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VIRAL SURVEY OF FATHEAD MINNOWS, GOLDEN SHINERS, AND WHITE SUCKERS FROM BAITFISH DEALERS IN WISCONSIN

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VIRAL SURVEY OF FATHEAD MINNOWS, GOLDEN SHINERS, AND WHITE SUCKERS FROM BAITFISH DEALERS IN WISCONSIN

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ABSTRACT

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Fathead minnows, golden shiners, white suckers, and other species of baitfish were sampled to determine the presence of target and unknown viruses. All fish sampled were obtained from four Wisconsin baitfish dealers and included imported, cultured, and wild-harvested baitfish. A total of 4,318 fish comprising 82 distinct lots were tested. A lot consisted of 60 fish of the same species that shared a common water source. One or more viruses were detected in 44% of the lots tested (36 of 82 lots). There were 39 total virus isolations. The viruses isolated included: Golden Shiner Virus, Fathead Minnow Nidovirus, and 15 unknown viruses. Fathead minnows were statistically more likely to be infected with a virus than all other species tested (P=0.021). Virus prevalence between seasons was statistically different, with 67% of all virus isolations (26 of 39 lots) occurring during the summer (P=0.009). There was no statistical difference in virus prevalence between baitfish dealers (P=0.372), import states (P=0.438), or between cultured and wild-harvested fish (P=0.236).

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INTRODUCTION

The baitfish industry plays an important role in the state of Wisconsin. The impact of this industry is both economic and ecological (Litvak and Mandrak 1993). Baitfish wholesale and retail values are difficult to obtain because of inconsistencies in catch reporting and difficulties in regulating individual baitfish harvesters (Litvak and Mandrak 1993). Among six Midwestern states (Minnesota, Wisconsin, Ohio, Michigan, Illinois, and South Dakota), baitfish sales exceed $145 million annually (Meronek et al. 1997a). In Wisconsin, annual sales of baitfish average about $30 million, though these estimates are likely conservative since reports from baitfish dealers are often inaccurate and incomplete (Meronek et al. 1997a). Because of the large economic impact the baitfish industry has in Wisconsin, the effects on the natural aquatic environment may be catastrophic. Baitfish may harbor numerous pathogens, viral and otherwise, that have the potential to infect other species of fish and aquatic wildlife.

The three species of baitfish sold in the highest quantities in the state of Wisconsin are: fathead minnows (*Pimephales promelas*), white suckers (*Catostomus commersonii*), and golden shiners (*Notemigonus crysoleucas*) (Meronek et al. 1997b). Baitfish dealers in Wisconsin maintain their supply of these baitfish through one of two means: (1) via importation from baitfish farms and/or wholesale dealers from other states, or (2) via wild harvest within the state. Once brought on site, these populations of baitfish are generally held in ponds or large tanks for later transportation to retail buyers.

These three imported baitfish species are native to Wisconsin; however, the pathogens they carry may not be. Although it is illegal in Wisconsin to release live bait into any body of water, introduction of baitfish commonly occurs. This may be intentional or accidental through the release of unused live bait into aquatic environments by anglers, a practice commonly referred to as "bait bucket transfer" (Litvak and Mandrak 1993, Ludwig and Leitch 1996). This practice can lead to negative ecological effects on the recipient ecosystem through the introduction of new parasites and diseases (Courtenay and Taylor 1986). One such example was the introduction of a protozoan parasite, *Oviplistophora ovariae*, from infected golden shiners from baitfish farms into native populations of golden shiners in Kentucky, Missouri, and Arkansas (Summerfeldt and Warner 1970, Litvak and Mandrak 1993, Pekkarinen et al. 2002). Furthermore, it is also believed that Largemouth Bass Virus was first introduced to wild populations of largemouth bass (*Micropterus salmoides*) in Florida waters via the release of ornamental fish (Grizzle and Brunner 2003).

Baitfish that are moved across state lines into new watersheds could potentially introduce viruses from an endemic area where fish have co-evolved with a particular pathogen, to new bodies of water where fish hosts may have little natural resistance (Goodwin et al. 2004). Unfortunately, comprehensive viral surveys of baitfish are absent in the current scientific literature. Therefore, it is important to establish which viruses (known and unknown) are present in these baitfish species. This data will allow for a better assessment of the potential risks that viruses could have on native populations. Moreover, results of a large-scale viral survey will help to shape future regulatory

policies in regards to the interstate transport of baitfish between states and allow managers to better assess the risks involved with such transfers.

The transport of baitfish across Wisconsin state lines is currently regulated by the Wisconsin Department of Agriculture, Trade, and Consumer Protection (DATCP) which acts under current Wisconsin law (Administrative Code ATCP 10.62 (1) (a) 4) requiring that all baitfish dealers importing live fish from other states must first obtain the proper import permits and health certificates. Health certification for these permits requires that fish pass a visual disease inspection by a dealer-hired veterinarian or certified fish health biologist. An exception to the visual inspection occurs for fish species that are listed on the Animal and Plant Health Inspection Service's (APHIS) Viral Hemorrhagic Septicemia Virus (VHSV) susceptible species list and which are obtained from VHSV- positive waters (Wisconsin Administrative Code ATCP 10.62). Tissue samples from these fish are required to be submitted for more rigorous laboratory testing to rule out possible VHSV infection. Any other diseases identified during this VHSV screening are considered non-reportable. This law was enacted in order to protect both the state's aquatic resources, as well as, the fish farm industry from VHSV infection.

In addition to this state import law, a federal act called the Lacey Act (Title 50, Part 16, United States Code, Section 3372) was created to provide greater federal oversight of interstate and foreign importation of wildlife. The Lacey Act makes it unlawful to import, receive, and acquire, in interstate or foreign commerce, any fish transported in violation of any law or regulation of any State.

The opportunity to obtain the baitfish sampled in this study resulted from a legal settlement and subsequent plea agreement by four Wisconsin baitfish dealers who were

convicted of illegally importing baitfish into Wisconsin. These dealers failed to obtain the proper permits for transporting fish across state lines and were tried and convicted in September of 2009 of violating the federal Lacey Act. As a condition of their probation, each dealer agreed to submit to monthly testing of baitfish intended for import into the state and twice-yearly on-site visits and sampling. The fish from this sampling underwent complete pathogen testing for viruses, bacterial pathogens, and parasites by the U.S. Fish & Wildlife Service's La Crosse Fish Health Center in Onalaska, WI.

Description of Baitfish Species

Fathead Minnows (*Pimephales promelas*)

Fathead minnows are an important baitfish species in the state of Wisconsin and rank first in baitfish sales in the state (Meronek et al. 1997b). In 1992, it was estimated that over 70,000 gallons of fathead minnows were sold in Wisconsin, second only to Minnesota (Meronek et al. 1997b). Depending upon the size of the minnow, this is the equivalent of between 84 and 414.4 million fathead minnows sold annually in Wisconsin (Meronek et al. 1997b). This information was gathered from surveys of retail bait dealers in Wisconsin and extrapolated based on the estimated number of total active bait retailers in the State (Meronek et al. 1997a).

Fathead minnows seem to display a preference for waters that are subject to large amounts of silting, but are widely distributed in North America and can be found in lakes, ponds, and streams (Becker 1983). In fact, as a result of the baitfish trade, fathead minnows have been so widely dispersed, especially in the southwestern United States, that it has become difficult to determine their original range (Eddy and Underhill 1976). Because of their small size and abundance, fathead minnows make ideal forage fish for

piscivorous fish and birds (Becker 1983). As a result of increased restrictions on the interstate transport of baitfish due to the risk of disease transmission, many baitfish dealers in Wisconsin have begun harvesting fathead minnows locally from lakes and ponds within Wisconsin during the summer months (Meronek et al. 1997a, Horne et al. 2010). To keep up with demand, however, they must also continue to import from baitfish farms or hatcheries in other states.

White Suckers *(Catostomas commersonii)*

White suckers account for the second largest volume of baitfish sold in Wisconsin at just over 11,000 gallons sold in 1992 (Meronek et al. 1997b). White suckers are native to Wisconsin and are common in most streams and lakes. They appear to be tolerant of a wide range of environmental conditions, including highly polluted and turbid waters (Becker 1983). Similar to other species in the family *Catostomidae,* white suckers are characterized by a protruding, sucking mouth and large, fleshy lips that are ventrally positioned to facilitate bottom feeding (Herald 1979).

White suckers are an important forage fish. In the wild, they are commonly fed upon by musky, walleye, northern pike, bass, and other top fish predators, birds, and occasionally bear and other mammals (Becker 1983). The Wisconsin Department of Natural Resources uses both wild harvested and imported white suckers as forage for game fish reared in the state's hatcheries. Prized by anglers because of their large size, white suckers are often propagated in ponds to ensure a steady bait supply (Phillips et al. 1982); however, like fathead minnows, they are commonly imported from southern baitfish farms and dealers in Midwestern states.

Golden Shiners *(Notemigonus crysoleucas)*

Golden shiners are common throughout Wisconsin's freshwater habitats and are generally found in slower-moving pools and sloughs where vegetation is dense (Becker 1983) and the water is clear or slightly turbid (Pflieger 1975, Tomelleri and Eberle 1990). Similar to fathead minnows, golden shiners have been so widely distributed by the baitfish industry that it is difficult to discern their native range (Eddy and Underhill 1976). Unlike most other fish species, golden shiners appear to be the only fish that are able to survive in waters that contain very low levels of oxygen during the winter months (Becker 1983). They also seem to be more tolerant of higher temperatures, with studies showing 34°C as the upper tolerance limit (Phillips et al. 1982, Becker 1983). When paired with other stressors such as physical disturbance and poor water quality, however, higher water temperatures can result in mortality (Becker 1983).

Golden shiners also serve as forage food for game fish like crappie, bass, and muskie, and for many aquatic birds like grebes, mergansers, herons, and kingfishers (Becker 1983, Tomelleri and Eberle 1990). They are prized as baitfish, especially in colder months. Because their natural reproductive rates in northern climates are not adequate to supply the demand by anglers, golden shiners are commonly raised on southern farms and are shipped to northern dealers (Becker 1983).

Target Viruses

The baitfish in this study were screened for the following viruses: Viral Hemorrhagic Septicemia Virus (VHSV), Spring Viremia of Carp Virus (SVCV), Infectious Pancreatic Necrosis Virus (IPNV), and Largemouth Bass Virus (LMBV). These viruses are certifiable pathogens and are included on the U.S. Fish & Wildlife Service's (USFWS) list of reportable pathogens and are included as part of the general

fish health screening protocol for all salmonid and non-salmonid fish species (AFS-FHS 2010). In addition, VHSV and SVCV are also listed on the World Organization for Animal Health's (OIE) reportable pathogen list and represent viruses that are of international concern. Three additional viruses: Golden Shiner Virus (GSV), Fathead Minnow Nidovirus (FHMNV), and Bluegill Virus (BLGV) are not included on the USFWS or OIE certifiable pathogen list, but were targeted in this study because they are considered to be significant pathogens associated with clinical disease and/or mortality.

Viral Hemorrhagic Septicemia Virus

Viral Hemorrhagic Septicemia Virus (VHSV) is a rhabdovirus known to cause extensive mortalities in both wild and cultured fish (Bain et al. 2010). Once considered limited to European freshwater fish (Bain et al. 2010), VHSV was first isolated in the United States in 1988 from North American adult coho (*Oncorhynchus kisutch*) and chinook (*Oncorhynchus tshawytscha*) salmon in Washington State (Meyers and Winton 1995). Previously thought to have been transferred via an exotic import from Europe, it was determined that the viral strain (genotype IVa) isolated from these North American salmon represents a new strain that is genetically distinct from the European VHSV (Meyers and Winton 1995). Since then, surveys of marine fish indicate that VHSV is now widely distributed among many species of fish in the North Pacific and North Atlantic oceans (Bain et al. 2010). VHSV (IVa) has also been detected in Pacific herring (*Clupea pallasii*), a common prey item of salmon. These herring are likely the source of VHSV (Iva) isolated from adult salmon in Washington State (Meyers and Winton 1995). VHSV (IVa) is highly pathogenic, causing mortalities approaching 100% in juvenile

salmon and mortality rates of 25-75% in adult salmon, depending upon environmental stressors (Wolf 1988, Meyers and Winton 1995).

In 2005, a second novel genotype of VHSV (IVb) was detected in freshwater drum (*Aplodinotus grunniens*) and round goby (*Neogobius melanostomus*) in the Great Lakes after an extensive die-off in Lake Ontario (Bain et al. 2010). In 2006, VHSV IVb was isolated from yellow perch (*Perca flavescens*) after a large fish kill in Lake Erie (Kane-Sutton et al. 2009). Since then, VHSV (IVb) has been detected in many more species of freshwater fish (Bain et al. 2010) and has been found in all of the Great Lakes, as well as inland lakes in Michigan, New York, Wisconsin, and Ohio (USDA 2009).

VHSV is shed via the urine and reproductive fluids of infected fish and is transmitted horizontally through the water column (Wolf 1988, Meyers and Winton 1995, Kane-Sutton et al. 2009). It is believed that the main route of entry of the virus is through the gills; however, a recent study suggests that fin bases may also serve as a major portal for Novirhabdovirus, a type of rhabdovirus that infects salmonids (Kane-Sutton et al. 2009). Vertical transmission of the virus has not been documented and is considered extremely rare or nonexistent (Wolf 1988); however, a recent challenge study conducted to test the efficacy of iodophor disinfection of fish eggs has shown that VHSV can be isolated from northern pike (*Esox lucius*) eggs exposed to VHSV via immersion treatment for up to 4 days post infection (Tuttle-Lau et al. 2009). Oral transmission of VHSV has occurred though the experimental feeding of infected trout to young northern pike (Kane-Sutton et al. 2009).

Water temperature and age of the fish are critically important in the transmission of the virus (Wolf 1988). Transmission occurs readily between 1°C and 12°C, but rarely

above 15°C (Wolf 1988) and the highest mortalities are generally observed at 8 to 10°C (Noga 1996). Though all ages of fish are susceptible, young fish tend to be more susceptible to infection, probably due to the lack of immunity that older fish acquire following prior viral exposure (Bergmann and Fichtner 2008).

Spring Viremia of Carp Virus

Spring Viremia of Carp Virus (SVCV) is a rhabdovirus that causes acute viral infections in cyprinids, primarily common carp (*Cyprinus carpio*) (Wolf 1988). Infections generally occur in the spring and are responsible for high numbers of mortalities in both adult and juvenile carp (Wolf 1988). SVCV has been isolated in carp diagnosed with infectious dropsy, a disease associated with petechial skin hemorrhaging and abdominal swelling (Goodwin 2002). The virus is widespread in European carp culture (Ahne et al. 2002), but was not detected in North American fish until April of 2002 when it was isolated from koi being raised on a fish farm on the Eastern coast of the United States (Goodwin 2002). During the same period, late April through early June of 2002, SVCV was detected for the first time in North American wild fish when it was isolated from moribund carp following a fish kill in Cedar Lake in northwestern Wisconsin (Dikkeboom et al 2004). Since that time, there have been isolated outbreaks of SVCV in wild fish populations. In 2007, SVCV was detected in common carp from Pool 8 of the Upper Mississippi River following a fish kill (Puzach, La Crosse Fish Health Center, U.S. Fish & Wildlife Service, personal communication). In June 2008, SVCV was detected in wild fish from two different locations in Ohio. One case involved samples of largemouth bass and bluegill from Pleasant Hill Lake that tested positive following a routine wild fish survey (Puzach, La Crosse Fish Health Center, personal

communication). The second case involved a lot of wild emerald shiners that were, again, sampled as part of a routine wild fish survey of the Ohio River (Puzach, La Crosse Fish Health Center, personal communication).

SVCV is transmitted horizontally in fecal casts, urine, and gill mucus of infected fish (Fijan 1999), but external parasites such as leeches and *Argulus* spp may also act as vectors (Ahne et al. 2002). Clinical signs associated with SVCV can be apparent both internally and externally. Internally, infected fish show damage to kidney, spleen, and liver tissue that leads to hemorrhaging of the internal organs, impaired immune function, and loss of osmoregulatory function (Ahne et al. 2002). External signs of infection may also include skin darkening, abdominal distension resulting from buildup of excess ascites fluid, and petechial hemorrhaging of the skin (Fijan 1999).

Fathead Minnow Nidovirus

Fathead Minnow Nidovirus (FHMNV) is a recently characterized virus belonging to the order *Nidovirales* (Batts et al. 2007, Batts et al. 2012). Little is known about this virus which was originally placed into the *Rhabdoviridae* family of viruses (Iwanowicz and Goodwin 2001). After more recent characterization and sequencing, it has since been placed into to the family *Coronaviridae* and genus *Bafinivirus.* It is most closely related to another recently describe virus, white bream virus (WBV), the only other known species of *Bafinivirus* (Batts et al. 2012). FHMNV was first isolated in 1997 from wild-type and rosy red fathead minnows in ponds on an Arkansas baitfish farm (Iwanowicz and Goodwin 2001). These minnows were a mix of wild and farm raised minnows from various, unspecified regions of the United States (Iwanowicz and Goodwin 2001). The virus was isolated from fish that died over a two month period at

water temperatures between 15°C - 20°C (Iwanowicz and Goodwin 2001). In addition to mortality, infected fish exhibited behavioral pathology (swimming in circles, listlessness, and loss of equilibrium) and petechial hemorrhages of the eyes, skin, and muscle (Iwanowicz and Goodwin 2001).

Golden Shiner Virus

Golden Shiner Virus (GSV) is a reovirus that was first detected in 1977 in golden shiners showing morbidity and mortality at an Arkansas hatchery (Brady and Plumb 1991, Goodwin et al. 2006). GSV is one of more than 30 viruses in the genus Aquareovirus that have been detected in a large number of species including fish, shellfish, crustacean, birds, insects, and plants throughout the world (Lupiani et al. 1995). The role that reoviruses play in the pathogenesis of their aquatic hosts is not well understood (Rangel et al. 1999). While aquareoviruses are generally associated with subclinical infections (McEntire et al. 2003), GSV has been isolated from fish displaying clinical disease including petechial hemorrhages in the cornea, dorsal musculature, skin, internal fat, and intestinal mucosa, as well as hepatitis and pancreatitis (Lupiani et al. 1995, Rangel et al. 1999). It has also been associated with behavioral pathology including listlessness, swimming near the surface, diving in response to disturbance, and spiraling (Wolf 1988). In addition to clinical signs of disease, GSV has also been associated with fish mortality (Goodwin et al. 2006). One such event was a fish kill involving grass carp (*Ctenopharyngodon idella*) in China during the 1970's (McEntire et al. 2003). Another mortality event occurred in March 1998 after golden shiners on a fish farm began dying at a rate of 100-1000 per day over a 2-3 week period (McEntire et al. 2003). Then, in April and May of 1998, a second fish farm reported a similar pattern of

golden shiner mortalities. GSV was later isolated from these fish (McEntire et al. 2003). Early experimental infection studies in golden shiners have shown low mortality rates and GSV has been frequently isolated from apparently healthy fish, however, it is believed that subclinical GSV infections may transition into clinical disease with subsequent mortality when fish are placed into crowded or environmentally poor conditions (Brady and Plumb 1991, Goodwin et al. 2006).

Early studies with experimental infections of GSV in golden shiners showed that transmission occurs horizontally (Wolf 1988) and the optimal water temperature for virus replication is 28°C (Brady and Plumb 1991). More recent evidence, however, has shown that GSV can actively replicate at water temperatures as low as 15°C (McEntire et al. 2003). This experimental infection study of GSV in golden shiners confirmed that replication of the virus does occur in golden shiners, but no attempts have been made to fulfill Koch's Postulates (Shors 2008).

Infectious Pancreatic Necrosis Virus

Infectious Pancreatic Necrosis Virus (IPNV) is a birnavirus that is particularly devastating to young salmonid fishes, causing widespread mortalities in fingerling and fry (Wolf 1988, Reno 1999, Bergmann and Fichtner 2008). Though rare, IPNV is occasionally isolated in adult fish and from non-salmonid species, but these infections tend to be subclinical (Bergmann and Fichtner 2008). Survivors of infection can remain asymptomatic carriers of IPNV for extended periods of time and may serve as reservoirs of infection to other fish (Munro et al. 2010).

IPNV is transmitted laterally through ingestion of infected urine or feces, and direct contact with sexual products. Infection may also occur via vertical transmission

from parent to egg during spawning (Wolf 1988, Munro et al. 2010). Infection can cause changes in behavior such as whirling, sinking to the bottom, and rapid, shallow respirations and external clinical signs such as skin darkening, abdominal distention, and hemorrhages (Wolf 1988).

Largemouth Bass Virus

Largemouth Bass Virus (LMBV), an iridovirus, was first detected in adult largemouth bass (*Micropterus salmoides*) associated with a large fish kill involving over a thousand fish in a South Carolina reservoir during the summer of 1995 (Plumb et al. 1996). LMBV is the only virus known for causing morbidity and mortality in wild largemouth bass populations (Piaskoski et al. 1999, Plumb et al. 1999).

The virus is transmitted horizontally through the water column and has been found in the cutaneous mucus of infected fish, allowing for possible transmission from infected to healthy fish during cohabitation (Grizzle and Brunner 2003). Oral transmission of LMBV was demonstrated via ingestion of infected prey fish (Woodland et al. 2002). Vertical transmission has not been demonstrated (Plumb et al. 1999). Fish infected with LMBV tend to appear normal with no external lesions, but may exhibit loss of equilibrium (Plumb et al. 1996). Internally, infected fish exhibit a red, inflamed, and enlarged swim bladder sometimes containing yellow fluids (Plumb et al. 1996, Plumb et al. 1999).

Bluegill Virus

Bluegill Virus (BLGV) is a newly characterized virus belonging to the family *Picornaviridae*. It was first isolated by the Wisconsin Department of Natural Resources and the La Crosse Fish Health Center in 2001 following a bluegill mortality event in

Montana Lake in northeastern Wisconsin (Barbknecht 2009). Since 2001, BLGV has been isolated from a number of locations in Wisconsin, Illinois, and Ohio (Barbknecht 2009) and has been detected in a wide range of host fish including largemouth bass, black crappie, green sunfish, pumpkinseed, and walleye (Lasee, La Crosse Fish Health Center, personal communication). Fish infected with the virus have displayed clinical signs of infection including hemorrhaging and swelling of the internal organs (Barbknecht 2009).

Research Objectives

The primary objective of this study was to perform a broad viral survey of the baitfish species that are most commonly imported and sold in the state of Wisconsin: fathead minnows, golden shiners, and white suckers. Since no viral surveys of this type exist in the current scientific literature, the results of this study will be helpful in estimating the overall prevalence of key viruses in these baitfish species, influencing regulatory policy regarding interstate baitfish movements, and detecting novel viruses. An experimental infection study of fathead minnows with golden shiner virus was performed in order to determine the virulence of GSV (a point of debate within the current scientific literature) and to fulfill Koch's Postulates for GSV in order to show a clear association between GSV infection and disease and mortality in fish. The specific objectives of this study were to:

1. Determine the presence of known viruses in imported and wild-harvested baitfish from the four baitfish dealers that were illegally importing baitfish into Wisconsin.
2. Detect and isolate unknown viruses through the observation of clinical signs, viral isolation on differential cell lines and incubation temperatures, and descriptions of cytopathic effect.

3. Determine the pathogenicity of Golden Shiner Virus (GSV) via experimental infection.

METHODS

Fish Collection

Fish were sampled at the request of U.S. Fish & Wildlife Law Enforcement and La Crosse Fish Health Center personnel. Up to two lots of fish were selected for testing from each bait dealer once per month, up to twelve times per year. For purposes of this study, a lot constituted 60 fish of the same species that shared a common water source. In a few cases, 60 fish of a single species could not be obtained. In these cases, fish of multiple species were tested as a single, mixed lot, but kept separated by sample tube. In other cases, when less than 60 fish were available, a lot was comprised of the number of fish that were available. Requested lots represented fish intended for import into the state of Wisconsin from bait suppliers in other states or from fish recently imported and held at the Wisconsin wholesale facility. Additionally, onsite inspections of each of the four baitfish dealer facilities were conducted annually, at which time four additional lots of fish were obtained. During these onsite visits, fish sampled were often a mix of both imported cultured fish from out of state and wild harvested fish from within Wisconsin.

The three species of baitfish that are most commonly imported and sold in Wisconsin were targeted for this study: fathead minnows (*P. promelas*), golden shiners (*N. crysoleucas*), and white suckers (*C. comersonii*). Because wholesale baitfish dealers occasionally harvest a mix of different species from wild populations, other species or "river mixes" of baitfish were also sampled during site visits when available.

Fish were obtained by two methods; either live shipments direct from the dealer or importer, or collection by fish health staff during semi-annual site visits. For live shipments, fish were sent to the La Crosse Fish Health Center via overnight express shipment in oxygenated water and plastic bags. The bags were packed on blue ice in coolers provided by the fish health lab. During site visits, fish health staff determined which lots of fish were to be sampled. This included both imported fish as well as wild harvested fish from Wisconsin waterways. Fish were euthanized and tissue samples were collected onsite. Resulting samples were then transported on ice to the lab for further processing.

Tissue Sampling

All live fish were euthanized using an overdose (>200 mg/L) of Tricaine Methanesulfonate (MS-222) (Argent Chemical Laboratories), an FDA approved fish anesthetic. Each fish was visually inspected for external signs of clinical disease such as hemorrhages and/or the presence of lesions. The presence of clinical signs was documented and photos taken.

Lots of sixty fish were pooled into groups of five (therefore a single lot consisted of 12 sample tubes) and all instruments were disinfected between pools using Extra™ brand disinfectant. Using a clean scalpel, each fish was incised laterally to expose the internal organs. Kidney and spleen tissue were taken from each fish and placed into a tube containing 3 ml of HBSS (Hanks Balanced Salt Solution with 2.75% antibiotics added). The antibiotics in the HBSS media were: 2% Penicillin/Streptomycin, 0.4% Gentamycin, and 0.25% Nystatin (an anti-fungal agent) and were added in order to kill any bacteria or fungus that may have contaminated the sample. The total amount of

tissue per tube did not exceed 1.5 g or a 1:10 ratio of tissue to media (AFS-FHS 2010). For fish measuring less than 3 cm, the entire viscera was removed for sampling, but every attempt was made to avoid this as whole viscera samples often create toxicity to the cell monolayer. In some cases, liver tissue was taken to supplement the kidney and spleen tissue in order to achieve the proper tissue to media ratio. (AFS-FHS 2010) (Fig. 1). Diluted tissue samples were homogenized using a stomacher (Stomacher 80 Biomaster-Seward) and maintained at 4°C until further processing.

Virology and Cell Culture

In order to maximize virus recovery, all tissue samples were processed and plated onto cell cultures within 48 hours of collection (AFS-FHS 2010). Homogenized tissue samples were centrifuged at 4°C for 15 min at 4800 RPM. One ml of supernatant was removed and added to a sterile tube of 1 ml HBSS. Once diluted, each tube was incubated for two hours at 15°C or up to 24 hours at 4°C in order for the antibiotics in the solution to kill or inhibit the growth of bacteria or fungi that may have been present in the sample. After incubation, the diluted tissue samples were centrifuged at 4°C for 15 minutes at 4800 RPM and plated onto the appropriate cell lines for tissue cell culture (AFS-FHS 2010) (Table 1).

Three cell lines and three incubation temperatures were used for the detection of viruses: Epithelioma Papulosum Cyprini (EPC) cells, derived from common carp cells (incubated at 15°C and 20°C), Chinook Salmon Embryo (CHSE) cells (incubated at 15°C), and Bluegill Fry (BF-2) cells (incubated at 25°C). Cell culture plates (24-well) were seeded with the appropriate cells 24 to 48 hr before sample inoculation. Once a confluent monolayer of cells was confirmed, 0.1 ml of diluted tissue sample from each

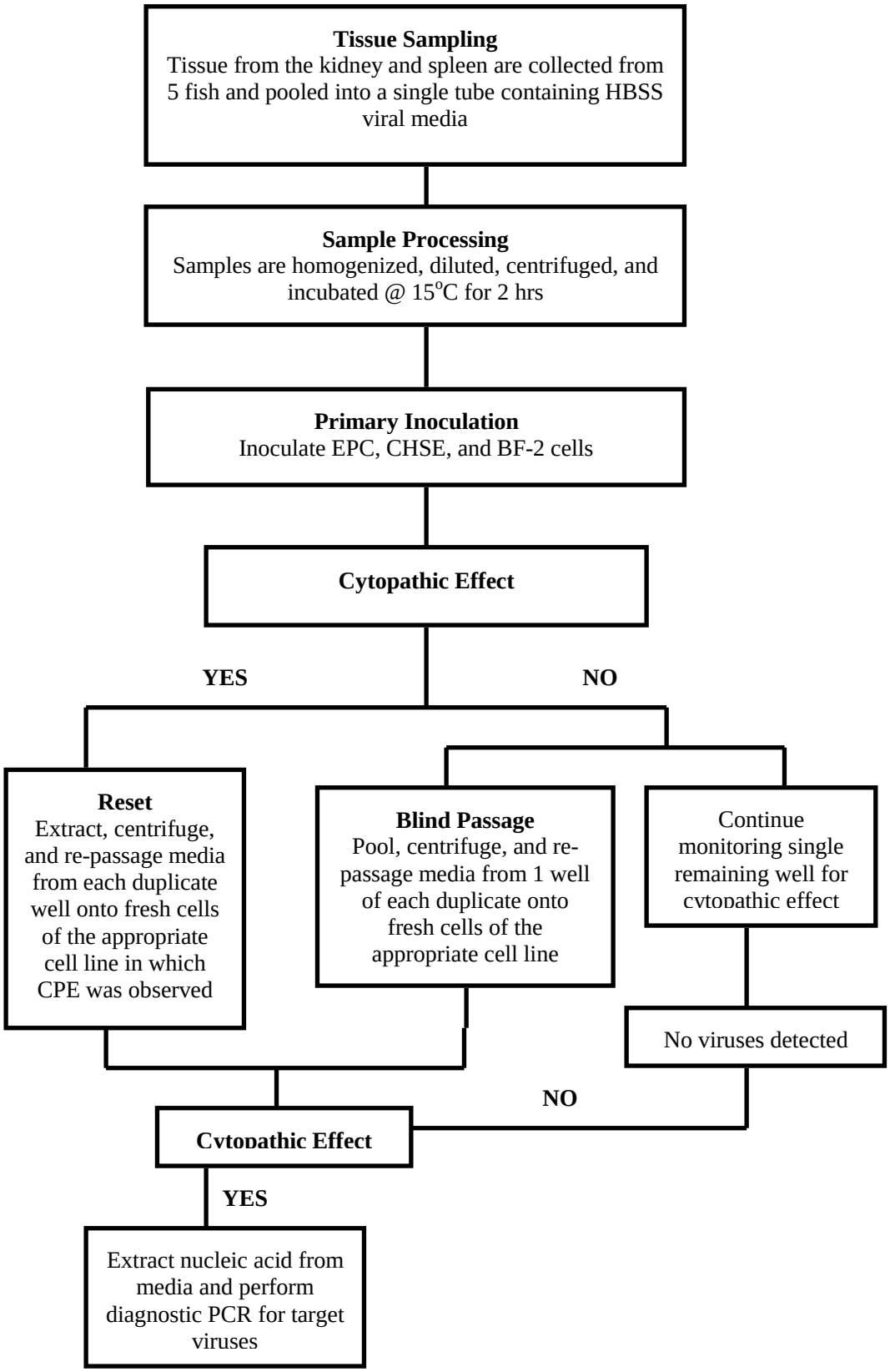

FIGURE 1. Reference flow chart outlining sample collection, processing, tissue culture and identification and confirmation of target viruses. Modified from Fig. 4 in Barbknecht 2009.

TABLE 1. Cell lines and incubation temperatures used in the detection of target viruses in Wisconsin baitfish.

Virus	Cell Line	Incubation Temp (^{o}C)
Viral Hemorrhagic Septicemia Virus	EPC	15
	CHSE	15
Spring Viremia of Carp Virus	EPC	20
Fathead Minnow Nidovirus	EPC	15, 20
	CHSE	15
Golden Shiner Virus	EPC	15, 20
	CHSE	15
Infectious Pancreatic Necrosis Virus	CHSE	15
	EPC	15
Largemouth Bass Virus	BF-2	25
Bluegill Virus	BF-2	25

tube was plated, in duplicate, onto the appropriate plates and rocked continuously for 1 hr to aid in viral adsorption. After one hour, 0.5 ml of Eagle Minimum Essential HCO_3 Medium (Sigma) at room temperature was placed into each sample well. A clear, adhesive, plastic film was placed aseptically over each plate in order to minimize media evaporation. Each plate was incubated at the appropriate temperature and monitored twice weekly via phase contrast microscopy for signs of cytopathic effect (CPE) for a total of 28 days. Viral CPE was photographed.

Any sample wells exhibiting CPE were diluted at a 1:5 ratio with HBSS and re-inoculated onto 24 hour old cells of the appropriate cell line, then incubated and

monitored for CPE for an additional 14 days. Alternatively, any wells from the original plates that did not exhibit CPE by day 14 post-inoculation were blind passed. Blind passage involved removing media from one well of each duplicate, pooling it with up to four additional sample wells, diluting at a 1:5 ratio with HBSS, and re-inoculating the pooled sample onto a 24-48 hour old monolayer (AFS-FHS 2010). The total combined incubation time for each pool of 5 fish was at least 28 days. If no CPE was observed during the 28 d observation period, samples were reported as negative and all plates and original tubes were autoclaved and disposed. Any wells suspected to contain virus were diluted at a 1:5 ratio and re-inoculated (up to two times) in order to replicate CPE (reset). Re-inoculated plates were monitored for 21 d for the original sample plates and 14 d for resets and blind passes. Any presumptive positive wells were held at 4°C. Standard polymerase chain reaction (PCR) procedures following the AFS Blue Book guidelines were performed to confirm and identify all target viruses. GSV, FHMNV, and BLGV were confirmed using PCR primers and methods specific to each virus (AFS-FHS 2010) (Table 2, Fig. 1).

Golden Shiner Virus Experimental Infection

To determine the virulence of golden shiner virus (GSV), an experimental infection of fathead minnows with golden shiner virus was performed. GSV isolated from fathead minnows from a previous case and confirmed via PCR was used as the stock virus solution. The fathead minnows used for the study were obtained from the Genoa National Fish Hatchery. This lot of fathead minnows was previously tested by the La Crosse Fish Health Center to ensure the fish were free of viruses, bacterial pathogens, and parasites. The study consisted of a negative control group and two treatment groups

TABLE 2. Viral pathogens, primer pairs and expected end products for target viruses.

Pathogen	Forward primer	Reverse primer	Product size
VHSV	GGGGACCCCAGACTGT	TCTCTGTCACCTTGATCC	811 bp [a]
SVCV (1st round)	TCTTGGAGCCAAATAGCTCARRTC	AGATGGTATGGACCCCAATACATHACNCAY	714 bp [a]
SVCV (2nd round)	TCTTGGAGCCAAATAGCTCARRTC	CTGGGGTTTCCNCCTCAAAGYTGY	606 bp [a]
IPNV	AAAGCCATAGCCGCCCATGAA	TCTCATCAGCTGGCCCAGGTAC	174 bp [a]
LMBV	GCGGCCAACCAGTTTAACGCAA	AGGACCCTAGCTCCTGCTTGAT	248 bp [a]
GSV	GGTGACGTGTTGGGATACAG	TTGATCTAGTCGCGTTACGG	129 bp [b]
FHMNV	TTTTGTTGAATTTATAGCTCTT	TGGCCATATCCTTAAGGG	277 bp [c]
BLGV	CTCGATAGTGTATGACTCGGACC	CATGGGGTTCAACACTCACA	180 bp [d]

References: [a] AFS-FHS 2010, [b] A. Goodwin, University of Arkansas, personal communication, [c] W. Batts, Western Fisheries Research Center, personal communication, [d] Barbknecht 2009

in replicate, two low viral concentration groups (3.4 X 102 pfu/ml) and two high viral concentration groups (3.4 X 107 pfu/ml). The experimental unit was a 5-gallon tank, each with 10 fish, and each fitted with a carbon filtration pump. The average daily water temperature was recorded at 15°C.

Each fathead minnow was anesthetized with MS-222, injected intraperitoneally with 100 µl of the appropriate amount of virus diluted in HBSS (Hanks Balanced Salt Solution with antibiotics) and then placed into a corresponding tank. Ten control minnows were injected with 100 µl of HBSS and placed into a single control tank. Fish were observed daily for 30 days for signs of clinical infection and mortality. Mortalities were removed daily and kidney and spleen samples were collected and processed using the same procedure describe previously. After 30 days, any surviving fish were euthanized with MS-222 and tissue samples were collected. All samples were processed within 48 hrs and inoculated onto EPC cells at 15°C and 20°C and CHSE cells at 15°C and were observed using an inverted microscope with phase contrast twice weekly for signs of CPE. If CPE was observed, sample wells were recorded as positive and confirmed by PCR. After 28 days, aliquots were taken from each positive well, as well as CPE negative wells and the presence of GSV confirmed using PCR.

Characterization of Unknown Viruses

A 1 ml aliquot of liquid suspension from cell culture for each of the fifteen isolations of unknown virus were shipped overnight on ice to the University of Minnesota Veterinary Diagnostic Laboratory and examined using transmission electron microscopy (TEM) (Dr. Anibal G. Armien and Donsanjiv Ariyakumar, MS). For negative staining, cell culture supernatant was processed using an on-grid centrifugation method (airfuge)

and stained with 1% Phosphototungstic Acid. Four unknown virus isolates were randomly amplified, cloned, and sequenced at the University of Minnesota Veterinary Diagnostic Laboratory in order to further identify these viruses (directed by Nicholas Phelps, M.S.).

Two unknown virus isolates were also sent to the University of Wisconsin-La Crosse Microbiology Department for further analysis and identification (directed by Dr. Michael Hoffman).

Statistical Analysis

The Pearson Chi–Square test was used to determine if there was a statistical difference in the prevalence of overall viruses between baitfish hosts, importation state, source (wild harvest or cultured) or season. The prevalence of GSV, FHMNV, and unknown viruses was also compared by fish host. P=0.05 was used as the level of significance.

RESULTS

Viral Survey

Virus Isolation

A total of 4,318 fathead minnows, white suckers, golden shiners, and other species of baitfish from 82 lots were tested for the presence of viruses (Table 3). Thirty-six of the 82 lots (44%) were positive for one or more viruses. The viruses isolated included Golden Shiner Virus (GSV) (Fig. 2), Fathead Minnow Nidovirus (FHMNV), and a number of unknown (non-target) viruses (UNK) (Fig. 2, Table 4). The most frequently isolated virus was GSV; it was detected in 20 lots of baitfish including fathead minnows, golden shiners, white suckers, creek chubs (*Semotilus atromaculatus*), and emerald shiners (*Notropis atherinoides*). GSV accounted for 51% of all viruses isolated. FHMNV was detected in four lots of fish, including fathead minnows and creek chub and accounted for 10% of all viruses isolated during the study.

Unknown viruses were isolated from 15 separate lots and are undergoing further characterization using electron microscopy and genetic cloning/sequencing at the University of Minnesota Veterinary Diagnostic Laboratory, St. Paul, MN, and the Microbiology Department at the University of Wisconsin-La Crosse, La Crosse, WI. Unknown viruses were detected in fathead minnows, white suckers, northern redbelly dace (*Phoxinus eos*), and a single lot of mixed, wild species. Unknown viruses accounted for 39% of all viruses isolated.

The prevalence of virus among host species was significantly different (P=0.021).

TABLE 3. Fish species examined, total number (n), number of lots, and percent (%) of total lots of Wisconsin baitfish tested for the presence of virus.

Species	Total (n)	Number of Lots Tested	% of Total Lots Tested
Pimephales promelas Fathead Minnow	2,210	37	51%
Catostomus commersonii White Sucker	1,204	20	28%
Notemigonus crysoleucas Golden Shiner	551	12	13%
Semotilus atromaculatus Creek Chub	125	3	4%
Notropis atherinoides Emerald Shiner	60	1	1%
Phoxinus eos Northern Redbelly Dace	10	1	1%
Other Species	158	8	2%

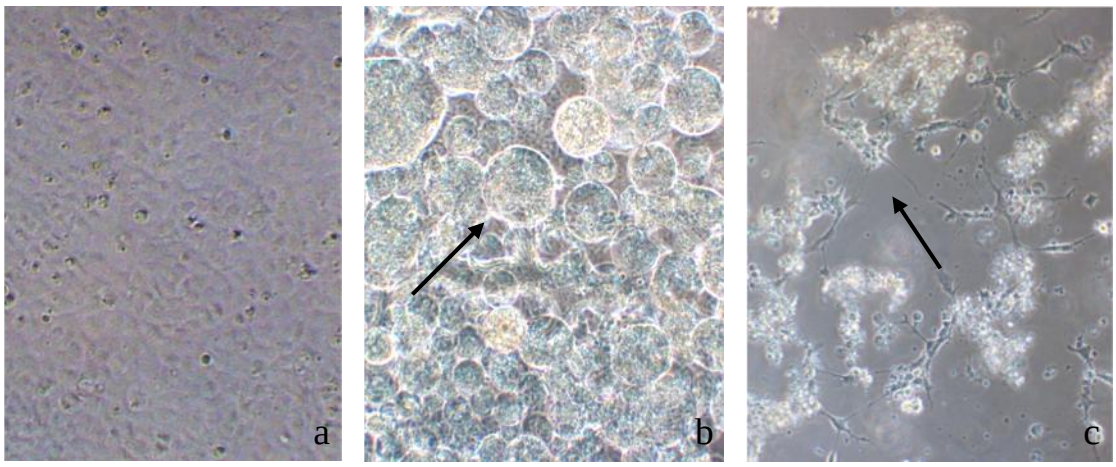

FIGURE 2. Cytopathic effect (CPE) caused by two different viruses on Epithelioma Papulosum Cyprini (EPC) cells: **(a)** Non-infected EPC cells exhibiting a confluent monolayer. **(b)** Golden Shiner Virus with large syncytia evident (arrow). **(c)** An unknown virus showing refractile cells, cell lysis, and destruction of the cell monolayer (arrow).

TABLE 4. Number of positive isolations of Golden Shiner Virus, Fathead Minnow Nidovirus, and unknown viruses within various species of Wisconsin baitfish tested.

Species	Golden Shiner Virus	Fathead Minnow Nidovirus	Unknown Viruses	Total
Pimephales promelas Fathead Minnow	12	3	9	24
Notemigonus crysoleucas Golden Shiner	5	0	0	5
Catostomus commersonii White Suckers	1	0	4	5
Semotilus atromaculatus Creek Chub	1	1	0	2
Notropis atherinoides Emerald Shiners	1	0	0	1
Phoxinus eos Northern Redbelly Dace	0	0	1	1
Mixed Species Lot	0	0	1	1
Total	20	4	15	39

For purposes of this study, virus prevalence is defined as the number of virus positive lots/total lots tested. Twenty-three of 37 lots (62%) of fathead minnows tested positive for at least one virus, five of 12 lots (42%) of golden shiners were positive for one or more virus, and white suckers tested positive in 5 of 20 (25%) lots tested. No link could be drawn, however, between the presence of any particular virus (GSV, FHMNV, or unknown virus) and host species. The P-values for each virus were: GSV (P=0.06), for FHMNV (P=0.476), and for unknown viruses (P=0.274).

Virus prevalence between seasons was statistically significant (P=0.009). A majority of positive virus isolations (67%, 26 of 39 virus isolations) were detected during

the summer months. Among the isolations, GSV was detected in 14 lots of fish, unknown viruses were detected in 8 different lots of fish, and FHMNV in 4 lots of fish. Summer was the only season in which FHMNV was isolated. The season with the next most frequent virus isolations was spring (21%, 8 of 39 virus isolations). During this period, GSV was detected in 5 lots and unknown viruses in 3 lots. Three unknown virus isolations occurred during the winter months (7%, 3 of 39 virus isolations) and this was also the only season that GSV was not detected. Two lots were positive for virus during the fall months (5%, 2 of 39 virus isolations): this included a single isolation of GSV and one unknown virus isolation (Fig. 3).

In evaluating seasonal virus prevalence by host species, fathead minnows had the greatest number of positive virus isolation across all seasons with 19% of lots positive during the spring months, 33% during the summer months, 3% in the fall, and 8% in the winter. Viruses from white suckers were only isolated in the summer, whereas golden shiners had a 3% virus prevalence in the spring, 8% in the summer, 3% in the fall, and no virus isolations in the winter months. Viruses in all other species were isolated during the summer only. (Fig.4)

There were no statistically significant differences in virus prevalence among baitfish dealers (P=0.372) (Table 5) or among import states (P=0.438). Baitfish were imported from three different states: Arkansas, Minnesota, and South Dakota. Ten of 17 (59%) of imported lots form Arkansas were positive for one or more virus, 18 of 41 (44%) lots from Minnesota were positive, and 3 of 8 (38%) lots from South Dakota tested positive (Table 6).

There was no statistical difference in virus prevalence between wild-harvested fish and cultured fish (P=0.236). Of the 66 lots of cultured fish that were tested, 31 were positive (47%) for at least one virus. Among wild-harvested fish, 5 out of 16 lots (31%) tested positive (Table 7).

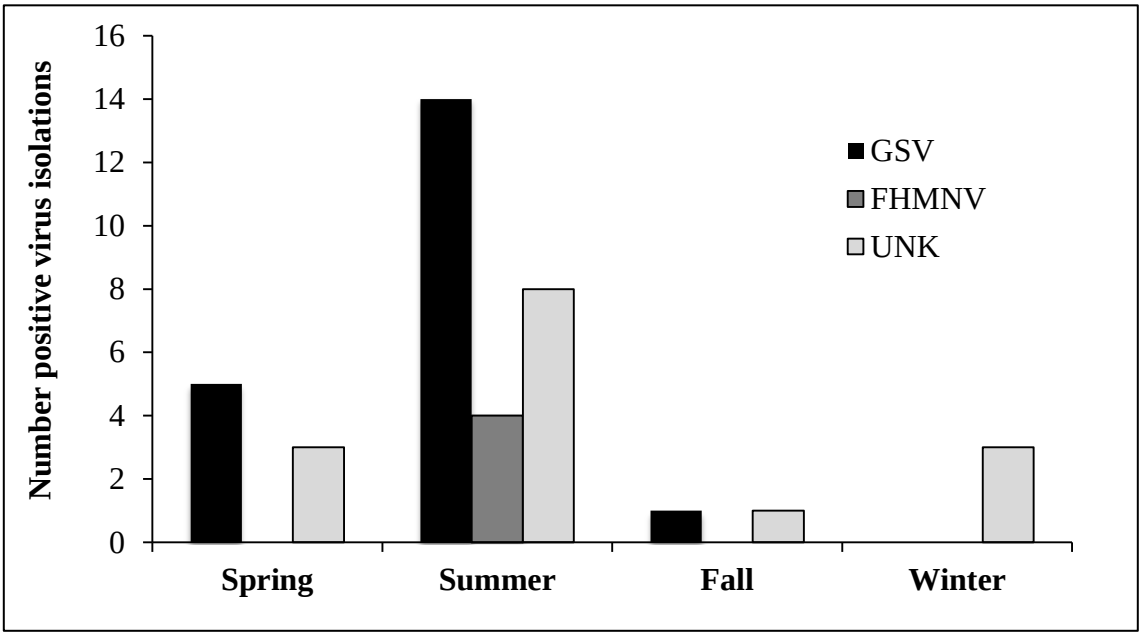

FIGURE 3. Number of positive isolations of Golden Shiner Virus (GSV), Fathead Minnow Nidovirus (FHMNV), and unknown viruses (UNK) during each season from all species of Wisconsin baitfish tested between February 2010 and December 2011.

Clinical Signs of Disease

Of the lots that tested positive for GSV, about half (10 of 20 lots) contained fish that displayed clinical signs: gill lesions and hemorrhages of the fin bases, vents, gill opercula, eyes, and skin (especially on the ventral surface) (Fig. 5). The species that showed clinical pathology were fathead minnows, golden shiners, and white suckers. GSV was also detected in fish without outward clinical signs including: creek chubs,

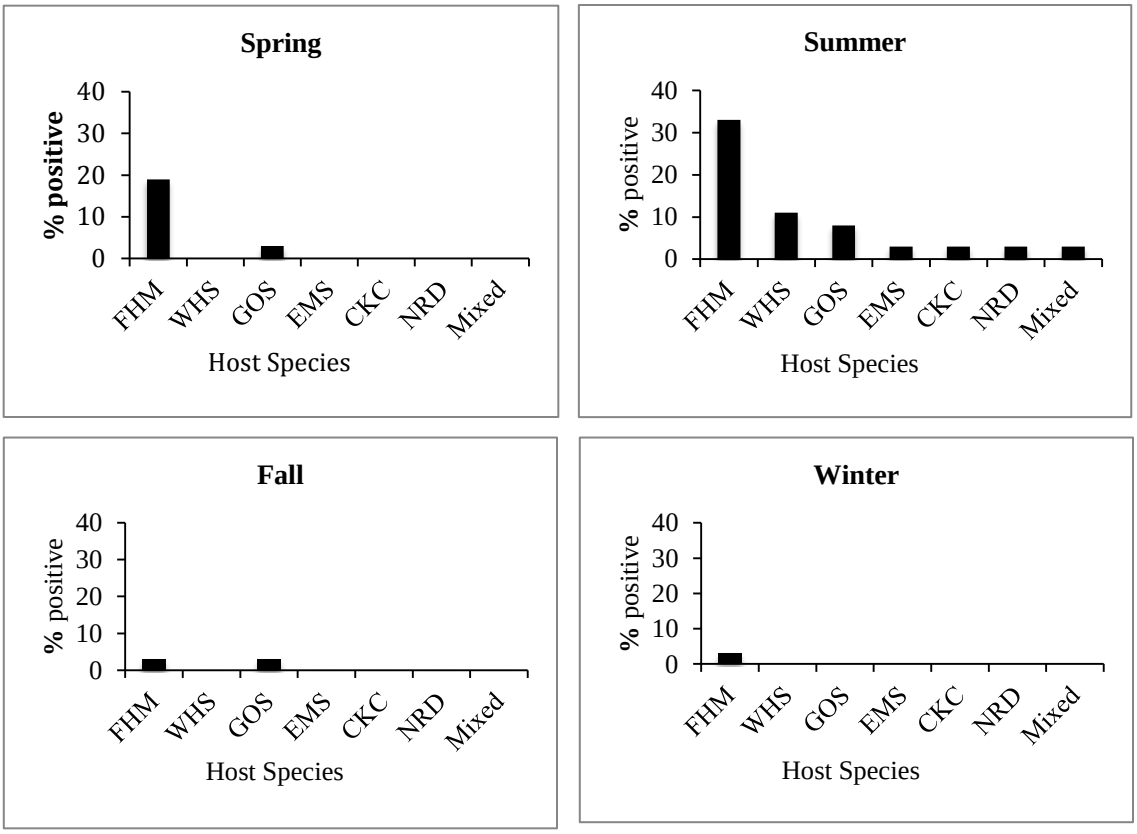

FIGURE 4. Percent (%) total seasonal virus prevalence for each host species from fish tested from four Wisconsin baitfish dealers. (FHM= fathead minnow, WHS= white sucker, GOS= golden shiner, EMS= emerald shiner, CKC= creek chub, NRD= northern redbelly dace).

emerald shiners, golden shiners, and fathead minnows. One of four lots that tested positive for FHMNV displayed external clinical pathology including skin lesions and hemorrhaging of the ventral surface and fin bases (Fig. 6).

Fish from nine of the 15 lots in which an unknown virus was isolated displayed clinical signs of disease. Those species included fathead minnows and white suckers. Clinical signs observed included hemorrhaging of the mouth, eyes, fin bases, gills, and skin. Northern dace, as well as three lots of fathead minnows and two lots of white

TABLE 5. Baitfish dealer and import state, number of virus positive lots, total number of lots tested, and % positive lots of fish tested from four Wisconsin baitfish dealers.

Dealer	Number of Positive Lots	Total Lots	% Positive
Dealer #1	5	19	26%
Minnesota	5	19	26%
Dealer #2	6	12	50%
Minnesota	6	12	50%
Dealer #3	10	23	44%
Arkansas	7	12	58%
South Dakota	3	8	37%
Wild Harvest	0	3	0
Dealer #4	15	28	54%
Arkansas	3	5	60%
Minnesota	7	10	70%
Wild Harvest	5	13	39%

TABLE 6. Number of virus positive lots, total lots tested, and percent (%) positive by import state for all imported fish obtained from all four Wisconsin baitfish dealers.

State	Positive Lots	Total Lots	% Positive
Arkansas	10	17	59%
Minnesota	18	41	44%
South Dakota	3	8	38%

TABLE 7. Number of positive lots, total number of lots tested, and percent (%) positive among cultured fish and wild-harvested fish obtained from Wisconsin baitfish dealers and importers.

	Positive Lots	Total Lots	% Positive
Cultured	31	66	47%
Wild Harvested	5	16	31%

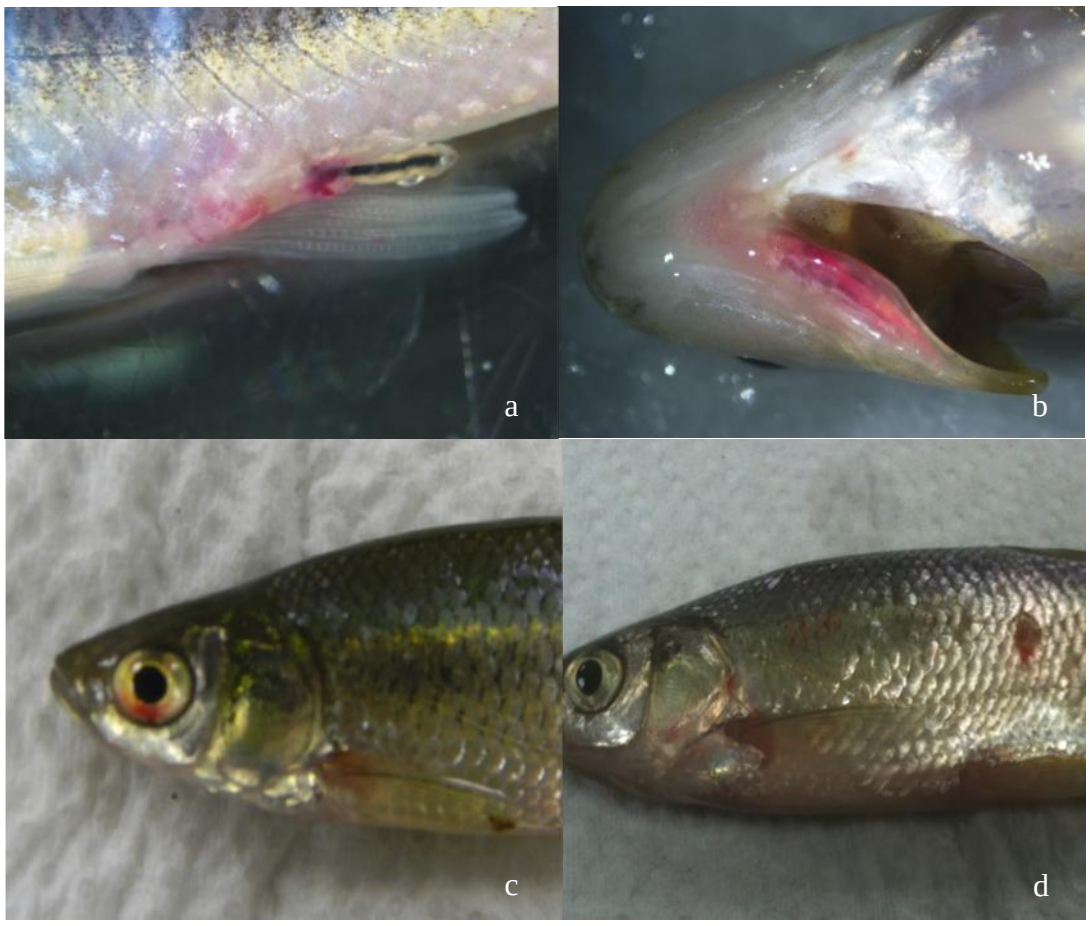

FIGURE 5. Clinical signs observed in fathead minnows and golden shiners from lots testing positive for Golden Shiner Virus (GSV): **(a)** Fathead minnow with hemorrhages at the vent. **(b)** Fathead minnow (ventral side facing up) with hemorrhages on the gill operculum and base of the mouth. **(c)** Golden shiner with hemorrhages in the eye and at the base of the left pectoral fin. **(d)** Golden shiner with a dermal lesion and hemorrhages of the skin, fin bases, and operculum.

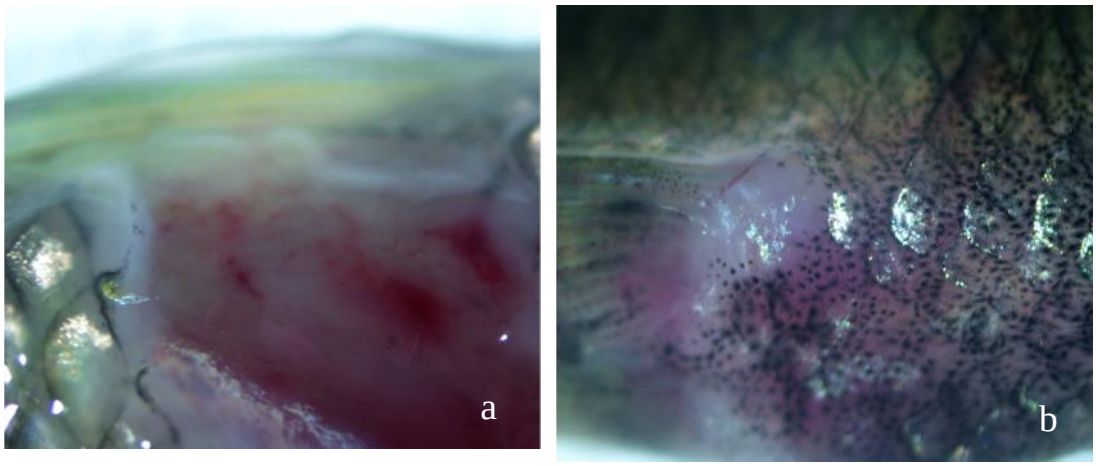

FIGURE 6. Clinical signs observed in fathead minnows from a lot testing positive for Fathead Minnow Nidovirus (FHMNV): **(a)** External lesion on the lateral surface, just below the dorsal fin. **(b)** Hemorrhages at the base of the pectoral fin.

suckers tested positive for an unknown virus but did not display any visible external signs of pathology. (Fig.7).

Characterization of Unknown Viruses

Initial results of TEM revealed that four of the isolates harbored small, round, non-enveloped virus particles measuring approximately 32-33 nm in diameter, consistent with the dimensions and morphology of positive sense, single-strand RNA viruses in the family *Picornaviridae* (A. Armien, University of Minnesota, Veterinary Diagnostic Laboratory, personal communication) (Fig. 8).

A negative stained sample isolated from white suckers revealed enveloped viral particles, ~130 nm in diameter, that were morphologically consistent with negative sense single stranded RNA viruses in the family *Paramyxoviridae* (A. Armien, University of Minnesota, Veterinary Diagnostic Laboratory, personal communication).

After TEM visualization, the samples consistent with a picorna-like virus were confirmed as two distinct novel picornaviruses using random amplification, cloning, and sequencing (N. Phelps, University of Minnesota, Veterinary Diagnostic Laboratory, written communication). These novel viruses are preliminarily being named Fathead Minnow Piconavirus (FHMPV). Following initial phylogenic analysis, the first novel *Picornavirus* (FHMPV-1) was found to have a 28% identity with Bluegill Virus (BLGV), while the second novel *Picornavirus* (FHMPV-2) showed a 52.7% identity with BLGV. These viruses are currently undergoing further sequence analysis to further determine their identity (N. Phelps, University of Minnesota, Veterinary Diagnostic Laboratory, written communication).

Using the FHMPV sequence data from both of these novel viruses, specific primers were designed to amplify the 3D gene of FHMPV. One primer set was designed to amplify a general region of this virus, while a second was designed to amplify a more specific region, thus distinguishing between the two novel isolates. Using these primers, 5 of the 15 lots of unknown viruses tested positive for one of these novel viruses (FHMPV-1), while 3 of the 15 lots tested positive for the second novel picornavirus (FHMPV-2).

Negative stain TEM visualization of the remaining unknown viruses has indicated the presence of another picorna-like virus in two samples. Four samples isolated from white suckers, northern redbelly dace, and a lot of mixed, wild-harvested fish, were negative-stained and visualized using TEM. These samples revealed viral particles measuring 80-120 nm in diameter and were morphologically consistent with viruses in the family *Orthomyxoviridae*. (Table 8).

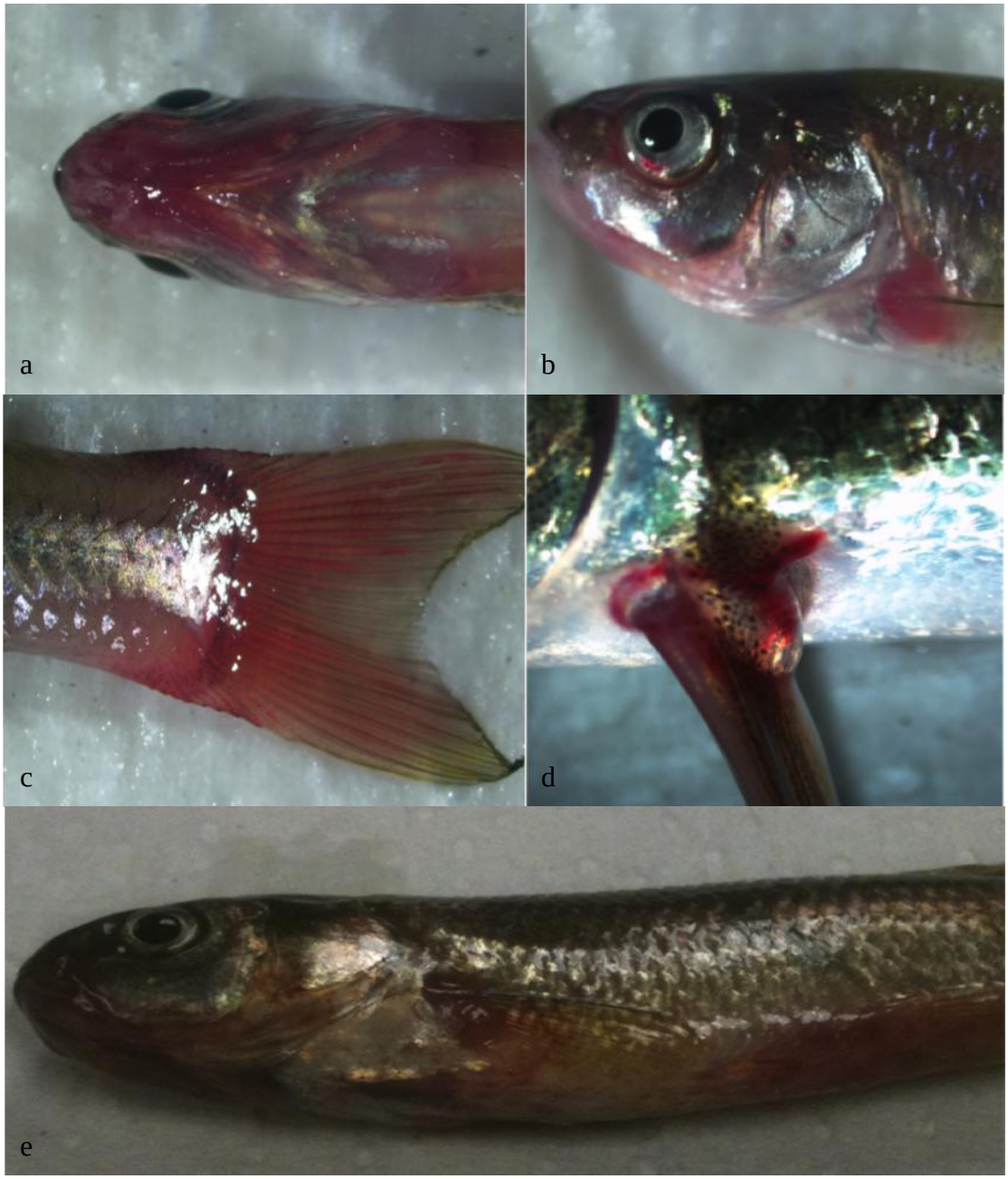

FIGURE 7. Clinical signs observed in fathead minnows and white suckers from lots testing positive for an unknown virus. **(a)** Fathead minnow with severe hemorrhages on the ventral surface. **(b)** Fathead minnow with hemorrhages in the eye and at the base of the pectoral fin. **(c)** Fathead minnow with severe hemorrhages in the caudal fin. **(d)** White sucker with hemorrhages at the base of the left pectoral fin. **(e)** Fathead minnow with hemorrhages along the ventral surface, at the fin bases, and below the mouth.

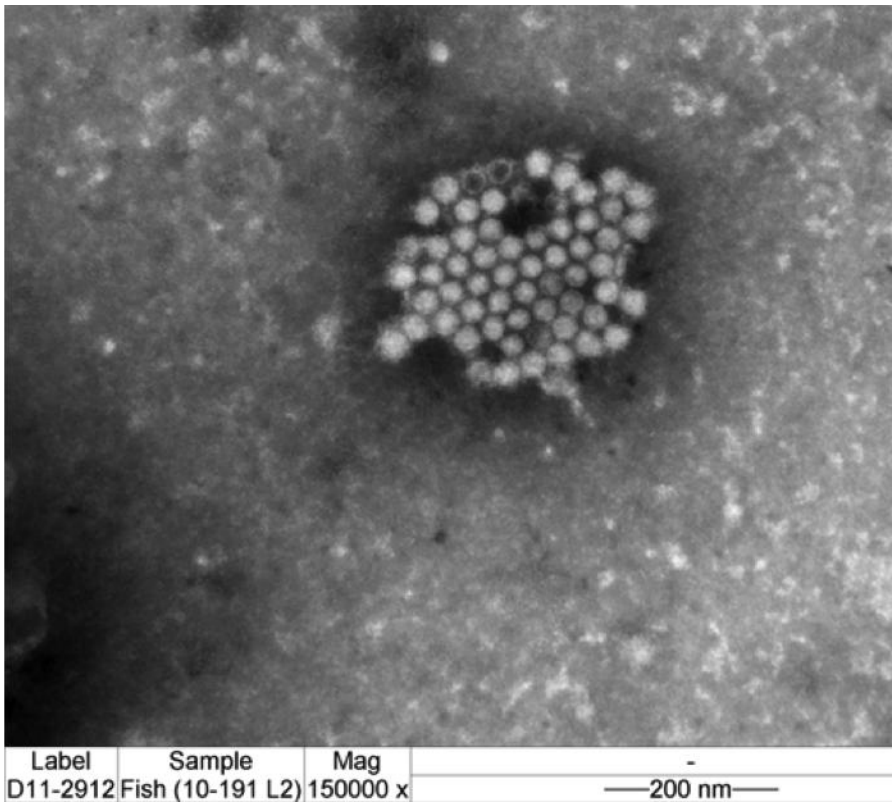

FIGURE 8. Transmission electron micrograph of a picorna-like virus isolated from fathead minnows obtained from a Minnesota baitfish importer. (Photo by A. Armien, University of Minnesota)

Golden Shiner Virus Experimental Infection

The fathead minnows experimentally infected with GSV experienced mortality and clinical pathology during the 30 day observation period. Confirmation of GSV infection was achieved through observation of syncytial CPE on EPC cells and subsequent PCR testing against a GSV positive control. These results indicate a fulfillment of Koch's Postulates for GSV and illustrate a clear relationship between GSV and mortality and disease in fathead minnows.

TABLE 8. Summary of unknown viruses isolated from baitfish obtained from four Wisconsin baitfish dealers (FHM= fathead minnow, WHS= white sucker, NRD= northern redbelly dace, MS=mixed species).

Virus	Size (nm)	Cell Line/ Incubation Temperature	Species
FHMPV-1	30-32 nm	EPC @ 20^0C	FHM
FHMPV-1	30-32 nm	EPC @ 20^0C	FHM
FHMPV-1		EPC @ 20^0C	FHM
FHMPV-1		EPC @ 20^0C	FHM
FHMPV-1		EPC @ 20^0C	FHM
FHMPV-2	30-32 nm	EPC @ 20^0C CHSE @ 15^0C	FHM
FHMPV-2	30-32 nm	EPC @ 20^0C	FHM
FHMPV-2		EPC @ 20^0C	FHM
Picorna-like	30-32 nm	BF-2 @ 25^0C	FHM
Picorna-like	30-32 nm	EPC @ 20^0C	WHS
Paramyxo-like	130 nm	EPC @ 20^0C	WHS
Orthomyxo-like	80-120 nm	EPC @ 20^0C	WHS
Orthomyxo-like	80-120 nm	EPC @ 20^0C	MS
Orthomyxo-like	80-120 nm	EPC @ 20^0C	WHS
Orthomyxo-like	80-120 nm	EPC @ 20^0C	NRD

Mortality

Of the two treatment groups (low and high viral concentration), each consisting of 20 fathead minnows, eight fish (40%) from each treatment group died prior to the end of the thirty day observation period. The first two mortalities, one from each treatment group, occurred on day 20. The remaining mortalities occurred on day 22, day 23, and day 26. Additionally, two minnows from the control group (20%) died on day 26. (Fig.9)

Clinical Signs of Disease

Fish from both treatment groups displayed clinical signs of infection. Fish from the high concentration treatment group that died during the 30-day period showed hemorrhages at the base of the pelvic and pectoral fins and skin darkening. Mortalities from the low concentration group showed hemorrhages at the fin bases and significant hemorrhaging of the ventral surface and mouth. Fish from the high concentration group that survived until the end of the 30 days showed skin darkening and displayed a loss of equilibrium. In addition, one fish from the high viral concentration group was also observed with a one-inch long mucoid fecal cast extending from the vent on day 21, but it is unknown if this fish was among the mortalities or survived until the end of the study. Surviving fish from the low concentration group showed skin darkening and hemorrhages at the pelvic and pectoral fin bases. No internal signs of disease were noted on any of the fish.

A single fish from the control group was observed with hemorrhaging on the ventral surface, mouth, tail, and fin base on day 27. On day 29, however, signs of hemorrhages were no longer apparent. Of the two mortalities from the control group, no

signs of disease were observed.

Tissue Cell Culture

All 20 viral samples from the high treatment group showed signs of CPE in tissue cell culture consistent with GS. A subset sampling of these cell culture supernates were tested via PCR to confirm positive virus isolation. Of the 20 fish from the low treatment group, 16 samples showed CPE consistent with GSV in cell culture. Samples from all four wells not showing CPE (after 28 days), and samples from 16 wells that did display CPE, were also tested using PCR. All samples were positive for GSV. None of the ten sample wells from the negative control group showed signs of CPE. Additionally, samples from the two fish that died prior to the end of the initial study observation period were tested via PCR and were negative for GSV.

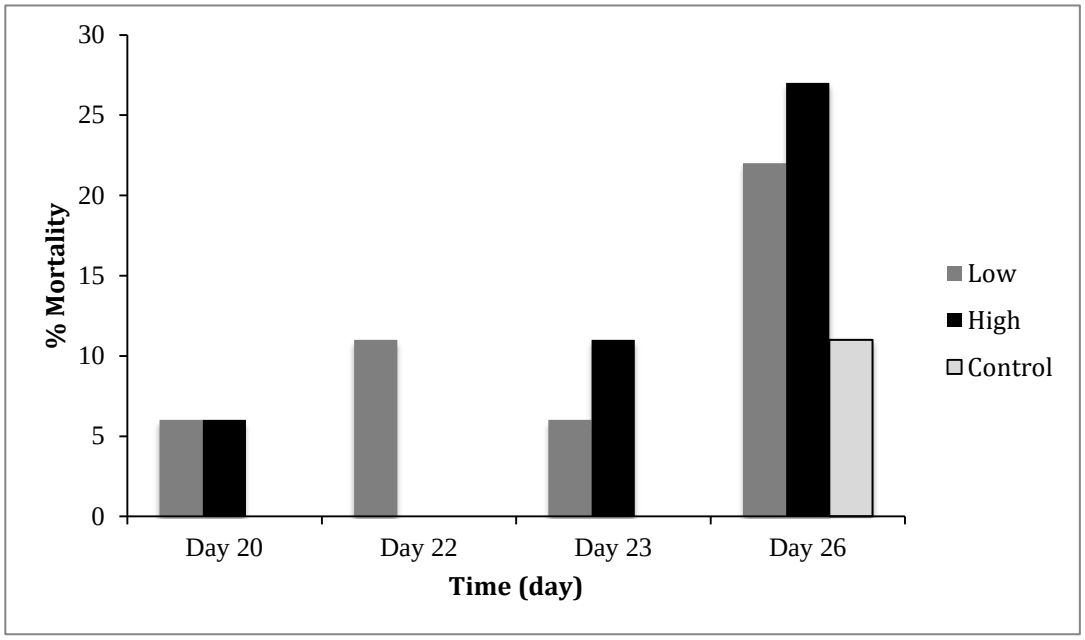

FIGURE 9. Percent (%) mortality during a 30 day observation period for each treatment group (low virus concentration, high virus concentration, and negative control) of fathead minnows experimentally exposed to Golden Shiner Virus.

DISCUSSION

Two primary objectives of this study were to survey baitfish from four Wisconsin baitfish dealers in order to determine the presence of target viruses and to detect any unknown, or non-target, viruses that might be present in these fish. A third important objective was to determine the virulence of Golden Shiner Virus through the fulfillment of Koch's Postulates. In analyzing these data, it was possible to evaluate the significance of certain factors that may have played a role in the presence or absence of viruses in these baitfish.

Fathead minnows were positive for one or more viruses 62% of the time (23 of 37 lots tested), a much higher rate than any of the other species tested. Fathead minnows are the most commonly purchased baitfish in the state of Wisconsin (Meronek et al. 1997b) and rank third in total baitfish sales in the United States (Horne et al. 2010). All of the fathead minnows tested during this study were obtained from aquaculture farms where the fish are intensively pond-reared (Horne et al. 2010). Pond culture methods often entail crowded conditions, variable water quality, and frequent handling and transport. These stressors are key in the expression and transmission of viruses among fish and the emergence of new viral pathogens (Johansen et al. 2011). Furthermore, cultured fathead minnows may be reared in aquatic environments that are different from their natural habitats (Crane and Hyatt 2011). Culture environments are crowded and stressful, which can lead to an increased risk of pathogen exposure, rapid transmission of disease, and frequent

disease outbreaks (Small and Bilodeau 2005). Stressors like frequent handling and transport, crowding, temperature fluctuations, and poor water quality are associated with increased secretions of cortisol, a stress hormone, which has been linked to immunosuppression and a compromised ability to combat infections (Davis et al. 2003, Small and Bilodeau 2005, Crane and Hyatt 2011). These stressors likely play a key role in the high virus prevalence within fathead minnows in this study.

Seasonality influenced the prevalence of baitfish viruses. Virus prevalence was higher in the summer and each of the viruses isolated during this study (GSV, FHMNV, and unknown viruses) have been found to optimally replicate at temperatures above 20°C (Brady and Plumb 1991, Batts et al. 2012, C. Puzach, La Crosse Fish Health Center, U.S. Fish & Wildlife Service, personal communication). Many aquatic viruses, including Spring Viremia of Carp Virus and Largemouth Bass Virus, display patterns of infection linked to seasonality (Ahne et al. 2002, Grant et al. 2003, Grizzle and Brunner 2003, Garver et al. 2007). In the case of LMBV, physiological stressors that become especially pronounced during the summer, such as elevated water temperature, low dissolved oxygen, and increased angling pressure, can all increase the likelihood of mortality in infected fish (Grant et al. 2003). Fish respond to a viral infection with the production of interferons, humoral antibodies, or specific cellular immunity (Ahne et al. 2002). This immune response is suppressed or delayed at various temperature ranges, depending upon the species of fish (Ahne et al. 2002, Grant et al. 2003). In SVCV, for example, clinical disease is prevalent in carp in water temperatures between 5°C and 10°C, but mortality occurs more readily when temperatures exceed 10°C and immune response in fish is impaired. It appears that in

the case of the baitfish sampled in this study, warmer water temperatures imposed a greater amount of physiological stress that may have led to an increase in viral prevalence.

Population dynamics may also play a role in the high viral prevalence during the summer. During the spring spawning season, adult fish are often more highly aggregated than at other times of the year (Lorenzen et al. 1991) and fish congregating at high densities often leads to increased viral transmission within the population. Once fry hatch, they are at an increased risk of viral infection due to a lack of acquired immunity (Bergmann and Fichtner 2008), leading to a high viral prevalence during late spring and early summer when these fish are most vulnerable. Furthermore, any surviving fish will likely clear the virus by fall or winter, thus acquiring some natural immunity and further limiting the spread of disease.

Viruses were isolated at a consistent rate across all three import states (Arkansas, Minnesota, and South Dakota) and among the four baitfish dealers. This is likely due to the frequent movement and transport of baitfish inherent to the baitfish industry. A single lot of fish imported from Minnesota may consist of a combination of fish from a number of states or dealers, along with fish that have been wild-harvested from within the state. Incomplete record keeping makes it difficult or impossible to determine the exact origin of most lots of baitfish and most of the Wisconsin baitfish dealers purchased baitfish from many of the same wholesalers and importers, likely making virus prevalence similar among dealers.

GSV infected each host species at similar rates. Since 1975, an increasing number of aquareo-like viruses have been described in species including fish,

shellfish, and crustaceans (Lupaiani et al. 1995). GSV infected a broad range of host species in this study, which is consistent with other studies (Lupiani et al. 1995, Rangel et al. 1999). It has been isolated by the La Crosse Fish Health Center in a wide range of species including fathead minnows, white suckers, emerald shiners, muskellunge (*Esox masquinongy*), silver carp (*Hypophthalmichthys molitrix*), and smallmouth buffalo (*Ictiobus bubalus*) (B. Lasee, La Crosse Fish Health Center, U.S. Fish & Wildlife Service, personal communication).

The unknown viruses detected in this study infected a range of host species. Two novel viruses, preliminarily identified as picornaviruses, were isolated from fathead minnows and share some genetic similarity to Bluegill Virus, which has been associated with disease and mortality in bluegill (Barbknecht 2009, N. Phelps, University of Minnesota, Veterinary Diagnostic Laboratory, written communication). Another picorna-like virus was isolated from both fathead minnows and white suckers. Other picorna-like viruses have been detected in a number of fish species including salmonids, sandbar shiners (*Notropis scepticus*), Australian sea bass (*Lates calcarifer*), Japanese parrotfish (*Calotomus japonicas*), red-spotted grouper (*Epinephelus akaara*), striped jack (*Caranx vinctus*), and rainbow smelt (*Osmerus mordax*) (Chittick et al. 2001). Previously uncharacterized picorna-like viruses have been implicated in disease and mortality within salmonids (Chittick et al. 2001). Understanding the patterns of disease caused by other picornaviruses may help illustrate the potential for these newly characterized baitfish picornaviruses to cause disease and mortality.

A paramyxo-like virus from white suckers and at least one orthomyxo-like virus from white suckers, northern redbelly dace, and a mixed lot of wild-harvested fish was detected in this study. Paramyxoviruses have been reported in carp, Chinook salmon, and Atlantic salmon and have been associated with gill disease in Atlantic salmon (Kvellestad 2003). Orthomyxoviruses, including Infectious Salmon Anemia Virus (ISA), have been associated with disease and mortality in Atlantic salmon and has been implicated in severe economic losses in salmon farming regions worldwide (Ritchie et al. 2009). Because of their wide host range, the impact of these unknown viruses could be catastrophic. It is unknown how many species these viruses could infect and how virulent they may be.

FHMNV was detected in fathead minnows and, reported for the first time in wild-harvested creek chubs. Because both species are in the Family Cyprinidae, it is not unreasonable to suspect that other species within this family may also be susceptible. More research needs to be done to determine the overall pathogenicity of this virus and to assess its natural range.

The prevalence of virus in cultured fish and wild-harvested fish was not statistically different. This may be due to the fact that baitfish from many different sources (both wild and cultured) are frequently combined and redistributed to many different locations. Dealers will often supplement their imported supplies of baitfish with wild-harvested fish and baitfish, in turn, are often released into the natural aquatic environment by anglers, thereby further increasing the risk of transmission of a virus between cultured and wild fish (Ludwig and Leitch 1996). It is impossible to know if these viruses evolved within cultured fish populations and then were

transmitted to wild fish populations, or if viruses in wild fish populations were introduced to farmed fish. Wild fish or eggs that are brought in to supplement cultured populations may have introduced viruses. What is certain is that the nature of baitfish sales, importations, and uses directly contribute to the increased transmission of viruses.

Golden Shiner Virus Experimental Infection

The objective of the experimental infection was to fulfill Koch's Postulates for GSV. Previously, Goodwin and Nayak (2006) reported that GSV had been isolated from moribund fathead minnows displaying clinical signs consistent with the virus, but that an association between the virus and disease could not be proven because GSV had also been isolated in other samples of apparently healthy fish. This study showed a clear association between mortality, clinical disease, and GSV and is an important consideration when making management decisions regarding the importation of infected fish across state lines.

Implications

The baitfish industry in the United States is vast, both in terms of the number of fish bought, sold, and transported, and the income generated. Millions of anglers use live bait for fishing and, consequently, the introduction of baitfish into lakes, rivers, and streams occurs readily. Although numbers are difficult to obtain, a study of interbasin bait transfers by Ludwig and Leitch (1996) suggests that the probability of a single angler on the Hudson Bay basin releasing live bait from the Mississippi River basin on a single day is 1.2/100. The fact that 44% of the lots of baitfish sampled during this study were positive for at least one virus suggests that the

likelihood for viral transmission to wild populations of fish is imminent and the consequences could be disastrous.

Because of the unpredictable nature of emerging viruses, the first line of defense must be effective surveillance (Woolhouse et al. 2005). Emerging pathogens often evolve when a virus is introduced to a novel host where no prior immunity exists (Wollhouse et al. 2005). This has been apparent with emerging viruses like VHSV, IPNV, and SVCV which have been implicated in widespread morbidity and mortality among cultured fish such as salmon and carp (Ortega et al. 1994). New genotypes of VHSV have evolved in different regions of the world (Meyers and Winton 1995). An emerging virus in common carp (*Cyprinus carpio*), Cyprinus herpesvirus-3 (CyHV-3), was first detected in 1998 and has been associated with major disease outbreaks and a mortality rate of 80-100% on carp farms along the Israeli coast (Ilouze et al. 2011). Since 1998, CyHV-3 has spread to Indonesia, Japan, Taiwan, and Thailand and has caused mass mortalities in carp and other closely related fish species in these countries (Ilouze et al. 2011). Factors such as wide host susceptibility and stability in the water column have provided CyHV-3 an evolutionary advantage that has allowed for the rapid dissemination. CyHV-3 could serve as an epidemiological model for other emerging fish viruses (Ilouze et al. 2011).

It has been established that a number of the aquatic viruses, such as VHSV and LMBV can be transmitted to fish via oral ingestion of other infected fish, but the efficacy of oral transmission of other aquatic viruses has yet to be determined. If predator fish can carry or become infected by eating infected baitfish, there may be

potential for other aquatic predators, such as birds and mammals to serve as potential vectors or viral reservoirs.

Regulatory Implications

The results of this survey should be used to assist in shaping future regulatory policy in regards to the sale and transport of baitfish both in Wisconsin and the United States in order to protect wild populations of fish from viral infections, disease outbreaks, and potential devastating losses. In order to limit the spread of disease to wild species of fish, it is critically important to make informed regulatory decisions regarding the movement of baitfish between states. Currently, GSV and FHMNV are not considered reportable pathogens by either the U.S. Fish & Wildlife Service or the World Organization for Animal Health (OIE), yet both viruses have been associated with clinical pathology, epizootics, and mortality. The Wisconsin Department of Agriculture, Trade, and Consumer Protection (DATCP), which is the agency responsible for the regulation of baitfish across state lines, currently requires that baitfish imported from aquaculture facilities and baitfish farms only need to be free of "visible signs of contagious or infectious disease" (unless they are known VHSV susceptible species and have been harvested from VHSV-positive waters). These importing facilities are only required to have fish visually inspected annually. While some of the virus positive fish from this study showed clinical signs of disease, others did not. Some fish showed obvious clinical signs of disease, but arrived with health certificates showing they passed a visual inspection. Other fish tested positive by tissue cell culture and did not have any clinical signs of disease. It is not possible to rule out viral infection based solely on visual inspection. The current regulations are

completely inadequate for identifying and stopping the spread of viral disease among these imported fish. Subsequently, many infected fish may have been introduced into rivers, lakes, streams, and ponds.

Enacting regulations in Wisconsin that are akin to regulations in Minnesota may help prevent to spread of viral pathogens into the state. In Minnesota, importing live fish for bait is illegal (Minnesota Department of Natural Resources 2011). This law was created in order to prevent the spread of aquatic disease. At the very least, more stringent laboratory testing should be required of all live fish entering the state. Additionally, the list of reportable pathogens should include Golden Shiner Virus and Fathead Minnow Nidovirus, as these pathogens have clearly been associated with significant disease and mortality in fish.

Future Research

Large-scale viral surveys of baitfish have not been previously performed because of the expense and labor-intensive work needed to complete such a study, as well as the difficulty in gaining access to such a large sample of fish directly from the baitfish dealer. Because all viruses isolated on CHSE cells were also detected on EPC cells at the same temperature, eliminating the CHSE cell line would assist in reducing the overall cost.

While it's been established that several viruses can infect multiple baitfish species, it is not known if these viruses can infect and cause disease in predator fish such as northern pike, walleye, and muskellunge. Experimental infection of predator fish to address this question would clarify the risk of these viruses to predator

populations and would provide knowledge that would aid in the better management of baitfish transport.

Additionally, because of the small sample size of wild-harvested fish in this study, a large scale viral survey of wild populations of baitfish would assist in cataloging which viruses are currently present within these wild populations. This would create baseline data with which to compare trends that may be apparent with future surveys and would allow for the evaluation of the natural ranges and species susceptibility of the unknown viruses detected in this study. It has already been demonstrated among other aquatic viruses, such as VHSV, that movement of these viruses from endemic areas to new watersheds can lead to the evolution of new, much more virulent strains that can infect a wide range of species. By understanding the natural ranges of the viruses isolated in this study, we can better manage or prevent the further spread of disease to wild populations. Finally, aquatic invertebrates serve as an important prey item for many fish species and assessing their ability to act as vectors of viral disease may help to further our understanding of the epidemiology of these aquatic viral diseases.

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