Management of Biological Invasions (2021) Volume 12, Issue 2: 363–377

Research Article

Detection of pathogens and non-target species in the baitfish supply chain

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Citation: McEachran MC, Mor SK, Phelps NBD (2021) Detection of pathogens and non-target species in the baitfish supply chain. *Management of Biological Invasions* 12(2): 363–377, https://doi.org/10.3391/mbi.2021.12.2.10

Received: 18 October 2020
Accepted: 28 September 2020
Published: 22 February 2021

Thematic editor: Matthew A. Barnes

Abstract

The movement of live fish for use as bait in recreational angling has been identified as a high-risk pathway for the spread of aquatic invasive species and disease in the Great Lakes region. To better understand the hazards present in Minnesota's live baitfish pathway, we employed both conventional and advanced diagnostic approaches to detect non-target species and pathogens in golden shiners (*Notemigonus crysoleucas*), a common baitfish sold in retail bait shops. Golden shiners were purchased from bait shops (n = 34 total; n = 15 sampled once, n = 19 sampled twice) in 2014 and 2015 across Minnesota. Of the 53 lots examined, non-target species were observed in 24/53 lots and included nine different fish species and one frog species, however, none of the non-target species found are listed as invasive in Minnesota. Nine parasite taxa were observed by wet mounts of the gills, epidermal mucus, and fin clips in 27/53 lots. The microsporidian parasite, *Ovipleistophora ovariae*, was detected in 24/53 lots by qPCR. While many bacterial species were identified by culture dependent and independent methods, two notable species including, *Aeromonas salmonicida* and *Yersinia ruckeri,* were confirmed in ten and five lots, respectively. Detection of replicating virus in culture was low, with only 2/53 lots positive for the golden shiner reovirus. No other viruses were detected with the standard culture-based assays; however, a total of eight novel viruses were detected by next-generation sequencing. These findings underscore the need for a proactive surveillance approach that includes advanced diagnostic tools for the detection of emerging aquatic pathogens, to better understand and manage the risks associated with the use of live baitfish.

Key words: cross-sectional studies, fish diseases, virology, whole genome sequencing

Introduction

Anthropogenic movement of live animals has been identified as a significant driver of fish and wildlife disease worldwide. In an increasingly globalized world, billions of animals and their accompanying microbes are translocated to naïve populations, driving host-switching, spillover between wild and domestic populations, and disease emergence (Daszak et al. 2000; Pavlin et al. 2009; Hulme 2009). The translocation of live fish is increasingly identified as a driver of disease emergence in aquatic systems (Peeler et al.

2011; Tompkins et al. 2015), with live fish comprising over 90% of live animal specimens imported into the United States (Smith et al. 2009), despite a lack of basic disease data for many aquatic pathogens and a generally poor understanding of the health status of fish populations (Jones 2000; Gaughan 2001).

The movement of live fish for use as bait in recreational angling has long been identified as a particularly high-risk pathway, whereby non-target species (e.g., aquatic invasive species (AIS) including fish, invertebrates, plants) and pathogens, may enter the live baitfish supply, be inadvertently purchased by an angler at a bait shop, and released intentionally or unintentionally into the destination waterbody (Litvak and Mandrak 1993; Ludwig and Leitch 1996; Goodwin et al. 2004). Although many states have regulations in place to limit the potential contamination of live baitfish supplies by non-target species and pathogens (e.g., visual and diagnostic inspection of farmed or wild harvested baitfish, prohibition of certain bait species, and special regulations for harvesting in AIS-infested waters), these hazards nevertheless still persist in the bait supply (Nathan et al. 2015; Boonthai et al. 2017, 2018). Therefore, it is critical for fisheries managers and bait producers to better understand what hazards are hitching a ride in the live baitfish pathway to inform risk assessment and management strategies that mitigate AIS and disease introduction.

Pathogens are particularly problematic as they can be present in asymptomatic fish at high prevalence, have little or no management options once released, and can cause long lasting population-level impacts on important fish species (Gaughan 2001; Copp et al. 2005a; Gozlan et al. 2006). Previous surveys of the retail bait supply have demonstrated the presence of fish pathogens and non-target species using culture-based and targeted PCR approaches to screen for specific hazards (McCann 2012; Nathan et al. 2015; Boonthai et al. 2017, 2018; Mahon et al. 2018), but the recent discovery of several novel baitfish viruses (Phelps et al. 2014; Haenen et al. 2016; Mor and Phelps 2016a, b; Munang'andu et al. 2017) and bacteria of concern (Mahon et al. 2018) in baitfish highlights the urgency of widening our focus when it comes to identifying hazards relevant to the live baitfish pathway.

Culture-based and molecular (e.g., PCR) assays are available for detection and diagnosis of several fish pathogens, and there is value in this type of approach, particularly for targeted surveys of high-priority pathogens such as viral hemorrhagic septicemia virus (VHSV), for which there are well-established diagnostic techniques (Warg et al. 2014a, b). However, many microbes are difficult or impossible to propagate in standard culture-based assays or have no available PCR assays developed, contributing to significant uncertainty in the baseline health status (Mokili et al. 2012; Munang'andu et al. 2017). Advances in diagnostic technology and bioinformatic techniques used for next-generation sequencing (NGS) now allow for non-specific

sequencing and analysis of DNA or RNA. NGS has been used to discover novel pathogens in many systems (Mokili et al. 2012; Hadidi et al. 2016; Munang'andu et al. 2017; Shi et al. 2018) and has allowed for an "eyes wide open" approach to hazard identification that is not limited to detecting pre-selected pathogens. The application of this approach as a screening tool is particularly useful in aquatic systems where very little is known, but the potential for pathogen distribution is high (Rodgers et al. 2011).

The primary purpose of this study was to employ both conventional diagnostic methods and advanced molecular approaches to detect the presence of non-target species and pathogens (parasites, bacteria, and viruses) in golden shiners (*Notemigonus crysoleucas*) sold in retail bait shops across the state of Minnesota. Golden shiners are small (~ 7–12 cm) cyprinid minnows, popular and economically important as a live baitfish species, and are an important ecological indicator (Stone et al. 2016). The importance of this pathway as a potential source of non-target species and pathogen exposure to important fish populations necessitates a rigorous approach to hazard identification to inform risk-based management.

Materials and methods

Collection of live baitfish from retail bait shops

Live golden shiners were obtained from bait shops (n = 34 total; n = 15 sampled once, n = 19 sampled twice) across Minnesota. Samples were collected during the summer season (May 2014–August 2014) and during the winter season (December 2014–February 2015). Bait shops were selected for inclusion if they were known to have golden shiners at the time of collection and to provide a statewide spatial distribution, as determined by Minnesota Department of Natural Resources (MNDNR) Conservation Officers (Figure 1). To mimic the purchase of bait by an angler, MNDNR undercover Conservation Officers conducted unannounced consumer visits (i.e., participation in the study was not disclosed). During each bait shop visit, a request was made to purchase "30 golden shiners", hereafter referred to as a "lot" (i.e., group of fish of the same cohort). The fish were transported in an air bag and delivered alive within 24 hours to the University of Minnesota Veterinary Diagnostic Laboratory (UMN VDL) for processing. Chain of custody and documentation was maintained from sample collection to reporting of diagnostic results. Following the conclusion of the study, uniformed Conservation Officers returned to each of the bait shops and requested sales records and receipts to verify the original source of the purchased fish.

First, a visual inspection of each lot was conducted to identify and separate any non-target species from the golden shiners. The number of all golden shiners and non-target species in each lot was recorded. All fish were then euthanized using an overdose of tricaine methanesulfonate (3.0g/L;

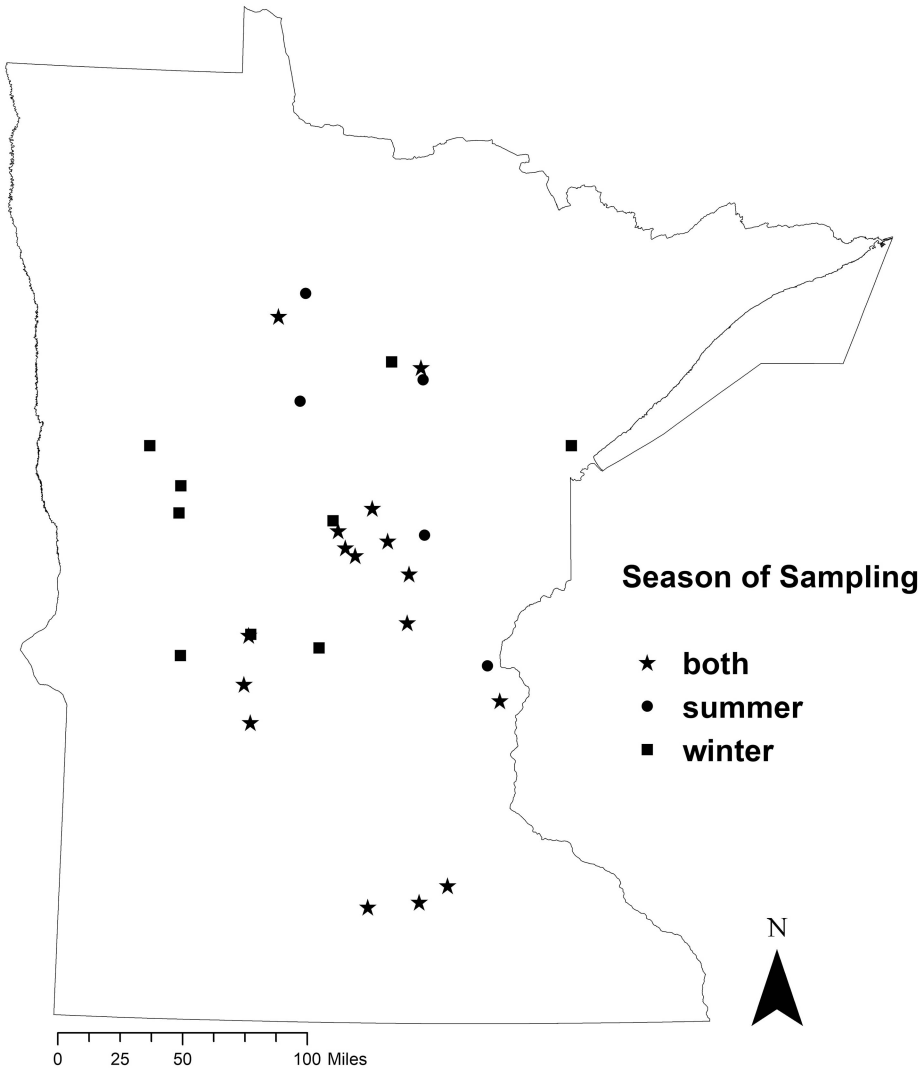

Figure 1. Map of Minnesota showing locations of sampled bait shops.

Sigma-Aldrich, St. Louis, MO), buffered with sodium bicarbonate (3.0g/L), adhering to the UMN VDL's animal care protocol for client-submitted fish. Up to 30 randomly selected golden shiners were saved from each lot for diagnostic testing. All other fish were discarded.

Detection of potentially harmful microbes

To determine the presence of external parasites, five golden shiners were selected at random from each lot and examined by wet mounts of gill tissue, fin clip, and epidermal mucus under light microscopy. External parasites were identified to family level and infection was categorized as no significant parasites, minor (< 10 parasites observed in five minutes/tissue type/lot), moderate (10–100 parasites), or severe (> 100 parasites). To detect the microsporidian parasite *Ovipleistophora ovariae,* ovaries of all mature females from each lot were aseptically dissected and preserved in 70% ETOH for analysis by qPCR. Adult males and juveniles were not examined because *O. ovariae* is known to primarily affect mature females with

oocytes (Phelps and Goodwin 2007). DNA was extracted from ~ 20 mg of ovarian tissue using the Qiagen DNeasy kit (Qiagen, Venlo, Netherlands) following the manufacturer's recommendation, except that the concentration of proteinase K was doubled and initial incubation was for 3 h. qPCR reactions were performed on an ABI 7500 Thermal Cycler (ThermoFisher Scientific, Waltham, MA) following the protocol described by Phelps and Goodwin (2007).

To determine the presence of bacteria, an aseptic swab of the anterior kidney was collected from all individual fish in the lot. Aerobic cultures were performed on sheep blood agar and tryptic soy agar and incubated at room temperature (~ 22 °C) for 48–72 hours. Bacterial identification followed the Clinical and Laboratory Standards Institute standard methods (Sneath 1986; Holt 2000; Markey et al. 2013). Briefly, isolates were evaluated based on morphologies after the initial incubation period and confirmed by bioassays (e.g., triple sugar iron agar and API 20NE; BioMerieux, Hazelwood, MO) or MALDI-TOF spectrometer (Bruker Daltonics Inc, Billerica, MA) using cutoffs of ≥ 1.8 for identification to genus and ≥ 2.0 for identification to species. If bacterial isolates could not be identified by either method, 16s sequencing was performed for confirmation (Weisburg et al. 1991).

We used paired t-tests to test for seasonal differences in number of non-target species, pathogen prevalence, number of mature females, bacterial taxa richness, and parasite richness from shops for which we had both summer and winter lots.

To determine the presence of viruses, virus isolation was conducted using pooled tissue homogenate of spleen, kidney, and heart from five fish (n = up to 6 pooled samples/lot; 5 fish/pool). Tissue homogenates were prepared and cultured according to the USFWS and AFS-FHS Blue Book recommended methods (American Fisheries Society-Fish Health Section & United States Fish and Wildlife Service 2016a) at 15 and 25 °C on epithelioma papulosum cyprini (EPC), fathead minnow (FHM), and chinook salmon embryo (CHSE-214) cell lines for 28 days, with a blind passage on day 14 if no cytopathic effects were observed. If cytopathic effects were observed during the cell culture incubation period, the sample was subjected to an additional passage on fresh cells (American Fisheries Society-Fish Health Section & United States Fish and Wildlife Service 2016a).

To further explore the viral diversity of the golden shiners, all lots collected during the summer season (n = 23 lots) were subjected to additional non-specific sequence amplification. Only summer lots were examined to reduce study costs. Following tissue homogenization, an equal aliquot from each pooled sample was once again pooled for a total of up to 30 fish per pool (n = 1 pooled sample/lot). Total nucleic acid extraction was performed following the protocol described by Mor and Phelps (2016a, b). Briefly, Trizol LS (Invitrogen, NY, USA) was used to extract total RNA followed by purification using a QIAamp Viral RNA Mini Kit (QIAGEN,

Table 1. Primers used for PCR amplification of RdRp gene of RNA viruses and replication gene of circovirus. The primers used for infectious myonecrosis virus and piscine mycocarditis-like virus can be found in Mor and Phelps (2016a) and Mor and Phelps (2016b), respectively.

Virus	Primers	Product size (bp)
Golden shiner astrovirus	F 5'-TGGTATGGDTTYCTVAAAAATGA-3' R 5'-GAAAGATGRTCATCMCCRTAGG-3'	566
Golden shiner picornavirus-1	F 5'-GTCTGGGCGTATTGTGAGGT-3' R 5'GACAAAGGGTTGCAGAGAGC-3'	793
Golden shiner picornavirus-2	F 5'-AAGACTCGGGTGGTTGACAC-3' R 5'-TTGGTCAGAAAGGGTTGGAG-3'	1033
Golden shiner betanodavirus	F 5'- ATTGGCCTGGTCTATGGTGA-3' R 5'-CAGTGATAGCACCGACGAGA-3'	805
Golden shiner circovirus	F 5'-TGGAGGAATTTACCCAGTGA-3' R 5'-ATCTCGCTGTTTGACGAGAC-3'	1049

Valencia, CA). Next-generation sequencing was performed on extracted RNA at the University of Minnesota Genomic Center for cDNA synthesis, library preparation and sequencing. Illumina TruSeq RNA v2 kit was used for library preparation followed by MiSeq 300 bp paired-end sequencing (Illumina, San Diego, CA). Analysis of the resulting sequence data was performed using CLC Genomics Workbench 7.5 (http://www.clcbio.com). The de novo assembly with default parameters was performed after trimming adaptor sequences and testing of sequence quality. Extracted contigs were analyzed using BLASTx in CLC Genomics. The ORFs were predicted using the ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The viral sequence was considered true if a contig length was ≥ 80% of the proposed genome in a particular virus family. The criteria proposed by International Classification of Taxonomy of Viruses was used to determine if detected viruses were a novel virus within a family. Virus-specific PCRs were developed for each virus that was identified by NGS in the summer samples, and lots (1 pooled sample/lot) from summer and winter seasons were screened by PCR to estimate prevalence (Table 1).

Results

Collection of live baitfish from retail bait shops

A total of 53 lots were collected during the study period from 34 bait shops across Minnesota (Supplementary material Table S1). Of the 53 lots, 23 were collected during the summer season (May 2014–August 2014) and 30 were collected during the winter season (December 2014–February 2015). Two sites were collected twice during the winter sampling period. There were no significant gross lesions, other than those typical of handling stress common in the bait trade (e.g., minor scale loss, minor dermal hemorrhage). Although in some cases intermediate suppliers were identified, the ultimate source of the golden shiners could not be confirmed during follow up interviews by the MNDNR Conservation Officers.

Golden shiners were present in all 53 lots, but the actual number of golden shiners varied widely (range 3–136, median 58). For four of the lots, only 30 golden shiners were counted and the total number in the lot was not

Table 2. Species composition of purchased golden shiner lots (n = 53) at retail bait shops in Minnesota from May 2014–February 2015.

Species	Number of lots	Number of individuals (in lots where present)		Allowed as bait in MN?	Aquatic invasive species in MN?
		Median	Range		
Golden shiner *Notemigonus crysoleucas*	53	58*	3–136	yes	no
Blackchin shiner *Notropis heterodon*	1	1	1–1	yes	no
Brown bullhead *Ameiurus nebulosus*	3	1	1–1	yes	no
Bullfrog *Rana catesbaiana*	1	1	1–1	yes	no
Fathead minnow *Pimephales promelas*	15	2	1–209	yes	no
Finescale dace *Chrosomus neogaeus*	12	1.5	1–32	yes	no
Mud minnow *Umbra limi*	1	2	2–2	yes	no
Spottail shiner *Notropis hudsonius*	2	22.5	12–33	yes	no
White sucker *Catostomus commersonii*	2	2.5	2–3	yes	no
Three-spine stickleback *Gasterosteus aculeatus*	4	1.5	1–32	no	no
Yellow perch *Perca flavescens*	1	2	2–2	no	no

* Median number of golden shiners were calculated using only 49 lots since the total number of golden shiners were not recorded in 4 lots.

recorded – these four lots have not been included in the median calculation. Ten different non-target species were found across all lots, with ≥ 1 non-target species in 24/53 (45%) of the lots (Table 2). One lot, purchased at an in-home bait shop and found through the online sales and exchange website Craigslist (http://www.craigslist.org), was highly contaminated with six non-target species (fathead minnows, n = 209; stickleback, n = 32; mud minnow, n = 2; finescale dace, n = 13; yellow perch, n = 2; and brown bullhead, n = 1) in addition to 114 golden shiners. The average number of non-target species present per lot did not differ by season for shops for which we had both winter and summer observations (paired t = 0.84563, df = 16, p-value = 0.4102). None of the non-target species (including the non-native stickleback) identified during the course of this study were prohibited or regulated AIS in Minnesota (MN Administrative Rule 6216.0250 and 6216.0260).

Presence of potentially harmful microbes

Of the 53 lots examined, nine external parasite taxa were observed in wet mounts of epidermal mucus, gill tissue, and fin clip in 27 lots, with infections ranging from minor to severe (Table 3). Twenty-six lots had no parasites observed. One lot of golden shiners had a notably severe fungal infection observed by light microscopy. The average number of parasite taxa identified was less than one, with a maximum of 4 different parasite taxa identified in a single lot. Parasite richness did not differ by season (paired t = −0.56569, df = 16, p-value = 0.5795).

Ovipleistophora ovariae was detected in mature female golden shiners from 24/53 (45%) lots by qPCR. This is the first record of *O. ovariae* in the state of Minnesota. The prevalence of *O. ovariae* within positive lots varied from 2/15 (13%) to 5/5 (100%) of mature females examined and was nearly 40% higher on average in the summer lots (t = 3.1155, df = 14, p-value = $7.595*10^{-3}$). Nearly all shops that were sampled in both seasons showed

Table 3. Prevalence of parasites observed in wet mounts of fin clip, epidermal mucus, and gill tissue from golden shiner lots purchased at retail bait shops (n = 53) in Minnesota from May 2014–February 2015. Severity was defined as having minor infection if < 10 parasites were observed in five minutes of examination/tissue type/lot, moderate infection if 10–100 parasites, and severe infection if > 100 parasites.

Parasite	Prevalence (n)			
	Minor	Moderate	Severe	Total
Chilodenella spp.	0	1.9% (1)	0	1.9% (1)
Dactylogyrus spp.	7.5% (4)	3.8% (2)	0	11.3% (6)
Epistylis spp.	3.8% (2)	0	0	3.8% (2)
Gydrodactylus spp.	7.5% (4)	0	0	7.5% (4)
Ichthyophthirius multifillis	1.9% (1)	0	0	1.9% (1)
Ichthyobodo spp.	1.9% (1)	0	0	1.9% (1)
Neascus spp.	15.1% (8)	7.5% (4)	0	22.6% (12)
Trichodina spp.	9.4% (5)	1.9% (1)	1.9% (1)	13.2% (7)
No significant parasites	–	–	–	49.1% (26)

decreased prevalence in the winter, but one shop showed an increase of 75%. Summer lots had approximately 7 more mature female golden shiners than winter lots (paired t = 4.7224, df = 14, p-value = $3.271*10^{-4}$).

There was a high diversity of bacteria identified during the course of this study (Table S1). Bacterial taxa richness as detected by culture and MALDI-TOF methods was slightly higher in the winter (mean = 6.88, sd = 2.06) lots than in the summer (mean = 5.71, sd = 2.95) lots in shops for which we had paired observations, though not significantly (paired t = −1.1464, df = 16, p-value = 0.2685). Notably, the important salmonid pathogens *Aeromonas salmonicida* and *Yersinia ruckeri* were confirmed to be present in ten and five lots, respectively. These bacteria are not regulated baitfish pathogens in Minnesota; however, they are in New York (Code 6 CRR-NY 188.2), and more broadly for salmonid species in Minnesota and elsewhere in the region (e.g., MN Statute 17.4984, Iowa Administrative Code 571.89.3).

Detection of replicating virus in culture was low, with only 2/53 lots positive for golden shiner reovirus. No other viruses were detected with the standard culture-based assays. However, a total of eight novel viruses were detected by NGS, including an astrovirus, betanodovirus, circovirus, fisavirus, two novel picornaviruses, and two now-described totiviruses, golden shiner totivirus (GSTV; Mor and Phelps 2016b) and piscine mycocarditis-like virus (PMCLV; Mor and Phelps 2016a). Two novel picornaviruses were identified, showing 22.26% identity with each other based on complete 3D (RNA dependent RNA polymerase) protein. On comparing with sequences available in GenBank, picornavirus 1 was highly divergent and showing only 21.05–26.75% identity with fish picornavirus sequences reported in GenBank. The picornavirus 2 was showing 21.62–53.82% identity with fish picornaviruses and highest 53.82% with recently reported Zebrafish picornavirus (AXQ03965). The golden shiner astrovirus type 1 and 2 sequences had a 64.11–65.40% amino acid identity based on the highly conserved complete RNA dependent RNA polymerase (ORF1b gene)

Table 4. Prevalence of viral detections by PCR during the summer (May–August; n = 23 lots) and winter (December–February; n = 30 lots) sampling seasons from golden shiners purchased at retail bait shops in Minnesota. Fisavirus was not tested (nt) by virus-specific PCR.

Virus	Prevalence (n)		
	Summer	Winter	Total
Astrovirus	67% (10)	90.0% (27)	82% (37)
Betanodavirus	7% (1)	0	2% (1)
Circovirus	53% (8)	90.0% (27)	78% (35)
Fisavirus	nt	nt	nt
Golden shiner picornavirus-1	60% (9)	43.3% (13)	49% (22)
Golden shiner picornavirus-2	7% (1)	0	2% (1)
Golden shiner totivirus	13% (2)	0	4% (2)
Piscine Mycocarditis-like Virus	13% (2)	10% (3)	11% (5)

protein. These sequences were 45% or less identical with other astrovirus sequences reported in the GenBank. The circovirus sequences were 50.25% and 30.85% identical with replication-associated and capsid proteins of circovirus detected in European catfish (*Silurus glanis*). The number of lots positive for each virus varied, but were generally consistent between sampling seasons as confirmed by virus-specific PCR (Table 4).

Discussion

This survey of golden shiners purchased from retail bait shops in Minnesota confirmed the presence of many microbes in the live baitfish supply, including some of significant concern. Many commensal microbes (e.g., trichodina) and opportunistic pathogens (e.g., *Aeromonas hydrophila*) were identified during the survey period and warrant no action – it is no surprise baitfish harbor a plethora of benign parasites, bacteria and viruses (Boonthai et al. 2017; Shi et al. 2018; Mahon et al. 2018). However, the detection of several pathogens of concern, underscores the need to better understand the risks that exist in the live baitfish pathway.

The detection of two bacterial pathogens, *A. salmonicida* and *Y. ruckeri*, in the live baitfish supply is potentially concerning given their disease-causing potential in many important fish species (Furones et al. 1993; Wiklund and Dalsgaard 1998) and the fact they were ranked among the higher-risk pathogens by a recent hazard prioritization framework (McEachran et al. 2020). *Aeromonas salmonicida* is the causative agent of furunculosis in salmonids (Schachte 1983) and ulcerative disease in goldfish (Humphrey and Ashburner 1993), though it may infect or be carried by many species. Detections of *A. salmonicida* in wild asymptomatic fish suggests that disease could occur with imperceptible impacts on wild fish populations (Mooney et al. 1995; Wiklund and Dalsgaard 1998), but the potential for introduction and harmful outbreaks via infected carrier fish released as leftover bait in novel waterbodies cannot be ignored. *Yersinia ruckeri* is also primarily associated with disease in salmonids, but outbreaks have occurred in non-salmonid fish species and recurrent outbreaks or novel introductions are often attributable to asymptomatic carrier fish (Furones

et al. 1993). Without a robust understanding of the current distribution and potential impacts of these pathogens in wild fish populations, detection of these pathogens in the bait supply warrants evaluation. Interestingly, both bacteria are regulated pathogens in Minnesota, but only if detected in salmonid species – no requirements currently exist to inspect live baitfish for these bacteria (MN Statute 17.4984). This highlights a common gap in regulations more often focused on the host species of concern, rather than pathogens or pathways that have the potential to impact multiple host species.

Ovipleistophora ovariae, found in nearly half of all tested lots, is a vertically-transmitted intracellular microsporidian parasite that causes degradation of developing eggs and progressively worsens with age, effectively sterilizing female golden shiners by age two (Phelps and Goodwin 2008; Stone et al. 2016). Bait producers have largely overcome the issue by using only one-year-old broodstock (Stone et al. 2016), but no research exists on population-level risks to wild golden shiners, and proactively preventing the introduction to naïve populations is justified. Despite the fact that *O. ovariae* is thought to be widespread in the golden shiner trade (Summerfelt and Warner 1970; Stone et al. 2016), this is the first known report in Minnesota. We acknowledge, however, that given the historically limited effort for fish health surveillance, we are likely underestimating the distribution of this parasite in wild populations.

The detection of novel and pathogenic microbes in the live baitfish supply highlights the importance of rapid, state-of-the-art diagnostic techniques to identify pathogens of concern that may infect fish without any external clinical signs, allowing them to pass undetected through the supply chain. While conventional culture- or PCR-based detection methods were effective in detecting some well-documented pathogens (e.g., *A. salmonicida, Y. ruckeri, O. ovariae*), for novel or emerging pathogens for which there are no established diagnostic methods, the critical need for technologies such as NGS become even more clear. Our study was also able to identify eight novel viruses as a result of an "eyes wide open" approach to diagnostics that allowed us to sequence viruses for which we had no prior knowledge. As technological and computational capacity improves and as the cost of NGS continues to drop, this approach could make significant contributions to the surveillance of emerging disease in poorly understood systems (Hadidi et al. 2016). Although our study demonstrates the ability of these technologies to detect new viruses, the potential risk to fish health presented by novel microbes is often unknown. Detection of novel or uncharacterized microbes, like those found in this study, should be augmented with robust, standardized assessment frameworks for interpreting the risks they present to wild or farm-raised fish, requiring a collaborative effort to understand the pathogenic capabilities of these viruses and the conservation implications of their introduction (Gaughan 2001).

The presence of non-target species and pathogenic microbes in the bait supply is certainly evidence that screening, diagnostic testing, and disease management are important, but we encourage caution when interpreting these findings. While there are clear advantages to the use of highly sensitive diagnostic methods (e.g., PCR, NGS), contamination between individuals within a lot or from environmental microbiota is possible. It is also possible that given the complex historical patterns of baitfish use in Minnesota, the pathogens and non-target species we found have already become widely established and any potential negative consequences already realized. However, the actual impact of introduced aquatic pathogens has been poorly explored in the scientific literature, given the limited surveillance of wild fish populations, poor understanding of disease dynamics in aquatic systems (Jones 2000), and difficulty in tracking live fish movements and introductions. Indeed, for this study the original source of the golden shiners could not be ascertained during follow up interviews at the bait shops and we cannot attribute the risk to any particular source population or producer. Future investigations are needed to determine if hazards present in Minnesota's live baitfish supply are from wild or farm-raised populations, or the result of illegal importation from out-of-state sources. Better understanding the disease status of recipient wild populations where pathogens may be introduced is also warranted to better define the risk of introduction, establishment, and potential impact. In addition, understanding disease dynamics under current and future scenarios will be essential to understanding risks, or lack thereof, associated with repeated pathogen introductions in the absence of additional risk-based management.

The presence of other bait species (i.e., fathead minnows, finescale dace) comingled with the golden shiners could be the result of mixing at the point of sale; however, the presence of non-bait species (e.g., yellow perch and stickleback) suggests that contamination at the original source of the fish also occurs. Bycatch of non-target species during wild harvest can be common with some techniques, but it presents a particular concern in wild-harvested bait where potentially invasive fish may be transported long distances overland and introduced to new environments (Mills et al. 1993; Ludwig and Leitch 1996; Drake and Mandrak 2014). Regardless of the source of contamination, the presence of non-target species at the point of sale highlights the potential risk of this pathway and a gap in current management efforts. Furthermore, the number of baitfish sold by retail sources is noteworthy. While we standardized our purchase request to 30 golden shiners at each location, the number provided varied dramatically. When non-target species were included with the purchase of golden shiners, the numbers of non-targets were often low, but in one purchase included more than 250 non-target fish of various species! There is a need to better understand this variability as these results emphasize that the risk of pathogen or non-target species introduction via the bait supply is not

uniform across bait purchases. Over-selling the number of baitfish, even for the intended species, may also lead to the unintended consequence of anglers releasing unused bait if they receive more than they requested or fish of a different species (Ludwig and Leitch 1996).

Retail bait shops are the final stage in the baitfish supply chain before the fish are purchased by an angler and transported to the destination waterbody, therefore representing an important point of control for limiting the risk of AIS and disease spread (Drake and Mandrak 2014; Connelly et al. 2018). The AIS-hazard analysis and critical control point (AIS-HACCP) program has been established to reduce the number of non-target species and disease at different points in the supply chain, including sale at bait shops (Gunderson and Kinnunen 2003). This is currently a voluntary program in Minnesota and individual species identification skills vary, limiting the current effectiveness of such measures. Efforts to develop and evaluate a standardized approach for third-party verification of AIS-HACCP that improves education and compliance with risk-mitigation strategies are in development; however, given the risks identified here and in related studies, consideration of such a program by managers and industry is warranted (Connelly et al. 2018).

These findings represent an important first step in identifying the hazards that should be considered in a complete risk analysis of the live baitfish pathway. Risk analysis can be a useful tool for quantifying the actual risk of a particular pathway and identifying management strategies to mitigate that risk (Kolar and Lodge 2002; Copp et al. 2005b; Vose 2008; Mandrak and Cudmore 2015). While identifying the harmful agents that are present in the live baitfish supply is a critical first step, a broader consideration of the whole bait pathway from source to hook is necessary to fully understand the risk that AIS and pathogens could be present in bait buckets. Understanding these risks would help managers balance the economic benefits and ecological risks of live baitfish use to inform risk-based management strategies.

Acknowledgements

The authors thank the Minnesota Department of Natural Resources Enforcement Division for supporting this project with sample collection and funding. We thank the Minnesota Veterinary Diagnostic Laboratory Bacteriology Section for their valuable contributions to this project. We also thank the two anonymous reviewers whose thoughtful comments greatly improved the manuscript.

Funding

Funding for this study was provided by the Minnesota Department of Natural Resources Enforcement Division. In addition, funding for this project was also provided by the Minnesota Environment and Natural Resources Trust Fund as recommended by the Minnesota Aquatic Invasive Species Research Center and the Legislative-Citizen Commission on Minnesota Resources.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Number of each baitfish species, bacteria and parasite taxa, and viruses present in each lot of baitfish purchased during the course of this study.

This material is available as part of online article from:
http://www.reabic.net/journals/mbi/2021/Supplements/MBI_2021_McEachran_etal_SupplementaryTable.xlsx