The role of the fish parasite *Myxobolus inornatus* in young-of-year Smallmouth Bass *Micropterus dolomieu* mortality in Pennsylvania

Identifying parasite distribution, intermediate host, and distribution of potential intermediate hosts

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INTRODUCTION

Prolonged incidence of disease-related mortality among young-of-year (YOY) Smallmouth Bass *Micropterus dolomieu* has caused a population decline and shifted population characteristics in a recreationally popular and locally economically important game fish in the middle and lower Susquehanna River and lower Juniata River (Smith et al. *In Press*). Mortality of YOY Smallmouth Bass was first documented in 2005 and again annually at varying degrees between 2006 and 2012 at the West Branch Susquehanna, Susquehanna, and Juniata rivers. *Flavobacterium columnare* (columnaris) and a number of motile aeromonad species have been isolated from the lesions of a high number of affected YOY Smallmouth Bass. Since 2010, visible lesions have been documented in other warm-water streams in the Susquehanna River Basin. Similar lesions were first observed outside of the drainage in the Allegheny, Schuylkill, and Delaware rivers during 2011. Many factors are of concern and may contribute to the observed lesions and population decline including higher than normal water temperatures, low dissolved oxygen levels, contaminants and infectious agents. Internal and external signs of parasite infections, both trematodes and myxozoans, are commonly observed in the diseased YOY Smallmouth Bass. However, previous sampling efforts for histopathological analyses have generally targeted fish with signs of disease (moribund, raised and/or eroded lesions) or were limited in sample size.

One factor of concern that had not been previously investigated was the presence of the myxozoan parasite *Myxobolus inornatus* (Walsh et al. 2012) within muscle and connective tissue. Myxozoan parasite infections can be responsible for important economic losses in fisheries and aquaculture industries (Sitjà-Bobadilla 2008). *Myxobolus cerebralis*, the parasite responsible for whirling disease in salmonids, has caused negative economic impact on recreational trout fisheries in Colorado (Nehring and Walker 1996) and other states where tourism was an important part of local and state economies (Mahoney and Hudson undated; Koel et al. 2006). Proliferate gill disease (PGD), caused by the myxozoan parasite *Aurantiactinomyxon ictaluri*, is the third most common disease diagnosis among commercially farmed Channel Catfish *Ictalurus punctatus* (Hanson et al. 2008). More recently, *Sphaerospora motemarini*-related mortality in juvenile Grey Snapper *Lutjnaus griseus* was predicted to have impacts in commercial and recreational fisheries for that species in the Gulf of Mexico (Holzer et al. 2013).

The role that the myxozoan parasites play in the YOY Smallmouth Bass mortalities remains unclear. Parasites may compromise YOY in a number of ways: high parasite loads may contribute to general stress, as well as immune suppression, resulting in increased susceptibility to opportunistic bacteria; sites of parasite entry or exit may cause wounds allowing for bacterial entry; or heavy parasite loads may increase sensitivity to water quality related stressors. To better elucidate the complex factors involved in the YOY disease syndrome, the distribution of the myxozoan parasite, identification of the intermediate host(s) and determination of the distribution of the intermediate host to infer risk to other populations are necessary. *Myxobolus inornatus* was first reported from Largemouth Bass fingerlings in a Montana hatchery in 1937 (Fish 1939). To our knowledge, the only other reports since then were from adult Smallmouth Bass in Lake Erie with identification based spore morphology (Dechitar and Nepszy

1988; Muzzall and Whelan 2011). Since then, the isolate from YOY Smallmouth Bass has been redescribed, genetically sequenced, and primer sets were developed (Walsh et al. 2012).

Objectives of Current Research

1). Distribution of the myxozoan infection in YOY Smallmouth Bass in Pennsylvania watersheds.
-To date, the sample sizes have been small and opportunistic. Sampling efforts for the 2013 season included random samples of at least 10 (preferably 20) YOY from a given site in order to gather larger samples sizes and to not specifically target diseased individuals.

2). Identification of oligochaete intermediate host(s) for the myxozoan parasite(s) in YOY Smallmouth Bass.
-Myxozoans commonly utilize oligochaete worms or other invertebrates, such as polychaetes and bryozoans, as intermediate hosts. Preliminary research by USGS during 2011 utilizing internal funding identified myxozoan DNA in some oligochaete worms (one is an invasive species); however, the sample size was extremely small. Further investigation is necessary to identify intermediate host(s) to better describe the systematics, life cycle and infectivity of *M. inornatus* as there is little known about this species.

3). Determine the distribution of oligochaete intermediate host species.
-Documenting the distribution of the intermediate host(s) would help to understand the potential for expansion of the parasite's range. In addition, identifying the water quality, climatic and flow regimes associated with populations of the intermediate host could help focus host-control initiatives, if applicable.

4). Determine the role of this parasite in YOY Smallmouth Bass infections and mortalities.
-Documenting the presence of the parasites in fish with lesions, the extent of resulting tissue damage and the presence or absence of opportunistic bacterial infections will help identify the risk factors for YOY mortality. Identification and genetic sequencing of the parasite allows for the development of techniques to study early infection. For instance, during routine histopathological analysis of YOY collected in 2011 and 2012, inflammation was observed under the skin and within the muscle prior to the observation of myxozoan cysts. This inflammation may be related to the early life stages of the myxozoan parasite or the presence of other pathogens. *In situ* hybridization methods will be used to detect DNA of immature stages of the parasite and commonly isolated bacteria, and will help to clarify the role of the various parasites/pathogens in the mortalities.

METHODS

<u>Fish collections</u>
YOY Smallmouth Bass were collected from different reaches of select large rivers within Pennsylvania as part of the Pennsylvania Fish and Boat Commission's (PFBC) directed surveys to document densities of YOY black bass. Locations used for specimen collection included areas where disease has been

documented in the past and areas where this condition has not been previously observed (Table 1). These included sites from each of the major drainages in the Commonwealth. Specimens were collected with back-pack electrofishing units (Smith-Root Model 12-B POW, Smith-Root, Vancouver, Washington; Coffelt Electronics Company, Inc. Model BP-1, or Appalachian Aquatics, Inc. Model AA-24, Appalachian Aquatics, Inc., Morristown, Tennessee) using alternating current (AC) or pulsed direct current (PDC) waveforms. A sample size of 20 specimens per site was targeted with a minimum number of 10. This was a random sample and did not specifically target affected fish. Specimens were euthanized using tricanemethanosulfate (MS-222, Argent Finquel®, Redmond, Washington) and preserved in Z-fix formalin solution (ANATECH, Ltd., Battle Creek, Michigan) for histopathological and *in situ* hybridization analyses.

Invertebrate collection

Collections were conducted in typical YOY Smallmouth Bass habitats and other large river habitats using a dome sampler (Gale and Thompson 1975). This sampler provides a comprehensive collection of small benthic macroinvertebrates to a depth of 5-10 cm within a 0.18 m^2 area, and allows for calculation of density of the species present. Two or three samples were taken from each collection site based on substrate availability. Samples were collected during late-May through early-June to capture species diversity during the timeframe when fish are believed to be infected with myxozoan parasites. Samples were live-sorted in the field or chilled in ice for laboratory sorting. Oligochaetes were preserved in 95% non-denatured ethanol for taxonomy and parasite analysis. Oligochaete worms were taxonomically identified to species or lowest achievable taxon based on condition of specimens. Worms were identified using a Leica AP08 Stereomicroscope (Leica Microsystems, Wetzlar, Germany) and Nikon Eclipse E200 and Nikon Labophot compound microscopes (Nikon Instruments, Inc. Melville, New York). Large tubificids were identified using stereomicroscopy while all other individuals were identified using simple wet mounts or temporarily mounted using CMC-10 mounting media (Masters Company Inc., Elk Grove, Illinois) so that anatomy and penal structure could be observed.

Histology

Whole bodies of YOY Smallmouth Bass were fixed in Z-fix formalin solution for at least 24 hours and decalcified using Cal-Ex (Fisher Scientific, Pittsburgh, Pennsylvania). Tissues were dehydrated, embedded in paraffin, sectioned at 5µm and stained with hematoxylin and eosin or Giemsa stain in preparation for histological evaluation (Luna 1992).

Molecular analysis

Pieces of muscle that exhibited inflammation characteristic of *M. inornatus* infection were placed in 96% alcohol for DNA extraction. Genomic DNA was extracted using a DNeasy® Tissue Kit (Qiagen Inc., Valencia, California) according to the manufacturer's protocol. The short subunit (SSU) rDNA gene cluster was amplified using polymerase chain reaction (PCR) primers that targeted myxozoan sequences. PCR products were cleaned with a QIAquick® PCR Purification Kit (Qiagen Inc.) and prepared for direct sequencing. A nonradioactive *in situ* hybridization technique (ISH; Antonio et al. 1998) is currently under development to determine the presence, tissue locations and associated response to *M. inornatus* in both fish and invertebrate tissues.

Invertebrate samples preserved in 95% ethanol from various sites were used for DNA extraction for both worm identification and myxozoan detection. Two extraction methods were tested including DNeasy® Tissue Kit (Qiagen Inc.) and Chelex® 100 (Bio-Rad Laboratories, Hercules, California). PCR was used to amplify myxozoan sequences with primers selected from Walsh et al. (2012), as were worm sequences with various oligocheate primers (Hallett et al. 2005). PCR products were cleaned with a QIAquick® PCR Purification kit (Qiagen Inc.) and prepared from direct sequencing. Sequences obtained were compared to existing myxozoan and oligocheate sequences using low complexity (E=0.01) National Center for Biotechnology Information (NCBI) BLAST searches.

RESULTS

Fish collections

Young-of-year Smallmouth Bass were collected between 15 July and 25 July at eight of 10 predetermined sites. High flow events in June and July in the upper Susquehanna River and West Branch Susquehanna River affected the abundance of YOY Smallmouth Bass at the time of sample collection. An adequate number of specimens (n=10-20) could not be obtained during initial surveys at the Delaware River at Matamoras, Susquehanna River at Mahantango Access, or West Branch Susquehanna River at Watsontown sites (Table 1). Additional effort at the Delaware River at Matamoras (16 August) yielded an adequate number of individuals for histological analysis.

Table 1. Waterbody, location, latitude, longitude, and status of young-of-year Smallmouth Bass *Micropterus dolomieu* and oligochaete worms (Annelida, Clitellata) collections.

Waterbody	Location	Latitude	Longitude	Fish Sample	Oligochaete Sample
Delaware River	Matamoras	41.38750	-74.71330	Y	Y
Lehigh River	Northampton	40.67799	-75.49262	Y	Y
Schuylkill River	Port Clinton	40.57722	-76.02530	Y	Y
Allegheny River	Buckaloons	41.83889	-79.25750	Y	Y
Susquehanna River	Laceyville	41.64200	-76.15700	Y	Y
Susquehanna River	Danville	40.96222	-76.63305	Y	Y
Susquehanna River	Mahantango	40.65389	-76.92138	N	Y
Susquehanna River	Harrisburg	40.31861	-76.89916	Y	Y
Juniata River	Thompsontown	40.55389	-77.24029	Y	Y
West Branch Susquehanna River	Watsontown	41.07944	-76.86083	N	Y

The catch per unit effort (CPUE; #/ 50m segment) of YOY Smallmouth Bass varied by site and ranged from 0.0 fish/ 50m (three sites) to 5.3 fish/ 50m at the Allegheny River at Buckaloons (Table 2). Smallmouth Bass with clinical disease (i.e., external lesions) were found at one of the 10 sites surveyed during 2013 (Table 2; Figure 1). No YOY Smallmouth Bass were caught initially at the Delaware River site and those values are reported for catch rate. Catchability of YOY Smallmouth Bass by prescribed sampling gear changes with ontogeny and disease impairment (i.e., larger fish are harder to catch,

disease fish are easier to catch) so catch rates for follow-up surveys are not reported to reduce bias. However, these fish were used to determine distribution of the parasite. Follow-up surveys were not conducted at the two remaining sites were bass were not collected.

Table 2. Catch, catch per unit effort (CPUE), number clinically diseased fish, prevalence of disease (parentheses), and presence of myxozoan parasites in young-of-year Smallmouth Bass *Micropterus dolomieu* at selected locations in Pennsylvania.

Waterbody	Location	Catch	CPUE (#/50m)	Diseased # (%)	Presence of Myxozoan
Delaware River	Matamoras	0	0.0	NF[1]	No
Lehigh River	Northampton	15	2.5	0	No
Schuylkill River	Port Clinton	25	4.2	0	No
Allegheny River	Buckaloons	32	5.3	0	Yes
Susquehanna River	Laceyville	23	3.8	0	Yes
Susquehanna River	Danville	9	1.5	0	No
Susquehanna River	Mahantango	0	0.0	NF	NF
Susquehanna River	Harrisburg	28	4.7	4 (14.3)	Yes
Juniata River	Thompsontown	5	0.7	0	Yes
West Branch Susquehanna River	Watsontown	0	0.0	NF	NF

NF = No fish collected

[1] Follow-up survey yielded enough fish for histological analysis

Invertebrate collections

Oligochaetes were collected from each of the 10 predetermined sites identified for YOY Smallmouth Bass collection (Table 1). A total of 16 taxa were collected across the 10 sites (Table 3). Diversity varied among sites from ten taxa at the Allegheny River at Buckaloons to two taxa each at the Susquehanna River at Mahantango and Schuylkill River sites (Table 3). Many of the specimens were immature at the time of collection and as such could not be positively identified to species. Additionally, 71 megadrile specimens (suborder Lumbricina) were found at five locations. These are generally considered earthworms and were not included in the analysis.

Molecular Analyses for Identifying the Intermediate Host

Primer sets are currently being evaluated for worm identification. Morphologically identified worms from the invertebrate collections (Table 3) will be used for determining parasite presence. Some problems with extraction methods have occurred and hence different extraction techniques are being evaluated for DNA quantity, quality and subsequent sequencing.

Only a small subset of taxonomically-identified worms has been available from 2013 samples. From this subset, none of the worms tested positive for *M. inornatus* DNA. Further taxonomic analyses have recently been completed on another subset of worms and hence these analyses are continuing.

Role of the parasite in disease and mortality

Very few YOY Smallmouth bass were collected in 2013 with clinical disease. Because so few fish were collected with observable lesions it was not possible to assess the role of this parasite in the disease syndrome or mortality during 2013. *M. inornatus* (or a similar-appearing myxozoan) was observed in fish at sites in the Susquehanna drainage and one fish from the Allegheny River. Of these, only fish from the Susquehanna River at Harrisburg had clinical disease. Myxozoan cysts were not observed at sites in the Delaware drainage.

At sites within the Susquehanna drainage fewer myxozoan parasite cysts and associated inflammation were observed in 2013 than were observed at sites with previously available data. Further analyses are necessary to determine if time of collection, annual differences water temperature, stream flow, or other water quality factors may influence the prevalence and severity of the infection.

DISCUSSION

Preliminary analysis conducted during 2011 identified myxozoan DNA in two oligochaete species whose DNA closely matched *Branchiura sowerbyi* and *Rhyacodiloides* sp. (Naididae). This suggested one or more intermediate worm hosts present in the life cycle of *M. inornatus*. For this reason, a more intense and consistent evaluation of the benthic worm populations, at multiple sites coinciding with fish collections, was conducted. Unfortunately, none of the worms tested in our samples appeared to host *M. inornatus*. This may be in part due to the small samples sizes of individual genera/species. Whirling disease is perhaps the most intensively studied myxozoan infection of fishes. The intermediate host for this disease is *Tubifex tubifex*. At sites in the Colorado River where population declines of trout have been attributed to whirling disease, studies have shown that the prevalence of infected tubificids (oligochaetes with hair chaetae) was only 1.2 to 2.8% (Zendt and Bergersen 2000). If similar (or less) prevalent infections of the intermediate host for *M. inornatus* occur, it is unlikely we would have detected it in the samples analyzed. In addition, there may be seasonal or even monthly differences in prevalence of the myxozoan in the worm host, hence, collections should probably occur at various times beginning earlier than the currently available collections.

Lumbriculus. variegatus has been documented to serve as an intermediate host for a number of myxozoan species (Özer and Wootten 2000; Urawa et al. 2011). *L. variegatus* was widespread among sites surveyed. *L. variegatus* was identified from six of the 10 locations surveyed during 2013. *L. variegatus* is considered a ubiquitous species and as such is commonly used as a test organism in laboratory studies including toxicity testing (Bailey and Liu 1980; Phipps et al 1993). It has a temperate Holarctic distribution with human introductions in the southern hemisphere (Pinder and Ohtaka 2004).

A variety of benthic worms in the subfamily Tubificinae were identified in the samples collected that have been shown to be intermediate hosts for myxozoan parasites of fishes, including the tubifcids both

with and without capilliform chaetae. Although these were all immature worms that could not be identified to genus, this group includes *T. tubifex* which is an intermediate host for numerous myxozoan parasites (Kent et al. 2001). *Limnodrilus hoffmeisteri*, another tubificid worm, was widespread during the survey and also serves as an intermediate host for a number of myxozoan parasites (El-Mansy and Molnár 1997; Hallett et al. 2006)

Branchiura sowerbyi has been reported as an intermediate host for a number of known fish parasites (Yokoyama et al. 1993; Yokoyama 1997; Székely et al. 1998; Kent et al. 2001; Caffara et al. 2009). *B. sowerbyi* is an aquatic invasive species native to Southeast Asia (Mills et al. 1993, Grabowski and Jablonska 2009, Raposerio et al. 2009) and believed to be introduced to North America through aquatic tropical plant and commercial pet trade (Paunovic et al. 2005, Raposerio et al. 2009). The first documentation found of *B. sowerbyi* in the Susquehanna River was in 1969, one mile downstream of Brunner Island (Wurtz 1970). However, macroinvertebrate investigations done in this reach of river rarely identified oligochaete worms to species level so exact dates of introduction of this species into the Susquehanna River is uncertain. *B. sowerbyi* is generally associated with warm-water conditions, including thermal effluent from power plants, with optimal reproductive and growth rates between 21 and 29^{o}C (Aston et al. 1982). At 25^{o}C, Aston et al. (1982) found that it was possible for a population of *B. sowerbyi* to double in as little as 1.58 weeks.

Like some members subfamily Tubificinae, members of the Naidinae including *Nais* sp. and *Dero digitata* have also been reported as intermediate hosts for important fish pathogens (Kent et al. 2001; Atkinson and Bartholomew 2009). One individual in this subfamily was found at each of two locations suggesting that the role that Naidinae may play as intermediate hosts is limited.

Some of the more ubiquitous worm species are tolerant of high levels of pollution. Brinkhurst (1980) categorized oligochaete assemblages including *T. tubifex*, *L. hoffmeisteri* and *Q. multisetosus* as pollution tolerant with a second assemblage containing *L. cervix*, *L. maumeensis*, and *B. sowerbyi* as slightly less pollution tolerant but reflective of strongly eutrophic conditions. Kaeser and Sharpe (2006) found that *L. hoffmeisteri* was present in both high-gradient and low-gradient Pennsylvania streams but increased in abundance as stream gradient decreased and fine sediment and organic pollution increased at a site. The presence of non-host worm species including *L. hoffmeisteri* may also be important. McGinnis and Kerans (2013) demonstrated that the abundance of *L. hoffmeisteri* was a factor contributing to occurrence of whirling disease at various locations due to habitat overlap with the target species (*T. tubifex*). Kaeser et al. (2006) identified the best factors for identifying the occurrence of *T. tubifex* infected with *Myxobolus cerebralis* was presence of organic point sources of pollution on low-gradient streams. Reductions of organic pollutants, lowering erosion, increasing stream flow, and removing objects that accumulate pockets of silt and other fine materials may help to control worm populations and subsequently disease (Brinkhurst 1996).

CONCLUSIONS

Based on sampling in 2013 and previous years, *M. inornatus*, a myxozoan parasite of muscle and connective tissue in YOY Smallmouth Bass appears to be widespread in the Susquehanna River basin,

present at a lower incidence and severity in bass from the Allegheny River, and to date has not been observed in samples from the Delaware drainage. Clinical disease was only observed at one mainstem site in the Susquehanna Basin in 2013; hence evaluating the potential role of the parasite in the disease syndrome of YOY was not possible. Despite the lack of visible lesions, there were signs of disease microscopically at the other sites including the presence of myxozoan cysts and inflammation in the muscle and connective tissue associated with myxozoan infections. We are uncertain if fish with myxozoan infections that did not have clinical disease would have developed clinical disease had they not been captured.

Table 3. Oligochaete worm taxa collected using a dome sampler (Gale and Thompson 1975) in representative young-of-year Smallmouth Bass *Micropterus dolomieu* habitat at ten locations across three major drainages in Pennsylvania. Phylogeny from Wetzel et al. 2009.

	Ohio	Delaware			Susquehanna				
					West Branch				
	Allegheny	Delaware	Lehigh	Schuylkill	Susquehanna	Juniata	Susquehanna River*		
Taxon	River	River	River	River	River	River	A	B	C
Lumbricina		6	28	33	1		3		
Lumbriculidae									
Lumbriculinae									
Lumbriculus variegatus	3	28	2	13	1				3
Tubificina									
Enchytraeidae	1								
Haplotaxidae									
Haplotaxis gordioides		6							
Naididae	1				1				
Naidinae									
Nais sp.			1						
Chaetogaster diaphanus					1				
Tubificinae									
Tubificinae w/ capilliform chaetae (immature)	1								22
Tubificinae w/o capilliform chaetae (immature)	15	2			7	26	33	32	
Aulodrilus pluriseta	1								
Branchiura sowerbyi						6			1
Ilyodrilus templetoni	2								
Limnodrilus cervix	6								
Limnodrilus claparadeianus	5								
Limnodrilus hoffmeisteri	17	14				10	108	1	10
Quistadrilus multisetosus					3				

* A = Laceyville site, B = Mahantango site, C = Harrisburg site

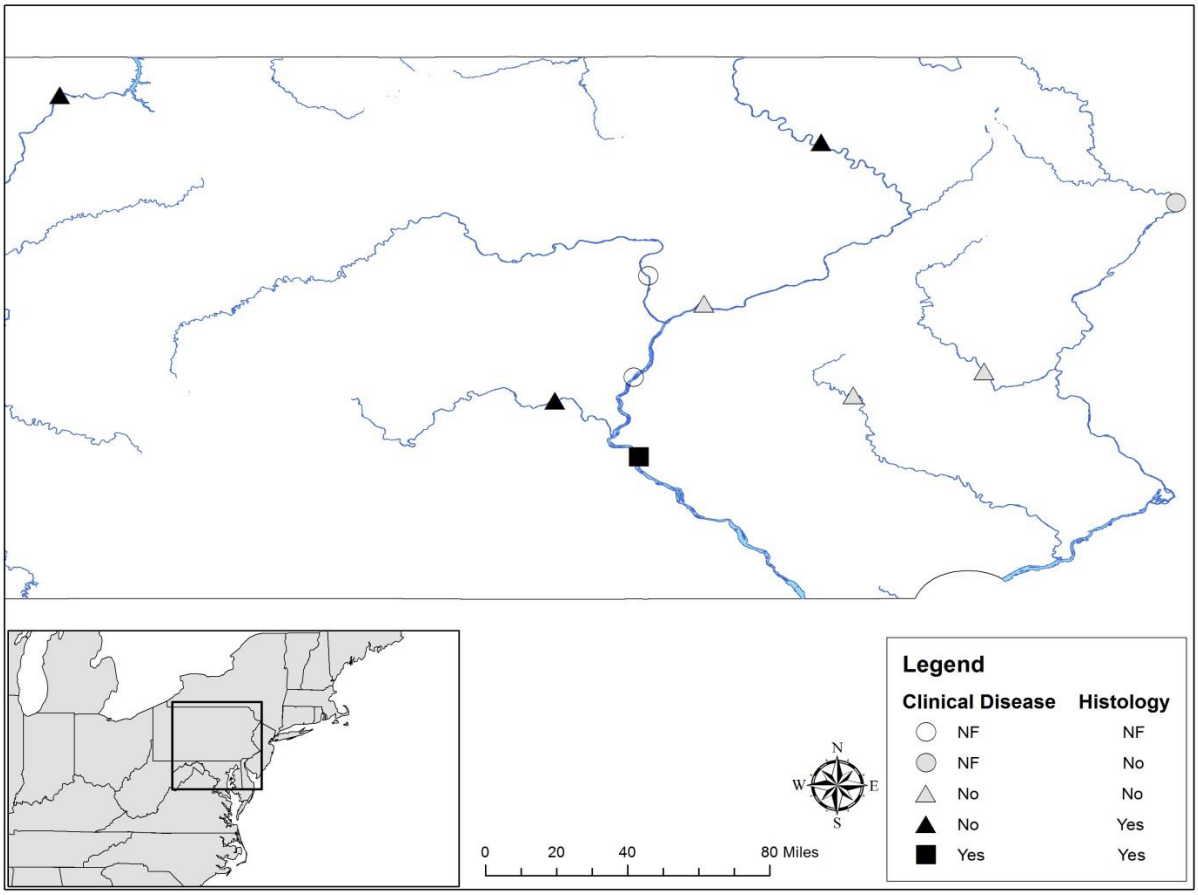

Figure 1. Distribution of *Myxobolus inornatus* in tissues of young-of-year (YOY) Smallmouth Bass *Micropterus dolomieu* during 2013. Circles indicate locations where no fish (NF) were captured during initial surveys. Triangles indicate locations where YOY Smallmouth Bass were found but did not display clinical symptoms (No) of disease. Squares indicate locations where YOY Smallmouth Bass had clinical signs of disease (Yes) during initial surveys. Gray-filled markers indicate locations where histological analysis did not document *M. inornatus* (No) while black-filled markers indicate locations where histological analysis documented *M. inornatus* (Yes). Initial surveys at the Delaware River at Matamoras yielded no YOY Smallmouth Bass; however, a follow-up collection produced enough fish for histological analysis.

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