to the time period with the greatest frequency of fatal severity infections. Fatal severity infections were seen occasionally after the peak, but not with equal frequency.

Condition factor

Condition factor was normally distributed (Shapiro-Wilk normality test, $W = 0.9945$, p-value = 0.9834). Analysis of condition factor of fish infected in the heart versus those not infected in heart revealed that infected fish have a higher condition factor than those not infected in the heart. Or, alternatively, fish with a higher condition factor are more often infected in the heart (Welch Two Sample t-test, p-value = 0.0015). When the cohorts were examined individually, this was barely non-significant for the spring cohort (normally distributed; p-value = 0.1403), and was very significant for the summer cohort (not normally distributed; Spearman's rank correlation rho, p-value = 3.779e-05). There was no significant difference in the condition factor of fish with low-grade (grades 1 and 2) versus fatal grade (grades 4 and 5) infections (normally distributed; Welch Two Sample t-test, p-value = 0.747). The mean condition factor of fish with fatal infections was higher in the spring cohort than in the summer cohort (p-value = 0.0017). There was no significant difference in the condition factor of those with low-grade infections between cohorts (p-value = 0.2659).

Histopathology

Mild to moderate degrees of autolysis were present across all slides. *Philometra saltatrix* females were often located at the base of the heart within the pericardial cavity, wrapped around the bulbous arteriosus, which is a location associated with less cardiac movement. This was also the most common site of inflammation and fibrosis. No evidence was found of direct parasite damage to the cardiac musculature itself, nor any evidence of myocardial invasion, attachment, pericardial rupture, or migration out of the

pericardial cavity. Immature, subgravid, and gravid female worms were all found within the pericardial cavity.

Grades 1-5 varied in the severity and extent of inflammation, pericardial fibrosis, physical condition of the parasites, and extent of adhesions between the heart surface (epicardium) and the pericardium, or the pericardium and the body wall. In both grade 4 and grade 5 infections, the epicardium and pericardium were diffusely affected, including that of the ventricle, atrium, and bulbous arteriosus. The epicardium and pericardium were, within the affected areas, fused and severely thickened (up to 3 mm) due to severe mesothelial hyperplasia and hypertrophy, abundant granulomatous inflammation, and immature fibrous connective tissue (Figure 4d, 4e). The epicardium had severe and diffuse papilliferous projections of mesothelium, which were compressed and contained few adult nematodes and occasionally, many larval nematodes.

Adult and larval nematodes were surrounded by many epithelioid to attenuated macrophages, few admixed multinucleated giant cells, small to moderate amounts of cellular debris, and moderate amounts of immature fibrous connective tissue. Gravid females were most commonly represented, as well as some subgravid females. Adult nematodes were often degenerate, with collapse and fragmentation of tissues, loss of differential staining, and replacement of structures by cellular debris or rarely mineral. Many larvae were located outside adult nematodes, embedded in granulomatous inflammation (Figure 4f). These larvae were often degenerate, with collapse and fragmentation of tissues, loss of internal structures with retention of a collapsed cuticle, and loss of differential staining. Some larvae were found within the remains of adults,

surrounded in granulomatous inflammation. The extent and thickness of fibrosis was severe, often with involvement of tissues surrounding the bulbous arteriosus.

In contrast, low-grade infections (grade 1 or 2) were associated with non-degenerate, recently viable parasites of all reproductive stages. These were not embedded in inflammatory cell matrix and were associated with mild inflammation, which was typically focal and restricted to the area immediately surrounding the parasite. Focal areas of pericardial mesothelial hyperplasia were present. Grade two infections involved a more reactive and thickened epicardium, with mild to moderate multi-focal granulomatous inflammation.

The degree of pericardial thickening in grade three infections was considerably increased compared to a grade two infection, with moderate to severe focal inflammation and moderate focal to locally-extensive pericardial fibrosis. Subgravid and gravid worms were seen embedded in a matrix of granulomatous inflammation. Parasites embedded in matrix were often in various states of degeneration. Discrete areas of inflammatory matrix were often associated with focal adhesions of the pericardium to the epicardium or body wall.

Slides of liver sections were markedly autolyzed due to the freezing and thawing process, and were not read. Slides of immature gonad were composed of moderately autolyzed ovarian tissue containing primordial follicles.
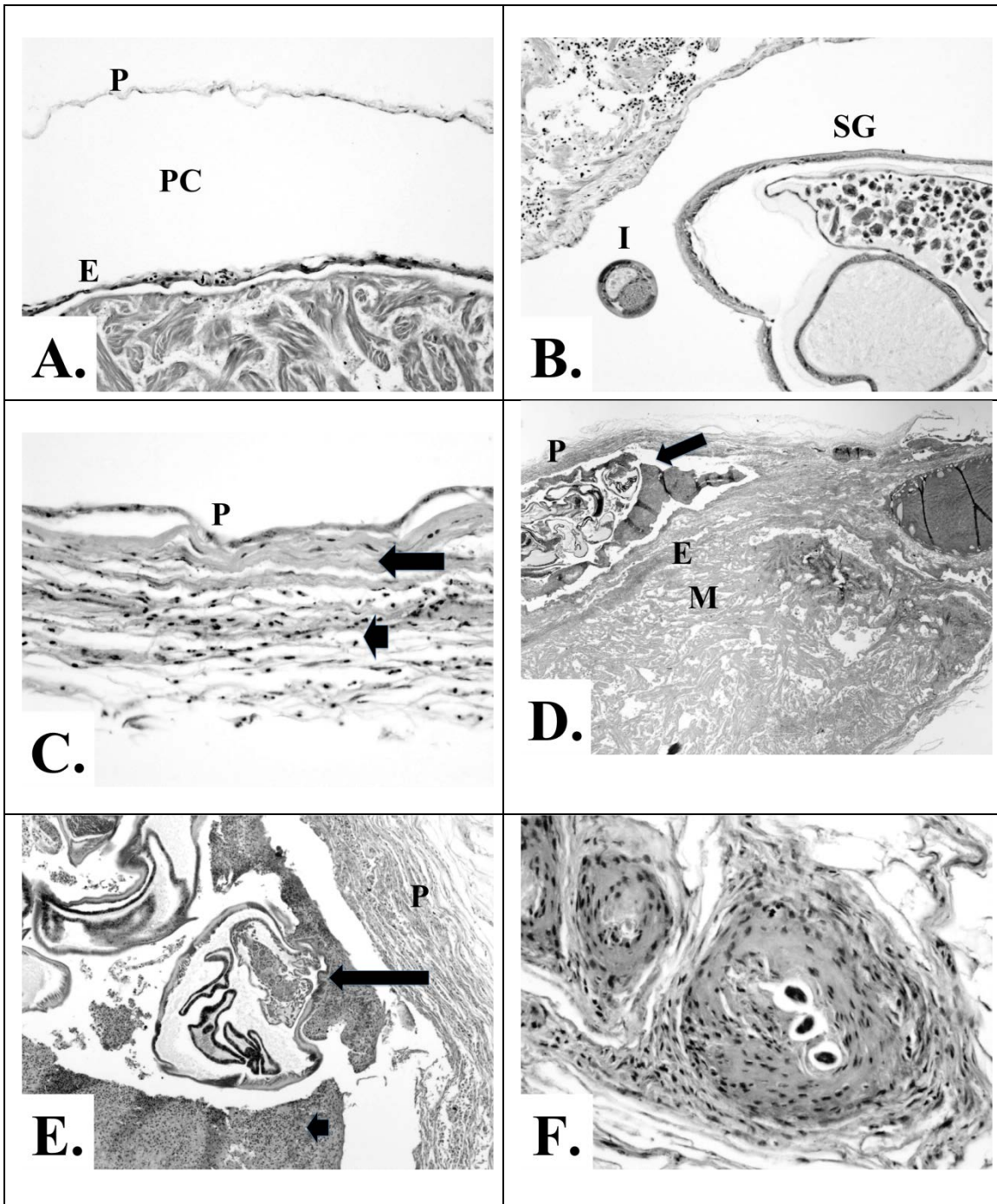
Figure 4. Representative histopathology photomicrographs. P = pericardium, PC = pericardial cavity, E = epicardium, M = myocardium. A. Normal heart, 20x magnification; B. Gross grade 2: immature female (I) and subgravid female (SG) *Philometra saltatrix*, no inflammation; C. Mild inflammation (short arrow) and fibrosis (long arrow); D. Gross grade 5: parasite embedded in diffuse and severe inflammation (arrow), severe fibrosis, 2x magnification; E. (Inset D) Gross grade 5: subgravid female with eggs in uterus (long arrow) embedded in severe inflammation (short arrow), severe fibrosis, 10x magnification; F. Gross grade 5: larvae granuloma, 40x magnification.

DISCUSSION

Pathology and pathophysiology

While *Philometra saltatrix* is responsible for considerable fatalities in juvenile bluefish, its effects are not due to direct tissue damage to the heart caused by the parasite. The severe cardiac pathology is instead due to the host's immune response to the dead parasite and/or release of larvae into the pericardial cavity. This pathology is in the form of varying degrees and extents of pericardial inflammation and fibrosis.

The heart of a fish is divided into three "chambers": a single atrium, a single ventricle, and the bulbous arteriosus (conus arteriosus). Blood from the hind end of the fish is drained through the liver to the atrium, which then empties into the ventricle. The ventricle, the muscular pump of the heart, pumps the blood forward to the gills for oxygenation through a fibroelastic tube called the bulbous arteriosus. The pericardium consists of two very thin layers. One layer, the visceral pericardium, is technically adhered very closely to the surface of the heart and the second layer, the parietal pericardium, makes up what is commonly known as the pericardium. The two layers attach back together at the base of the heart around the bulbous arteriosus. The space between these two layers is called the pericardial cavity. Normally, the parietal pericardium is a single cell layer thick but tough (Figure 4a), and the pericardial cavity contains a very small amount of clear, serous fluid, which acts as a lubricant (McGavin and Zachary 2007).

Histopathology

Histopathology served to further characterize the microscopic changes associated with pericardial infection and understand the pathophysiology behind the changes observed. Low-grade infections associated with live, non-degenerate worms induced minimal pathology, and would not produce systemic signs of disease. Low-grade infections were also associated with immature and subgravid female worms (Figure 4b). The most severe pathology was associated with gravid worms that had died in the pericardial cavity before or after releasing larvae into the pericardial cavity. Thus, the majority of the inflammatory response happens after the worm dies or releases larvae.

The presence of macrophages, multi-nucleated giant cells, and cellular debris indicates the inflammatory process seen is chronic in nature and directed at a large, difficult to breakdown nidus of inflammation. Cellular debris indicates a longstanding inflammatory response as well as bystander cell damage and cellular remodeling. Granuloma formation, or the layering of fibrous connective tissue around an area of inflammation, is a hallmark of chronic inflammation.

The presence of larval granulomas in some fish, or individual/clusters of larvae surrounded by granulomatous inflammation, were observed both free in the pericardial cavity indicating the release of larvae, and also within the degenerate cuticle of a dead gravid female, indicating that the inflammatory response continued after the death of the female (Figure 4f).

The matrix surrounding dead mature worms, formed by inflammatory cells and cellular debris, anchored the pericardium to the epicardium frequently and facilitated the organization of fibrosis and adhesions (Figure 4d).

Pathophysiology

A fatal pericardial infection with *Philometra saltatrix* is defined by the severity and extent of pericardial fibrosis present. Fibrosis is a natural response to chronic infection and inflammation. A foreign body, such as a large nematode or nematode larvae, which is incapable of being quickly broken down and cleared by the cells of the immune system, elicits a demonstrated chain of responses from the body. These include persistent active inflammation, tissue destruction, collagen deposition and fibrosis in an attempt to repair tissue.

Early on, the response is mediated by certain types of white blood cells designed for the chemical disintegration of foreign material. Over time, the predominant form of white blood cell changes to those involved in the consumption and sequestration of foreign material too large to be broken down. Chronic inflammation can be detrimental, however. The long-term presence of inflammatory cells in an area, as well as their chemical inflammatory mediators can cause damage to bystander tissues in the area. The body tries to protect itself from damage by walling off the foreign object and laying down connective tissue in the area to prevent further damage. It is important to note that fibrosis, or the synthesis of collagen fibers leading to connective tissue formation is an irreversible process. The degree and extent of the fibrosis, and how it affects nearby tissues, is central to gauging if an infection will be fatal.

The transformation of the normal, thin, elastic, pliable pericardium to a thickened, fibrous, inelastic pericardium due to chronic, severe inflammation can result in a condition called constrictive pericarditis. As the fish grows or requires increased cardiac

output, this inflexible pericardium restricts proper filling of the ventricle during diastole, or the filling phase of the heart. While the prognosis for constrictive pericarditis in mammals is generally poor (Asher and Klein 2002), the myocardium, or heart muscle itself, is generally unaffected in these situations. Therefore, systolic function of the ventricle is generally unaffected. Once the filling potential of the ventricle reaches its filling constraint, however, filling ceases and congestion of blood behind the heart occurs (Asher and Klein 2002).

Moderate to severe, or severe fibrosis of the pericardium, when diffuse, is considered fatal based on inadequate ability of the heart to fill with blood during diastole. Therefore, grade 4 or 5 infections, both grossly and histopathologically, are considered fatal based on the degree and extent of pericardial fibrosis. Focal fibrosis is more forgiving as the heart contracts and expands, and a fish with focal fibrosis of the pericardium may be able to grow and function normally. Therefore, those fish with focal fibrosis are not included in this calculation.

In mammals, constrictive pericarditis produces signs of exercise intolerance, poor peripheral tissue perfusion, hepatic congestion, and eventual heart failure due to the inability of the heart to meet the circulatory demands of the body. In fish, these changes would be manifested as decreased ability to hunt and forage, decreased ability to pursue normal migratory behavior, and increased susceptibility to predation. The effects would become more pronounced as the fish outgrows the size and cardiac output of its confined heart.

Using histopathology as the definitive scoring tool, the prevalence of fatal infection (grades 4 and 5) in all juvenile bluefish sampled from the HRE in 2010 was 25% (20/80). Of all infected fish, 37.04% (20/54) of infections were potentially fatal.

The 25% annual fatality rate is an approximation based on a relatively small sample size from a portion of the year (late July to October). Ideally, fish should be sampled over the entire calendar year sampling interval with a fixed number sampled from each sampling location. Although the collection netting technique used was designed to optimize random sampling of the fish population, one could argue this fatality rate may be artificially increased due to the inability of clinically-affected grade 4 and 5 infected fish to escape sampling nets. However, the presence of uninfected/score-0 fish in the nets (see Table 2), as well as grades 1-3, makes this unlikely. Also, the peak FPS occurred in mid to late August for the spring cohort and late September for the summer, while sampling continued after those dates. A higher frequency of severe infections would be expected at the end of the sampling period for both cohorts, along with a relative decrease in the frequency of grade 0-3 of that cohort, if the prevalence of clinically affected fish was increased. Instead, there is a decrease in the frequency of severe pathology, indicating either resolving infections or the deaths of severely affected individuals.

An important result of this study is the ability to employ a quick gross inspection technique of pathology in the field with confidence in the agreement between GPS and histopathology-verified FPS. There was always 100% agreement with the gross findings, and histopathology is likely to support or increase the grade of severity. Thus, for researchers assessing prevalence and severity of this infection in juvenile bluefish, it

would not be necessary to submit individual histopathology samples to arrive at a definitive severity grade. This means that the criteria included in the gross pathology scoring system, which are designed to estimate the severity and extent of inflammation, fibrosis, chronicity, and outcome, are also representative of the cellular changes.

The "grade 3" infection

Of the adjustments made to gross pathology scores, two grade one infections out of 19 were increased to grade two, which is not surprising based on the small amount of visible gross pathology. Also, two grade three infections out of seven were increased to grade four. This is interesting because the degree of cellular change was more advanced compared to the gross appearance. Yet, there were also grade three infections with a similar gross appearance which were less severely affected on a microscopic level. Across several of these grade three infections, a common finding involved degenerate worms encased in inflammatory matrix. In the progression of infection from gross grade one to grade five, the most severe inflammation and fibrosis is expected to be associated with dead gravid females in grade five infections. After death, the worms begin to degenerate and are removed slowly by the immune system.

However, in some grade three infections, the worms were degenerate and the inflammatory response less severe and less extensive. This could indicate that some gross grade three infections may be instead sequential to a grade five infection, reflecting a state of resolving infection. This is supported by the presence of larval granulomas and well-encapsulated degenerate worms, indicating a passage of time since the death of the gravid female. Fibrosis of the pericardium was still present but focal to multi-focal in

extent, which is expected because it is irreversible. Thus, both resolving infections of non-fatal severity and progressing grade two infections could have the same gross grade three pathology score, and would require histological examination. Progressing grade two infections would involve live or very recently dead worms.

This sharing of grade 3 GPS by progressing and resolving infections is likely responsible for the loss of significant positive correlation with increasing fork length and increasing final pathology score in the spring cohort. The spring cohort is likely the only cohort affected by this phenomenon because a longer window of the infection cycle is seen during the sampling period, and the severe summer cohort infections have not yet begun to resolve during the sampling period.

Parasite Ecology

Juvenile bluefish likely acquire the infection close to their spawning grounds through the consumption of a copepod carrying an infective larva (Moravec 2004; Bryan et al. 2008). Since a single adult arises from a single infective larva, multiple larvae are acquired to result in multi-worm infections. The difference in the sexual maturity level (immature, subgravid, or gravid) is a function of how recently the infective larva was consumed (assuming a constant rate of parasite maturation). All females found in the pericardial cavity were fertilized, indicating the presence of male nematodes in the fish's body as well. Recent research has shown males to be present in the body cavity and gonad in juvenile bluefish (Koske et al., unpublished research).

The weekly percent prevalence of infection seen over the course of the sampling period (Figure 3), which occurs in two major waves, reflects the staggered arrival of the

cohorts in the estuary. After the fish acquires the parasite, at least two months pass before infection is first visible in the pericardial cavity. For example, the summer cohort appears in the HRE in early August but the first infection in the summer cohort is not found until six weeks later, when prevalence suddenly approaches 100%. This is likely a function of the close time proximity in which members of the summer cohort become infected and the maturation time of the parasite. If the sampling period were extended earlier in the year, a similar trend would likely be observed in the spring cohort.

Given the fact that the parasite's reproductive strategy is likely linked to the off-shore spawning of adult bluefish (Clarke et al. 2006; Moravec 2004) and the parasite is likely acquired off-shore, not within the HRE, the parasite likely matures to its adult form during the fish's migration into, and early residence in, the estuary. This hypothesis is supported by the fact that a significant trend exists linking increasing fork length with increasing pathology score. If the parasite was acquired in the estuary, this trend would be obscured by recurring waves of grade one infections and the progression of those infections. Instead, there is a significant correlation in both cohorts with greater pathology scores and larger fork lengths with collection dates later in the year. These results point to a single infection event in each cohort. Also, evidence of re-infection, defined by the co-existence of immature females and degenerate gravid females in the pericardial cavity, was not observed during the entirety of this sampling period, decreasing the likelihood the parasite is acquired in the HRE.

The overall prevalence of infection in the pericardium of all fish sampled was 67.5% (54/80). Studies evaluating the prevalence of parasitic infection in a natural wild population are rare and thus comparable studies of other nematode parasites are few.

With a prevalence of 67.5%, it is unusual to be lacking reports of occurrence from the rest of the 1980s and 1990s. Cheung (1984) described an 80% prevalence in fish examined at the New York Aquarium; however, his sample size was not reported in the available abstract. Obviously, this parasite is widespread in the Hudson River estuary and not localized to specific geographical areas of the Hudson River.

Importantly, during this sampling window, both the spring and summer cohorts were equally infected in terms of prevalence and severity of infection. This indicates that the summer cohort is not preferentially infected, and mortality associated with this infection affects both cohorts equally. This is not in agreement with the proposition by Clarke et al. (2006) who hypothesized that the summer cohort may be preferentially infected due to a higher occurrence and intensity of *Philometra saltatrix* in the gonad of summer-spawning adults.

Because the exit of the summer cohort from the HRE was not observed during the sampling period, the summer cohort could possibly experience an increase in mortality not seen in this study. However, it is also possible that the spring cohort experiences mortality events as infection continues to progress when the fish have migrated back out to sea and are effectively lost to the study. Thus, the ability to definitively evaluate and compare mortality between cohorts in a natural setting may not exist. Experimental infection and monitoring over time would give a better indication of mortality rates in general, but the natural factors such as seasonal prey availability and water quality and temperature that may affect infection rates between cohorts would be lost in this study.

During their residence in the HRE, juveniles grow in length, mass, and condition. The smallest fish were not infected in the heart, and longer and heavier fish were more

likely to be infected in the heart. This reflects the time interval between initial infection and when the infection is grossly observable. Fish infected in the heart also had a higher condition factor, which is not surprising given that condition factor is dependent on both mass and length. Thus, the condition factor of the spring cohort is also expected to be higher than for the summer because of their greater relative length and mass.

Gross pathology scores also increase with fork length, which reflects the fish acquiring the parasite when small and having the infection develop and become more severe as it grows over the course of the year. The fact that there is a trend for high degree pathology in larger fish, in addition to the discovery that this correlates also with time of year, is indicative that larger fish are not preferentially affected, but rather that the score is the result of a natural long-standing infection. The final pathology score, which is adjusted to reflect the histopathology findings, also increases with length and mass for the same reason.

The histopathologic findings observed in 2010 juveniles of the HRE and larger juveniles sampled from the New York Bight (NYB) in 2009 are similar (Koske and Pinkerton 2010), although the prevalence of fatal infection in the NYB was only 9.09%. This possibly reflects a further loss of severely infected individuals after migration out to sea, or instead a reflection of the smaller sample size (n=33) in the NYB study.

In this sampling period, data on the spring cohort was collected for ten weeks, and 13 weeks for the summer cohort. However, of those 13 weeks, the summer cohort was infected for only six weeks. The prevalence and average pathology score data were taken from the entirety of the sampling period, not just the weeks where infection was present. Thus, the comparison between the results of a 10-week and a 13-week study showed

equal prevalence and pathology scores between cohorts. Without a sampling period that includes both the arrivals and departures of both cohorts from the HRE, it cannot definitively be said that one cohort is not preferentially affected over the other. However, within the sampling period of this study, no significant difference was observed between cohorts in the overall prevalence of pericardial infection, the prevalence of high versus low-grade FPS, or the number or prevalence of fatal infections.

Condition factor and infection

Results showed that fish infected in the heart had higher condition factors than those not infected in the heart. The formula used to calculate condition factor is dependent on both fork length and mass. Since condition factor increases as fish FL increases, the link between infection in the pericardium with both increased condition factor and increased FL is not surprising. While one might expect a parasitized fish to have a lower condition factor than a non-parasitized fish, the results show that fish are grossly infected in the pericardium only after reaching 97mm FL and a condition factor of 1.068. This could indicate two possibilities: 1) fish are infected early in life and infection becomes apparent only around the age when condition factor approaches 1.0; 2) the parasite might arrest in development within the fish until the fish reaches a size and condition appropriate for optimal parasite development.

This also indicates that a severe pericardial infection does not induce significant loss of condition while the fish resides in the estuary. Some loss of condition may be present and not reflected here, however, because the increasing length of the fish would mask any loss of mass due to the method of calculation. The fact that mass continues to

increase with increasing FPS also indicates that infection does not significantly impact fish mass. Generally, a parasite that routinely induced fatality or severe condition loss of its host would not be evolutionarily successful due to host loss. *Philometra saltatrix* infection may not even result in loss of condition. Knowing this infection can be fatal through cardiac insufficiency and heart failure, any difference in condition may only become apparent in severely infected fish just before the death of the fish, or not at all if the fish succumbed to predation. These fatalities, of course, would occur out at sea and are not measurable.

Suspected changes in the severely affected juvenile's ability to successfully forage, complete natural migrations, and escape predation are based on the pathophysiology of heart failure. Moles (2003) described decreased prey capture rates in Dolly Varden (*Salvelinus malma*) parasitized by the Philometrid nematode *Philonema agubernaculum* in the body cavity. Parasitized fish captured only 32% of available prey versus 64% by non-parasitized Dolly Varden. No difference was observed in the condition of the parasitized versus non-parasitized Dolly Varden. Moles (2003) hypothesized that while the effects of infection might be minimal when food supply is abundant, in a limited food supply environment, the non-parasitized fish likely fare better, and thus infection has the potential to alter predator-prey relationships.

Bluefish is a species heavily dependent on burst swimming and ambush during normal feeding. Eventual decreased cardiac output and oxygenation of peripheral tissues would lead to changes in cellular metabolism, thereby causing early muscle fatigue and affecting foraging ability. Since constrictive pericarditis compromises the cardiac output

of an affected fish, infection with *Philometra saltatrix* could also predispose affected fish to fatalities associated with low dissolved oxygen content (McGladdery et al. 1988).

CONCLUSIONS

Pericardial cavity infection by *Philometra saltatrix* is an important source of mortality in juveniles of the Hudson River Estuary, potentially fatal in 25% of all fish sampled. The pathologic consequences of infection, which include varying degrees of severity of constrictive pericarditis, have the potential over time to severely impact the foraging and migration capabilities of severely affected growing fish. The most severe pathology is induced after the death of the parasite and/or release of larvae into the pericardial cavity. The severity of infection can be accurately assessed by quick gross examination of changes seen in the character of the pericardium as described in this study.

The results of this study show that both spring and summer-spawned cohorts are equally affected in terms of prevalence of infection, severity of pathology, and prevalence of fatal infections. Thus, this study suggests that *Philometra saltatrix* is not responsible for the preferential decline in recruitment observed in the summer cohort.

Infection is likely acquired off-shore near spawning grounds and develops over the course of the fish's migration into the estuary. Over time, as the fish increases in size, mass, and condition, the likelihood of infection in the pericardial cavity as well as the severity of pathology increases. There was no evidence fish are re-infected in the estuary, decreasing the likelihood of involvement of a paratenic host in transmission of the infection in the estuary.

RECOMMENDATIONS

Further study is needed to definitively quantify the negative effect on recruitment caused by pericardial infection with *Philometra saltatrix*. Ideally, samples should be collected from various locations in the HRE over the course of an entire calendar year in order to observe the natural infection cycle in both cohorts.

Obtaining fresh samples of liver tissue at the time of processing could histologically indicate whether passive congestion of blood is occurring in the liver of grossly affected fish, which is a classic symptom of heart failure in mammals. This would enable reliable extrapolation of other classic signs of heart failure to fish.

Optimally, in order to chronicle the story of infection from beginning to end and note the timeline, as well as changes in health, growth rates, condition, and fecundity, experimental infection should be pursued in a controlled environment over multiple years. This would also enable the study of the health impacts on survivors of infection and the whole of cohorts infected versus not infected.

Finally, it is important to note that the prevalence of infection likely varies from year to year, depending on ocean current strengths, annual copepod crop, and water temperature, as maturation rate of Philometrid species larvae in experimentally infected copepods has been demonstrated to be heavily dependent on water temperature. Since the presence of this parasite was not noted during the late 1980s and 1990s, it is possible that infection prevalence has been steadily increasing since that time and *Philometra saltatrix* infection in the pericardial cavity could be an already emergent and unmonitored source of recruitment failure among juveniles of both cohorts. Fisheries biologists and

conservation scientists should be involved in field monitoring of infection prevalence,

and additional effort should be directed toward elucidation of the life cycle of this

parasite to help direct future intervention.

ACKNOWLEDGEMENTS

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