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An updated geographic distribution of *Myxobolus cerebralis* (Hofer, 1903) (Bivalvulida: Myxobolidae) and the first diagnosed case of whirling disease in wild-caught trout in the south-eastern United States

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Abstract

Myxobolus cerebralis (Bivalvulida: Myxobolidae), the aetiological agent of salmonid whirling disease, was detected in 2 river basins of North Carolina during 2015, which initiated the largest spatial–temporal monitoring project for the disease ever conducted within the south-eastern United States (focused mainly in eastern Tennessee and western North Carolina). A total of 2072 rainbow trout *Oncorhynchus mykiss*, 1,004 brown trout *Salmo trutta* and 468 brook trout *Salvelinus fontinalis* were screened from 113 localities within 7 river basins during June 2017 through October 2019. Infections were detected by pepsin–trypsin digest, microscopy and the species-specific nested polymerase chain reaction (PCR) in 19 localities across 6 river basins. Myxospore morphology was indistinguishable from the published literature. In 2019, five rainbow trout that symptomatic for whirling disease (sloping neurocranium and lordosis) were captured and processed for histopathology. Myxospores were detected in the calvarial cartilage of two deformed trout with associated erosion of the cartilage consistent with reported whirling disease lesions. This is the first report of *M. cerebralis* in Tennessee and the first histologically confirmed cases of whirling disease in southern Appalachian (south-eastern United States) rivers and streams and expands the distribution of *M. cerebralis* throughout western North Carolina and eastern Tennessee.

KEYWORDS
epidemiology, histology, myxobolus, myxozoa, salmonidae

1 | INTRODUCTION

The cartilage/bone-infecting myxozoan species, *Myxobolus cerebralis* (Hofer, 1903) (Bivalvulida: Myxobolidae), the causative agent of whirling disease, was first documented in naïve rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) (Salmoniformes: Salmonidae) exported from the United States to Germany (Hofer, 1903). These trout were alleged to be originally infected in Germany and developed the clinical signs of the disease (tail chasing, disequilibrium, erratic swimming, and skeletal and pigment abnormalities) that are obvious and can alarm anglers (Halliday, 1976; Hoffman, 1990; Lorz, Amandi, Banner, & Rohovec, 1989; Sarker, Kallert, Hedrick, & El- Matbouli, 2015). Hoffman (1970) suggested this parasite is likely endemic to brown trout *Salmo trutta* Linnaeus, 1758 (Salmoniformes: Salmonidae), which are endemic to rivers in Europe, western Asian and north Africa and are resistant to developing clinical signs of

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TABLE 1 Results of pepsin–trypsin digests (PTD) and nested polymerase chain reactions (PCR) by river basin in the present study

River Basin	Collections	Positive sites	Host	Total length (mm)	PTD results		
					N	M+	PCR+
Catawba River	11	1	BKT	133–145 (170 ± 6; 5)	1	0	0
			BNT	51–405 (184 ± 61; 104)	27	0	2
			RBT	63–415 (163 ± 45; 186)	41	0	2
French Broad River	54	11	BKT	27–370 (106 ± 55; 165)	42	0	1
			BNT	10–375 (166 ± 59; 562)	134	1	5
			RBT	22–540 (114 ± 62; 1,002)	238	1	8
Hiwassee River	4	1	RBT	30–229 (107 ± 50; 105)	21	0	1
New River	22	3	BKT	40–240 (116 ± 45; 174)	32	0	0
			BNT	54–291 (151 ± 54; 286)	75	0	1
			RBT	65–381 (170 ± 52; 190)	45	0	2
Savannah River	1	1	BNT	140–320 (226 ± 58; 13)	4	0	1
Upper Tennessee River	16	2	BKT	70–200 (128 ± 49; 24)	6	0	0
			BNT	90–290 (150 ± 67; 15)	3	0	0
			RBT	8–300 (103 ± 41; 521)	112	0	3
Yadkin Pee-Dee River	5	0	BKT	62–170 (90 ± 27; 87)	19	0	0
			BNT	69–228 (143 ± 61; 17)	4	0	0
			RBT	72–180 (89 ± 20; 26)	6	0	0

Note: Total length reported as range (mean ± standard deviation; sample size).
Abbreviations: BKT, brook trout, *Salvelinus fontinalis*; BNT, brown trout, *Salmo trutta*; M+, number of microscopy-positive pellets; *N*, number of pellets after pooling; PCR+, number of PCR-positive pellets; RBT, rainbow trout, *Oncorhynchus mykiss*.

whirling disease when infected with *M. cerebralis*. The translocation of salmonids, particularly the global dissemination of brook trout *Salvelinus fontinalis* (Mitchill, 1814) (Salmoniformes: Salmonidae), brown trout and rainbow trout, has led to *M. cerebralis* infecting a number of salmonid species and being documented in 26 countries and 24 states in the United States (Bartholomew & Reno, 2002; El-Matbouli, Fischer- Scherl, & Hoffmann, 1992; Halliday, 1976; Ruiz et al., 2017; Sarker et al., 2015). Whirling disease is one of the most economically and ecologically devastating diseases of salmonids, harming both wild and cultured stocks, and is of particular concern for stocks of brook and rainbow trout, both of which have been demonstrated to be more susceptible to developing clinical signs of whirling disease than brown trout (Bartholomew & Reno, 2002; Halliday, 1976; Nehring & Walker, 1996; Thompson, Nehring, Bowden, & Wygant, 1999). Despite being one of the most well-studied parasites of freshwater fishes, *M. cerebralis* was not documented in the south-eastern United States, south of Virginia, until 2015; however, wild salmonids still had not been diagnosed with whirling disease in the south-eastern United States (Ruiz et al., 2017).

Trout fishing in western North Carolina and eastern Tennessee brings millions of dollars and generates thousands of jobs for the region (Ahn, Steiguer, Palmquist, & Holmes, 2000). While brook trout is the only endemic salmonid species to rivers and streams in North Carolina and Tennessee, many systems maintain healthy populations of brown and rainbow trout (Flebbe, 1994; Larson & Moore, 1985). Habitat alteration in the early 1900s restricted the range of native brook trout and prompted the introductions of non-native rainbow and brown trout to support the regions trout fisheries (Larson & Moore, 1985). Brook, brown and rainbow trout are still stocked annually and the presence of all size classes of brown and rainbow trout in these systems combined with telemetry data which documents annual spawning runs suggests fish hold over after stocking and spawn successfully (Bettinger & Bettoli, 2004, 2002; Burrell, Isley, Bunnell, Van Lear, & Dolloff, 2000). The value of the trout fishery in this region makes monitoring the distribution and spread of *M. cerebralis*, an invasive pathogen that can potentially hurt trout stocks, vitally important to resource managers.

A large-scale surveillance programme in the south-east United States was initiated as a collaboration between North Carolina Wildlife Resources Commission (NCWRC), Tennessee Wildlife Resources Agency (TWRA) and the Southeastern Cooperative Fish Parasite and Disease Laboratory (SCFPDL) to document the distribution of *M. cerebralis* infection and screen/monitor wild salmonids in the south-eastern United States for whirling disease. Brook trout, brown trout and rainbow trout were sampled from 7 major river basins (Catawba, French Broad, Hiwassee, New, Savannah, Upper Tennessee and Yadkin Pee-Dee) in eastern Tennessee and western North Carolina and screened for *M. cerebralis* infection (Table 1; Figure 1; Table S1). From October 2018 through June 2019, five rainbow trout symptomatic for whirling disease were identified. Based on the results of parasitology and pathology on those fish, we herein report the first cases of whirling disease among wild-caught

salmonids in southern Appalachia (south-eastern United States) as well as expand the geographic distribution of *M. cerebralis* throughout western North Carolina and into eastern Tennessee.

2 | MATERIALS AND METHODS

2.1 | Fish collection and pepsin–trypsin digest

Rainbow trout (*n* = 2,072), brown trout (*n* = 1,004) and brook trout (*n* = 468) were collected from 113 localities, selected by the state agencies, across 8 river basins across North Carolina and Tennessee from 14 June 2017 through 3 October 2019 and processed using the pepsin–trypsin digest (PTD) method outlined in Markiw and Wolf (1974) and the *M. cerebralis*-specific nested PCR from Andree, MacConnell, and Hedrick (1998) as prescribed in the US Fish and Wildlife Service and American Fisheries Society Fish Health Section Blue Book (Table 1; Figure 1; Table S1) (USFWS & AFS-FHS, 2014). State agencies selected sites that had high recreational value, sustained natural reproduction of trouts, or were in under sampled systems, when possible trout of a size likely to belong to the year-1 class were prioritized for collection. Trout were collected using a backpack electrofisher and either bagged and shipped overnight on ice to Auburn University or bagged and frozen until trout could be retrieved from the state agencies. Upon arrival at the SCFPD, trout were measured, weighed and examined for deformities. The head of each trout was removed and bisected with one half being frozen as archive tissue and the other being frozen overnight prior to being subjected to the PTD per Markiw and Wolf (1974). Each PTD contained no more than five half heads (all of the same species) or 25 g of material, which resulted in 808 pellets. Each pellet was examined for 10 min using a Zeiss Axioskop with a 40 × objective and differential interference contrast (DIC) components.

2.2 | Myxospore morphology

Myxospore measurements were generated from 25 myxospores sourced from fresh PTD pellets at 630 × and 1,000 × using an Olympus BX53 with an ocular micrometre and differential interference contrast (DIC) components following guidelines in Lom and Arthur (1989). Myxospore micrographs were generated from the same PTD pellet measurement source from at 1,000 × using an Olympus BX53 with DIC components equipped with a Jenoptik Gryphax camera. Lugol's iodine and India ink were used to stain the iodiniphilic vacuole and mucous envelope, respectively, per Lom and Arthur (1989). To minimize movement of myxospores, a small drop of positive PTD pellets was placed on a coverslip, inverted and placed onto a thin layer of 1% agar (Lom, 1969).

2.3 | Nested PCR

All pellets were subsequently subjected to nested PCR. DNA was extracted from a 50 µl subsample of each pellet using the DNeasy

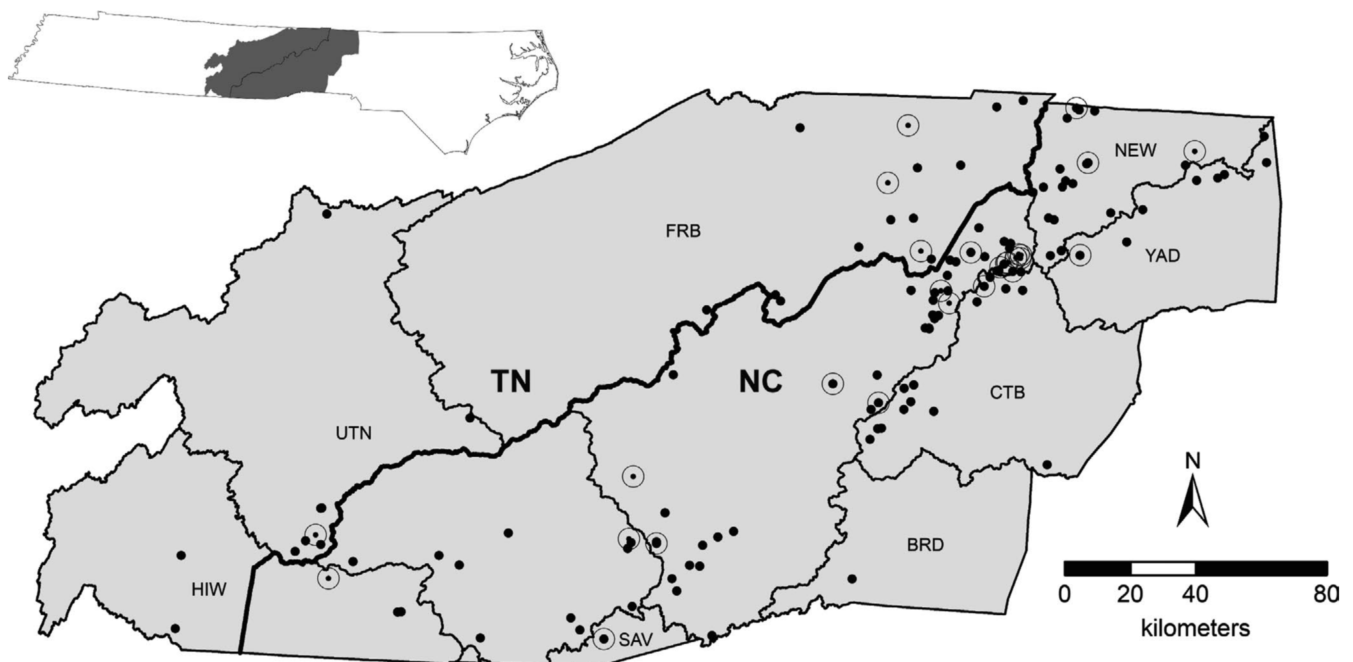

FIGURE 1 North Carolina and Tennessee (United States) collection localities comprising 8 river basins screened for infections of *Myxobolus cerebralis* (Hofer, 1903) (Myxozoa: Bivalvulida) in rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) (Salmoniformes: Salmonidae), brown trout *Salmo trutta* Linnaeus, 1758 (Salmoniformes: Salmonidae) and brook trout *Salvelinus fontinalis* (Mitchill, 1814) (Salmoniformes: Salmonidae). Encircled dots = infection localities. Bold line indicates state boundary. Bold text indicates state labels: NC, North Carolina; TN, Tennessee. HIW, Hiwassee; UTN, Upper Tennessee; SAV, Savannah; FRB, French Broad; BRD, Broad; CTB, Catawba; YAD, Yadkin Pee-Dee; and NEW, New

FIGURE 2–5 Rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) (Salmoniformes: Salmonidae), collected from Wildbur Tail Race and French Broad River Basin, displaying clinical signs of whirling disease, photographed using Olympus tough TG–6 digital camera. (2–3) Full body view of rainbow trout displaying a sloping neurocranium. (4) Higher magnification view of rainbow trout from Figure 1. (4) Higher magnification view of rainbow trout from Figure 2

Blood & Tissue Kit (Qiagen) according to manufacturer protocol, with the added step of microwaving the sample for one minute prior to the addition of the lysis buffers (ATL buffer and Proteinase K) and incubation. Following extraction, DNA was quantified using a NanoDrop–1000 spectrophotometer (Thermo Scientific, Nanodrop Technologies), diluted to 50 ng/μl and stored at −20°C. A region of the 18S specific to *M. cerebralis* amplified using primers Tr3–16 and Tr5–16 for the first PCR cycle and Tr3–17 and Tr5–17 for the second PCR cycle with thermocycle conditions that follow Andree et al. (1998). The PCR product was visualized using gel electrophoresis. Microscopy and PCR results by locality and river basin were recorded (Table 1; Table S1).

2.4 | Processing deformed trout

From October 2018 through July 2019, five rainbow trout were collected from the French Broad River Basin that displayed clinical signs of whirling disease: 4 with a sloping neurocranium (2 collected from Watauga River [36°21′18.1″N 82°12′26.6″W], 1 collected from Rocky Fork [36°02′13.7″N 82°34′29.2″W] and 1 collected from South Indian Creek [36°01′13.7″N 82°33′23.2″W]) and 1 with a lordosis (collected from Jonathans Creek [35°31′22.6″N 83°01′52.6″W]). Upon arrival at the SCFPD, these trout were measured, weighed and photographed (Figures 2–5). The deformed portion of each trout was removed and fixed in 10% neutral-buffered formalin (n.b.f.). After fixation in 10% n.b.f for 48 hr, fixed tissue was grossed into 1-cm-long portions to fit into tissue processing cassettes. Trunk sections too wide for cassettes were processed with the additional step of having the muscle dorsal and ventral to the vertebral column removed. Sections of the skull too large for cassettes were processed with the additional step of separating the neurocranium and branchiocranium. This yielded a total of 28 portions of tissue that were rinsed in distilled water for 2 hr, decalcified in EDTA for 1 month, dehydrated in an ethanol series, embedded in paraffin, sectioned at 4–7 μm and stained with Gill's 2 haematoxylin and eosin or giemsa per Luna (1968). Portions of deformed tissue (neurocranium or vertebral column) were serially sectioned for each fish and 20 slides were cut from each of the remaining portions of tissue (branchiocranium). This yielded 2,499 sections on 714 slides. The remainder of the skeleton was subjected to the PTD and nested PCR as described above.

3 | RESULTS

3.1 | Microscopy results

Myxobolus cerebralis infections were detected in trouts collected from the Catawba, French Broad, Hiwassee, New, Savannah, Upper Tennessee and Yadkin Pee-Dee river basins. A total of 1 of 249 (0.4%) pellets from brown trout (Elk River) and 1 of 453 (0.2%) from rainbow

trout (Doe River) from 2 localities in the French Broad River Basin were positive for infection by *M. cerebralis*. The remaining 153 localities within the Catawba, French Broad, Hiwassee, New, Savannah, Upper Tennessee and Yadkin Pee-Dee river basins were negative for *M. cerebralis* by microscopy (Table 1; Figure 1; Table S1). All pellets resulting from deformed trout were negative by microscopy.

3.2 | Myxospore morphology

Species identification was based on a comparison of the dimensions of myxospores sourced from PTD pellets compared to dimensions reported in Lom and Hoffman (1971) and Ruiz et al. (2017). Our myxospores, sourced from PTD pellets, were indistinguishable from those of Lom and Hoffman (1971) and Ruiz et al. (2017) and measured 7–10 (8.4 ± 0.9; *n* = 20) long, 7–10 (8.85 ± 1.1; *n* = 20) wide 6–8 (6.8 ± 0.8; *n* = 5) thick, with a mucous envelope on the posterior margin of the spore; with two polar capsules 5–6 µm (5.4 ± 0.5; *n* = 40) long, 3–4 µm (3.5 ± 0.5; *n* = 40) wide, with 5–6 polar filament coils (Figures 6–9). This constitutes the first morphological identification of *M. cerebralis* in Tennessee.

3.3 | Nested PCR results

All pellets were subject to the species-specific nested PCR and no microscopy-positive pellet was negative by nested PCR. A total of 1 of 106 (0.9%) pellets from brook trout, 9 of 249 (3.6%) from brown trout and 16 of 453 (3.5%) from rainbow trout from 19 localities across the Catawba, French Broad, Hiwassee, New, Savannah and Upper Tennessee river basins were positive for *M. cerebralis* by the species-specific nested PCR. Positive pellets by river basin and species are as follows: 2 of 27 (7.4%) from brown trout and 2 of 41 (4.8%) from rainbow trout from the Catawba River Basin (Linville River); 1 of 42 (2.4%) from brook trout (North Fork Ivy Creek), 5 of 134 (3.7%) from brown trout (Elk River, Plumtree Creek, Roaring Creek and Watauga River) and 8 of 228 (3.5%) from rainbow trout (Boone Fork, Doe River, Jonathans Creek, South Holston River and Watauga River) from the French Broad River Basin; 1 of 21 (4.8%) from rainbow trout (Dockery Creek) from the Hiwassee River Basin; 1 of 75 (1.3%) from brown trout (Little River) and 2 of 45 (4.4%) from rainbow trout (Big Horse Creek and Buffalo Creek) from the New River Basin; 1 of 4 (25.0%) from brown trout from the Savannah River Basin; 3 of 112 (2.7%) from rainbow trout (Caney Fork and North River) from the Upper Tennessee River Basin. Salmonids collected from the remaining 137 localities across the Catawba, French Broad, Hiwassee, New, Savannah, Upper Tennessee and Yadkin Pee-Dee river basins were negative by nested PCR for *M. cerebralis* (Table 1; Figure 1; Table S1). All PTD resulting from the deformed trout were negative by nested PCR.

3.4 | Histology

Two of the five deformed trout (Watauga River and French Broad River Basin), both with a sloping neurocranium, had myxospores consistent in with *M. cerebralis* infecting the deformed tissue (Figures 2–5). In infected trout, the calarial cartilage of the neurocranium (ventrolateral region of the brain case) was eroded and replaced by mature myxospores or a mass of epithelioid tissue (Figures 10–12). In both infected trout, myxospores were observed outside cartilage adjacent to an area of eroded cartilage containing myxospores (Figure 10). In one infected trout, a cavity of eroded cartilage was observed filled by epithelioid tissue with no myxospores present (Figure 12). No inflammatory response was observed in infected trout. Myxospores were not observed in the three remaining deformed trout (South Indian Creek, French Broad River Basin; Jonathans Creek, French Broad River Basin; Rocky Fork, French Broad River Basin).

4 | DISCUSSION

Currently, the protocol for the detection of infection by *M. cerebralis* comprises the species-specific nested PCR as a confirmatory test for microscopy-positive pellets (USFWS & AFS-FHS, 2014). The increase in diagnostic sensitivity from microscopy to molecular techniques has been reported widely (Andree et al., 1998; Schisler, Bergersen, Walker, Wood, & Epp, 2001; Cavneder et al. 2004; Arsan, Atkinson, Hallet, Meyers, & Bartholomew, 2007). Schisler et al. (2002) evaluated a single round PCR for detection of *M. cerebralis* in wild salmonids and observed that the PTD was positive in only 68% of PCR

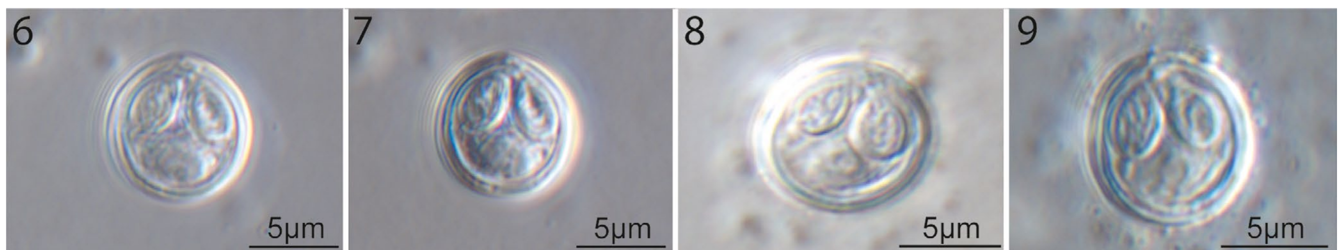

FIGURE 6–9 Myxospores of *Myxobolus cerebralis* (Hofer, 1903) (Bivalvulida: Myxobolidae) collected from rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792) (Salmoniformes: Salmonidae) from Doe River and French Broad River Basin, Tennessee, photographed with differential interference contrast optical components

positives. Andree et al. (1998) found that the PTD was only positive in 14% of nested PCR-positive samples while screening wild salmonids. Consistent with published results, we observed increased diagnostic sensitivity using the nested PCR with the PTD, only detecting *M. cerebralis* morphologically in 10.7% of nested PCR positives. Kelley et al. (2004) used experimentally infected salmonids to test diagnostic sensitivity of 5 protocols and observed similar sensitivity from PTD, nested PCR and QPCR approaches; however, these fish were exposed to 2000 triactinomyxons in 450 ml of stagnant water, which is unlikely to occur in naturally infected salmonids. This raises the concern that these results are an artefact of high-intensity infections unlikely to be encountered in wild salmonids. The lack of a true sensitivity analysis of the nested PCR and QPCR protocols prevents a comparison of the sensitivity of the two approaches. The primary advantage of molecular approaches is the ability to detect all life stages of *M. cerebralis*, as the PTDs inability to detect early infections may result in false negatives. This is particularly valuable to resource managers surveying wild populations of salmonids that may harbour low-intensity or presporogonic infections. Our results further support that the nested PCR should be a required diagnostic test for *M. cerebralis*, rather than a confirmatory test, to prevent the further dissemination of infected salmonids.

The deformed trout detailed herein comprise the first histologically confirmed cases of whirling disease in wild-caught salmonids in the south-eastern United States. In the two infected trout we detailed herein, the observed lesion was consistent with that documented in the literature and understood as symptomatic of whirling disease (Halliday, 1974; Hoffman, Dunbar, & Bradford, 1962; Plehn, 1904). The PTD and PCR of the vertebral column, ribs and fins of these fish were negative for *M. cerebralis*; however, as this system (Watauga River) has historically been positive for *M. cerebralis* and anglers reports indicate the presence of deformed fish this likely represents an artefact of the infection being limited to the calvarial cartilage of these fish, which was serially sectioned and not included in the PTD or PCR. The presence of mature myxospores, lack of developing trophozoites and presence of an epithelioid response suggested that these particular salmonids were infected for at least 8 months prior to capture (Hoffman et al., 1962). It is important to note the origin of these trout cannot be confirmed. These trout were collected from a site stocked annually, which indicates there is potential that these diseased fish may have been infected when they were stocked into the system; however, this information demonstrates that whirling disease positive salmonids are present in the south-eastern United States which is valuable information to resource managers as they increase efforts to contain the spread of *M. cerebralis* infections and increase angler awareness in the region.

One of our samples indicated that trout can clear infections by *M. cerebralis*. We observed myxospores loose in tissue adjacent to eroded cartilage containing myxospores in eroded cartilage and cavities of eroded cartilage indicative of *M. cerebralis* infection that were devoid of myxospores or developing trophozoites, suggesting that the host could have cleared the infection and simultaneously, perhaps, shed myxospores (thereby allowing for horizontal dispersal without host mortality). This observation is significant because the only other reported dispersal mechanism for *M. cerebralis* is post-mortem decomposition of the fish or by passing myxospores through the digestive tract of another vertebrate that ate the infected trout (El-Matbouli & Hoffmann, 1991; Taylor & Lott, 1978).

Results of the surveillance programme expand the geographic distribution in the south-east United States. *Myxobolus cerebralis*

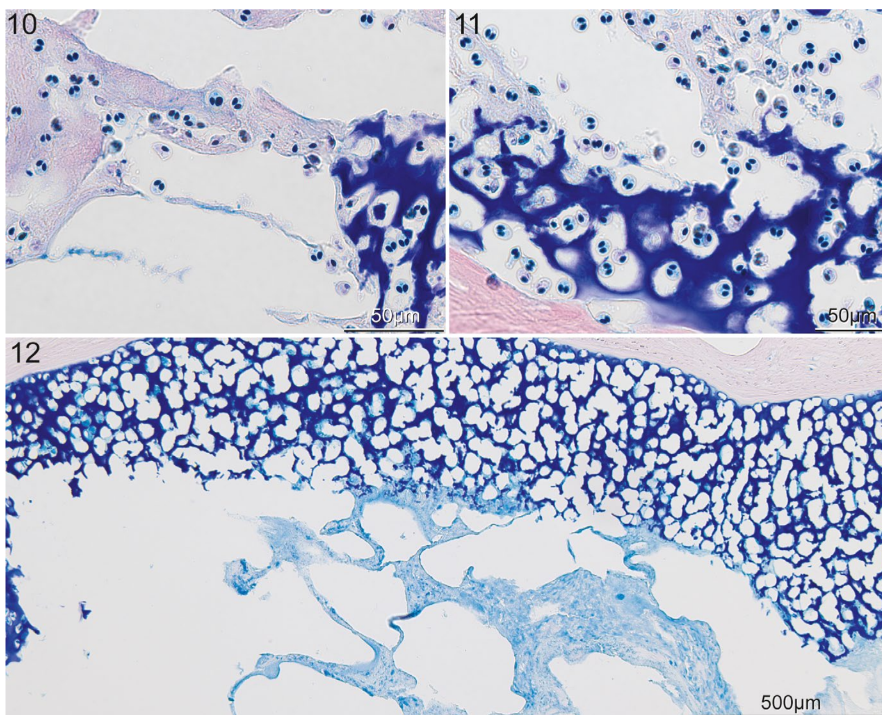

FIGURE 10–12 Transverse histological sections (giemsa) of rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) (Salmoniformes: Salmonidae), neurocranium infected with *Myxobolus cerebralis* (Hofer, 1903) (Bivalvulida: Myxobolidae). (10) Loose myxospores in epithelioid tissue adjacent to eroded infected cartilage. (11) Infected cartilage showing mature myxospores in eroded pockets or cartilage and mature myxospores in epithelioid tissue. (12) Cavity in eroded cartilage with typical epithelioid host response lacking myxospores

positive sites presented in Ruiz et al. (2017) were limited to 2 river basins (French Broad and Yadkin Pee-Dee river basins) in western North Carolina. We detected *M. cerebralis* in 6 river basins (Catawba, French Broad, Hiwassee, New, Savannah and Upper Tennessee) in North Carolina and Tennessee including the first detection of *M. cerebralis* in Tennessee and positives from three sites that were negative (Linville River, North Fork Ivy Creek and Caney Fork) in Ruiz et al. (2017). As fish were pooled per AFS-FHS Blue Book, prevalence cannot be determined; however, infections were more commonly detected in pellets sourced from rainbow (3.5%) and brown (3.6%) trout. Sites from the French Broad River Basin were most commonly positive for *M. cerebralis* infections; however, this may be an artefact of the increased sampling effort in this river basin and the yearly sampling of sites which are known to be positive for *M. cerebralis*. Our results clearly show that the pathogen is established in particular rivers but remains relatively geographically restricted there. Future monitoring efforts will document the containment or spread of infections among Appalachian salmonids.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The manuscript is absent of shared data.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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