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PREDATOR-PREY: INTERACTIONS BETWEEN THE SPINY WATERFLEA (*BYTHOTREPHES LONGIMANUS*) AND PUMPKINSEED SUNFISH (*LEPOMIS GIBBOSUS*)

By

Jaime F. LeDuc

A THESIS

Submitted in partial fulfillment of the requirements for the degree of

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This thesis has been approved in partial fulfillment of the requirements for the Degree of MASTER OF SCIENCE in Biological Sciences.

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DEDICATION

To Rainy

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ABSTRACT

Small pumpkinseed sunfish (*Lepomis gibbosus*), were found to be capable of removing the spine of *Bythotrephes longimanus,* an invasive cladoceran. Because fish consumption may be important in the dispersal or control of *Bythotrephes*, aquarium feeding experiments were conducted to 1) establish if the spine removal behavior of the pumpkinseeds was locally unique; 2) quantify how frequently pumpkinseeds exhibit the behavior; 3) determine if pumpkinseed handle *Bythotrephes* more quickly than other species of fish; and 4) verify if *Bythotrephes'* resting eggs pass through the digestive systems of pumpkinseeds in viable condition. The experiments revealed that pumpkinseeds (45-70 mm TL) from two geographic regions were more successful (100%) at removing *Bythotrephes'* spine, and handled *Bythotrephes* more quickly than yellow perch (*Perca flavescens*) (49-57 mm TL) and smallmouth bass (*Micropterus dolomieu*) (50-57mm TL) used in the study. Of 244 live *Bythotrephes'* resting eggs fed to the pumpkinseeds, 104 (42.6%) passed through their digestive systems. From those eggs, only 10 successfully hatched. Preliminary enclosure experiments were carried out and indicated that pumpkinseeds will consume *Bythotrephes* in natural settings. These findings provide new evidence that certain fish, with specialized morphology for prey manipulation, have the ability to influence the distribution and establishment of *Bythotrephes*.

INTRODUCTION

The spiny waterflea (*Bythotrephes longimanus*), a predacious aquatic crustacean, was first discovered in Lake Ontario in 1982 and was established in all of the Great Lakes by 1987(Lehman 1987). It is commonly agreed that the Eurasian native traveled to the Great Lakes in ship ballast water (Berg *et al.* 2002), and rapidly colonized inland lakes aided by transportation in contaminated bait-buckets, on boat and trailer surfaces, in live wells, on nets, and on various other fishing and boating gear. *Bythotrephes* has spread along a temperature- defined latitudinal band ranging from New England and Quebec to Minnesota and Manitoba (Branstrator *et al.* 2006, Kerfoot *et al.* 2011), and is now among the most notable species in zooplankton communities within northern Minnesota, Michigan, and Ontario. Considering the consensus that humans are largely responsible for the spread of *Bythotrephes* (MacIsaac *et al.* 2004, Muirhead and MacIsaac 2005, Weisz and Yan 2010*,* Yan *et al.* 2011), its distribution will likely grow because control measures have not yet been fully implemented across its range.

Zooplankton are a food source that sustains juveniles and small individuals of many fish species. As a predatory cladoceran and competitor with fish, *Bythotrephes* can drastically affect native zooplankton communities. Post-invasion effects include a decline in species richness (Yan *et al.* 2002, Strecker *et al.* 2006, Barbiero and Tuchman 2004), alterations in zooplankton body sizes and structures (Bungartz and Branstrator 2003, Hobmeier *et al.* 2011 oral presentation), and a reduction in total zooplankton abundance (Lehman and Cáceres 1993, Boudreau and Yan 2003). There is also a concern that *Bythotrephes* can reduce energy transfer to higher trophic levels. *Bythotrephes* invasions, in most occurrences, result in a decline in the abundance of herbivorous cladocerans and a shift of zooplankton communities to assemblages dominated mainly by copepods and other larger zooplankton (Strecker *et al.* 2006, Hobmeier *et al.* 2011). The change induced by *Bythotrephes* can reflect an elevation in the trophic position of zooplankton and fish species, and may lead to substantial increases in contaminant concentrations of top predators (Rennie *et al.* 2010).

Bythotrephes has various traits to increase survival and minimize pressure from fish predation. Reproducing sexually, they can generate thick-shelled diapausing eggs that are highly resistant to desiccation, chlorine and salt water and can withstand 100°F water for up to 10 minutes (Personal communication with Donn Branstrator-University of MN-Duluth). Under experimental conditions, *Bythotrephes* resting eggs were successfully cultured after passing through the gut of yellow perch (*Perca flavescens*), bluegill (*Lepomis macrochirus*), and smallmouth bass (*Micropterus dolomieu*) (Jarnagin *et al.* 2000). Dispersal by recreational fishing has been linked to use of baitfish because diapausing eggs can be excreted into live wells or bait buckets and transferred to different fishing destinations (Kerfoot *et al.* 2011).

Perhaps *Bythotrephes* most prominent adaptation is its spiny appendage, a spike that extends caudally with pairs of lateral barbs. Though the spine constitutes up to 80% of total body length, it provides protection against predation by small fish, which could explain why long spines are present at birth (Compton and Kerfoot 2004). A variety of fish have difficulty ingesting *Bythotrephes,* exhibiting behaviors like repeated rejection and recapture, aggressive flaring of buccal and opercular cavities, whole-body convulsions, and learned aversion (Barnhisel 1991a, Barnhisel 1991b, Barnhisel and Harvey 1995, Barnhisel and Kerfoot 2004, Compton and Kerfoot 2004). Small fishes that fail to learn to avoid *Bythotrephes* can suffer structural damage from spines perforating their stomach wall and lower intestines (Compton and Kerfoot 2004).

Gut analysis has provided some evidence that certain species of large fish will prey heavily on *Bythotrephes*. Fish that are efficient at feeding on *Bythotrephes* likely possess a mouth and gillraker region adapted to small or hard bodied prey. Gill raker number, length, and separation are often positively correlated with planktivory in fishes (Macneill and Brandt1990). In North America, *Bythotrephes* has been found in large numbers in the stomachs of fishes such as alewife (*Alsoa Pseudoharengus*) and *Coregonus* species that possess these traits (Keilty 1990, Branstrator and Lehman 1996, Coulas et al. 1998). A number of analyses report boluses of tangled caudal spines in intestines (Coulas *et al.* 1998, Keilty 1990, Parker *et al.* 2001) that can impede food passage. *Bythotrephes* spines are largely indigestible, and could reduce growth by occupying space in fish stomachs without providing nutritional value (Stetter *et al.* 2005).

Fishes that feed on hard-bodied organisms or organisms with primary external defenses can spend considerable time and energy in handling individual prey, therefore the amount of time required to handle an item is critically important to the energy returned to the predator (Helfman *et* al. 2009). Barnhisel (1991a) calculated an 800% increase in the handling times of small rainbow trout (*Onchorhynchus mykiss*) fed fully spined *Bythotrephes* versus *Bythotrephes* with the spine removed. After repeated exposures, *Bythotrephes* was recognized and ignored by the fish. Taylor Jarnagin et al. (2004) concluded that because of aversion behavior to the defensive spine, it is rare for small fish to utilize *Bythotrephes* as a food source. Kerfoot et al. (2011) also documented a narrow size "window" for yellow perch to consume *Bythotrephes*. The small fish failed to consume *Bythotrephes,* whereas larger year-1 fish switched to larger, benthic prey species. There is considerable concern that spine-induced predator aversion will enable *Bythotrephes* to effectively compete with juvenile and small fish for food and decrease *Bythotrephes* predation risk (Barnhisel and Kerfoot 2004, Compton and Kerfoot 2004, Kerfoot et al. 2011).

This research examines a species of small fish that appears capable of overcoming the spine adaptation, *Bythotrephes'* main defense against other small planktivorous fish. In laboratory feeding experiments, pumpkinseed sunfish (*Lepomis gibbosus)* were observed to engulf an entire *Bythotrephes*, manipulate it to separate the spine from the body, then spit out the spine and ingest the body.

The pumpkinseed is a freshwater fish of the sunfish family (Centrarchidae) and is native to and most common in the Great Lakes Drainage and throughout the northeastern quarter of the United States (Huckins 1997). As adults, pumpkinseeds can consume large quantities of snails, a prey type that is ignored by many other species of fish (Mittelbach 1984). Aside from the sister species redear (*Lepomis microlophus*), more common in lower latitudes, pumpkinseed are the only known molluscivorous members of the endemic North American freshwater fish family Centrachidae (Wainwright et al. 1991).

PURPOSE

The objectives here are to 1) establish whether or not the spine removal behavior of pumpkinseeds is locally unique or general; 2) quantify how frequently pumpkinseeds exhibit the spine removal behavior; 3) determine if pumpkinseeds are faster than are other species of fish at ingesting *Bythotrephes*; and 4) verify if *Bythotrephes'* resting eggs pass through the guts of pumpkinseeds in viable condition. The results of this effort will provide progress toward the ultimate goal of determining how widespread the spine removal behavior is and to what degree particular fish, like pumpkinseeds, can limit the distribution of *Bythotrephes*. The hypothesis is that fish predation constraints complement the geographic temperature limitation observed by Kerfoot et al. (2011).

METHODS

Aquarium Experiments

The first fish observed exhibiting the spine removal behavior during this project were pumpkinseeds (<70 mm total length) seined from a small pond connected to the Keweenaw Waterway near Michigan Technological University. The objectives had been to replicate an aquarium experiment using methods outlined by Compton and Kerfoot (2004), examining the effects of the *Bythotrephes* spine length on fish handling time of *Lepomis*. Whereas Compton and Kerfoot (2004) had used bluegill (*Lepomis* *macrochirus*), the plan was to feed pumpkinseeds (*Lepomis gibbosus*) *Bythotrephes* with spines of various lengths (full spine, half spine and spineless) and to record the handling time. Within the first presentations of the preliminary feeding trial, a striking and important interaction was observed between pumpkinseeds and *Bythotrephes* that had not been documented in previous fish feeding experiments. The pumpkinseed could engulf an entire *Bythotrephes*, manipulate it to separate the spine from the body, then spit out the spine and ingest the body. Due to the specialized nature of pumpkinseed morphology and behavior, this is not unexpected in light of the snail manipulation abilities this species exhibited in a previous study (Huckins 1997, personal communication with Casey Huckins).

Additional pumpkinseeds (<70mm total length) were captured via beach seining from Portage Lake near the mouth of the Pilgrim River in September 2010. They were transported to a lab in a portable cooler, where they were cultured in aquaria on a diet of commercial flake food for approximately one month before feeding experiments began, on September 13, 2010. The Keweenaw Waterway cuts across the Keweenaw Peninsula and connects to Lake Superior at North and South Entries, with Portage Lake in-between. Portage Lake acts as a sink to *Bythotrephes*, as storm surges move water from Lake Superior through the North and South Entry and periodically reintroduce *Bythotrephes* into Portage Lake (Compton and Kerfoot 2004). Therefore it was possible, that pumpkinseeds in Portage Lake could encounter *Bythotrephes*, although incidence was infrequent at best.

One day prior to feeding experiments, eight pumpkinseeds (45-68 mm total length) were isolated individually into 2.5-gallon (9.5-liter) partitioned sections of aquaria and starved for 24 hours. *Bythotrephes* were collected from the Keweenaw Waterway at North Entry by towing a 0.5-m plankton net (350 µm mesh) along the edge of the Lily Pond retaining wall. Twenty four hours earlier, a storm surge by heavy Northwestern winds had pushed large numbers of *Bythotrephes* in with Lake Superior water. *Bythotrephes* were brought back to the lab in a 48-quart insulated transportable cooler. Because *Bythotrephes* is known to exhibit poor survival in lab settings, we frequently replenished the supply using periodic sampling.

Multiple adult *Bythotrephes* were offered to individual pumpkinseed using a forceps to insert the prey individually into the aquaria water. Feeding behavior at each presentation was noted; in particular recording whether or not the fish ingested the *Bythotrephes* whole or removed the spine. Several encounters were captured on video. Although the main focus had shifted to documenting and enumerating the spine removal behavior, the original experiment analyzing the effects of the *Bythotrephes* spine length on fish handling time spine length was also finished. Following the methods by Compton and Kerfoot (2004), *Bythotrephes'* spines were cut or removed by clipping the appendage to the desired length. One third of the *Bythotrephes* presented to fish in aquaria had a full spine, one third had a half spine, and the final one third were spineless. Using a stopwatch, handling times were measured as the period of burst buccal and opercular activity. Satiation was tested by feeding the fish their normal flake food, and observing if they ate or rejected the food.

In order to determine if the spine removal behavior exhibited by the 2010 Portage Lake pumpkinseeds was unique geographically, additional pumpkinseeds were captured from Pelican Lake, Minnesota, July 2011, using a 1.2 x 6.1 m (6.35 mm mesh) bag-less seine. *Bythotrephes* had not been documented in Pelican Lake, MN (communication with Minnesota Department of Natural Resources (MNDNR) fisheries personnel). The pumpkinseeds were transported back to the lab in an insulated 48-quart cooler, where they were cultured in glass 10-gallon aquaria on a diet of commercial flake food for a two-week acclimation period. One day prior to feeding experiments, seven pumpkinseeds (48-65 mm total length) were isolated individually into 2.5-gallon (9.5-liter) partitioned sections of aquaria and starved for 24 hours. Vertical and horizontal zooplankton tows using a 30 cm, 153 µm mesh plankton net were taken from open water on Rainy Lake, MN, off a 21 foot Crestliner and transported back to the lab in insolated 48-quart coolers. The *Bythotrephes* stock was frequently replenished using periodical sampling. In the lab, multiple adult *Bythotrephes* were offered to individual pumpkinseeds using a forceps to insert live *Bythotrephes* individually into the aquaria water. The feeding behavior of each presentation was described, including whether or not fish ingested the *Bythotrephes* whole or removed the spine. Again, handling times were measured as the period of burst buccal and opercular activity using a stopwatch.

Additional feeding studies were conducted using the same methods to test yellow perch (*Perca flavescens*) and smallmouth bass (*Micropterus dolomieu*) feeding behavior, which served as comparisons with pumpkinseed. Additional published bluegill (*Lepomis* *macrochirus*) handling time data for *Bythotrephes*, from Compton and Kerfoot (2004), were also obtained. New experiments with live bluegill did not take place. Morphologically generalized fishes, such as bass, bluegill and perch were chosen for comparison because snails are not common elements in their diet, unlike the specialized food choice of pumpkinseeds (Lauder 1983). Yellow perch, in particular, were chosen because they had been used in previous *Bythotrephes* aquarium feeding experiments (Barnhisel 1991b, Jarnagin 2000) and because *Bythotrephes* consumption by yellow perch had been documented locally (personal communication with Minnesota Department of Natural Resources fisheries personnel; Kerfoot et al 2011).

Yellow perch were beach-seined from Rainy Lake on August 11, 2011, using a 2.4 x 30.4 m (6.35 mm mesh) bag-less seine. The fish were transported back to the lab in an insulated 10 gallon circular water cooler and cultured in aquaria on a diet of commercial flake food for two weeks before lab experiments began. Small mouth bass were seined from Portage Lake on September 22, 2011, using a 1.8x 7.6 m (6.35mm mesh) bag-less seine. Those fish were transported back to the lab in an insulated 10 gallon circular water cooler and cultured in 10 gallon class aquaria for one week prior to feeding experiments.

During feeding experiments, multiple adult *Bythotrephes* were offered to individual yellow perch (N=7, 46-57mm total length) and smallmouth bass (N=7, 7-53 mm total length) using forceps to insert the prey individually into the aquaria water. Feeding behaviors were described. Whether or not the fish ingested *Bythotrephes* whole or removed the spine was noted. Handling times were measured as the period of buccal and opercular activity using a stopwatch.

Statistical Analysis of Aquarium feeding experiments

Spine removal success of individual fish was defined as the proportion of instances that the spine was removed, and was calculated as the number of instances each fish removed the spine divided by the number of observations of each fish being offered a spiny waterflea. Average prey handling times of each fish were calculated as the sum of the handling times of individual fish divided by the number of observations from each fish. Using R statistical software, a non-parametric Wilcoxon Rank Sum test was performed to compare the spine removal success between the two groups of pumpkinseed and also to compare the spine removal success by pumpkinseed for full-spined *Bythotrephes* and half-spined *Bythotrephes*. A Kruskal-Wallis Rank Sum test was used to compare the spine removal success between the three species of fish, and a one-way ANOVA with a Tukey's Post Hoc test was used to compare the average handling times between the three species of fish. ANOVA with a Tukey's Post Hoc test was also used to compare the individual handling times of pumpkinseed offered *Bythotrephes* with various spine lengths.

Resting eggs experiments

A resting eggs gut passage experiment was carried out in February of 2011 when *Bythotrephes* adults were not active in the water column. Females typically produce resting eggs in fall for overwintering. The mother dies and the eggs lie dormant on the bottom until temperatures rise to about 4 to 8 degrees Celsius. In the spring the eggs hatch into parthenogenetic females (Yurista 1992). Resting eggs were sorted from a cooler that contained *Bythotrephes.* The zooplankton sample was taken along the edge of the Lily Pond retaining wall at North Entry, September 21, 2011. The females dropped their eggs and then died, allowing the eggs to be easily suctioned off the bottom of the cooler with a turkey baster. According to Jarnagin et al. (2000), visual maturity classification of eggs is effective in predicting the ability of the egg to initiate development and hatch successfully. Mature eggs, which posses a distinct thick outer shell and are a golden color with dispersed droplets, survive best through gut passage. Only mature eggs were chosen for this experiment and were stored in a dark container at 4 °C in a walk-in storage cold room near the lab.

Eggs were placed inside wax worms fed to pumpkinseeds. Wax worms (*Galleria mellonella* and *Achroia grisella*) are commonly used by anglers to catch small fish through the ice and were purchased at a local bait shop. A razor was used to cut a slit in the outside exoskeleton of multiple wax worms. Between 6-12 resting eggs were placed in each of the individual wax worms through the slit and the number of worms inserted onto each worm was recorded. The slit was then glued shut using commercial super glue to keep the eggs from falling out during the feeding process.

Pumpkinseeds (N=6, 60-87mm total length) were housed in individual aquaria lined with a two-gallon clear plastic bag. The bag was used to decrease the likelihood of missing eggs that survived gut passage being lost in the aquarium corners. The fish were starved for 24 hours and then fed wax worms containing resting eggs. All wax worms were fed to pumpkinseed within 20 minutes of loading with eggs. After 72 hours, fish were removed from individual aquaria and the contents of each plastic bag were poured through a 75 µm sieve. Debris was examined under an Olympus SZ30 microscope fitted with an Olympus GSWH10X/22 ocular micrometer and resting eggs were tabulated.

A second gut passage experiment with resting eggs was conducted in early October 2011, when *Bythotrephes* was still active in the water column. Pumpkinseed had been beach-seined from Portage Lake near the shore at Michigan Tech using a 1.8 x 7.6 m (6.35mm mesh) bag-less seine and housed in aquaria for two weeks. *Bythotrephes* were collected with a 0.5-m plankton net (350 µm mesh), while walking along the edge of a retaining wall at the Lily Pond boat access site, then brought back to the lab. Fish were starved for 24 hours in plastic lined individual aquaria, and then fed spiny waterfleas with resting eggs. Again the contents of each plastic bag were poured through a 75 µm sieve and examined under a microscope for the presence of resting eggs.

The eggs that survived passage through the gut in the second experiment were transferred into a dark 200 ml bottle using a pipette and stored in a walk-in storage cold room until August 15, 2012, to test for viability. Egg hatching methods are outlined in detail by Jarnagin et al. (2000). The eggs were poured into a glass beaker and stored at room temperature under a fluorescent light that was on for 18 hrs during the day and off for 6 hr at night. This schedule continued for one week, before contents of the beaker were examined under a microscope.

Enclosure experiments

A seining program established in 1983 by MNDNR is carried out on Rainy Lake and Kabetogama Lake, MN, during four consecutive weeks every year in July. The main purpose of the MNDNR's seining program is to document what species of non-game fish are present and in what abundances, as well as abundance and size of game fish species. Twenty established seining sites on Rainy Lake and 15 on Kabetogama Lake are sampled every week as conditions allow. In 2011, Rainy Lake was sampled only three times, for a total of 36 hauls, due to a state government shutdown. Kabetogama was sampled three times for a total of 34 hauls. A 100ft long bag seine with ¼ inch mesh that was 8 feet tall was used on Kabetogama Lake and a 6 ft tall by 100ft long seine with ¼ inch mesh (no bag) was used on Rainy. In cooperation with the MNDNR's seining effort in 2011, an area where pumpkinseed and *Bythotrephes* were both locally present was sought. This would have helped in answering the question of whether or not pumpkinseed select *Bythotrephes* as a food source in natural settings; however, only one pumpkinseed was found throughout the entire sampling.

To determine if pumpkinseed prey on *Bythotrephes* in natural settings, enclosures were constructed to put pumpkinseed and *Bythotrephes* in the same place. An enclosure containing pumpkinseed and a known variety of zooplankton could theoretically determine if pumpkinseed prey on *Bythotrephes* when other prey items are available.

The main body of the enclosures were four six gallon Better Bottle© clear plastic carboys. Approximately two feet of white parachute chord tied to an anchor bag filled with rocks was attached around the brim of each carboy. A small piece of 250 µm mesh was placed over the bottle opening and cinched down with several rubber bands. Water was then suctioned in by holding the carboys underwater and repeatedly squeezing the sides. This was done to ensure that there was no zooplankton in the enclosure water prior to experiments.

Because pumpkinseeds could not be found locally in lakes that contained *Bythotrephes,* and because a permit could not be attained to transfer fish between lakes, a preliminary test experiment took place in Rainy Lake August 5, 2011, with resident yellow perch (49-53mm TL) and *Bythotrephes*. Five vertical openwater zooplankton tows were taken consecutively off a 21-foot inboard-outboard fishing boat and poured into individual one liter plastic bottles. One tow was preserved with 10:1 formalin sucrose solution as a reference to clarify the contents of the other tows. The contents of the remaining four tows were introduced separately into carboys. One perch was then added into each of three carboys. The remaining fishless carboy served as a control. The openings of the carboys were again covered with a small piece of 250 µm mesh cinched down with rubber bands. It was hypothesized that covering the opening of carboys with mesh would still allow some lake water to move in and out along with scents and pheromones that could be detected, making a more natural setting for the fish. The carboys were lowered into the water and anchored near a rock-filled crib dock.

After 24 hours, the enclosures were inspected with snorkeling gear. Large predatory smallmouth bass were seen staring at and lightly ramming into the clear plastic carboys.

One fish within the enclosure had died. After 48 hours, the contents of each carboy were poured into a 153 µm mesh plankton net and emptied into a 1 liter plastic bottle. To insure that contents were collected, the inside of the net was washed with tap water three times after each carboy was emptied, and again the remaining contents were emptied into the same plastic bottle. The fish were removed from the sample and preserved with 95% EtOH. The other contents were preserved in 10:1 formalin sucrose solution.

Enclosure experiments were also conducted in the fall, on September 23, 2011 and October 19, 2011, in Portage Lake. Pumpkinseeds where seined from the shore near Michigan Tech and transferred to lab aquariums for 72 hours- the holding time allowed for gut passage of pumpkinseed prey items. Carboys were filled with water on site, through 250 µm mesh netting and anchored in weeds. The carboys were placed in weeds so that the fish would be more hidden, which would reduce predator behavior and thus stress on the pumpkinseed. Pumpkinseeds were transferred to the enclosures 24 hours before zooplankton were added, to allow for acclimation. Five 90 m horizontal tows were taken from the Lily Pond boat access site and immediately transferred to the carboys in individual plastic 1 liter bottles. After the contents of the plankton tows were added, the carboys were anchored back in the weeds for 48 hours. The contents of the carboys were then emptied into plastic 1 liter bottles and preserved in 10:1 formalin sucrose solution. Pumpkinseed were preserved in 95% EtOH

Gastrointestinal tracts of fish were cut open using a razor and contents were identified and quantified with an Olympus SZ30 microscope fitted with an Olympus GSWH10X/22 ocular micrometer. Stomach contents were identified to the lowest taxonomic level practical and the number of *Bythotrephes* spines, barbs, eggs, and body parts were recorded to estimate the number of individual *Bythotrephes*. Contents of the reference bottle in the second experiment were identified to the lowest taxonomic level. The contents of the reference bottles in the first and third experiments were not analyzed.

Cisco/whitefish stomach analysis

To complement existing literature of fish predation on *Bythotrephes*, lake whitefish (*Coregonus clupeaformis*) and cisco (*Coregonus artedi*) were sampled from Rainy Lake in cooperation with the MNDNR. The MNDNR designed a monitoring program to document the population characteristics of lake whitefish in Rainy Lake. The program involves using the Fish Community Index Netting (FCIN) protocol developed by the Province of Ontario (LNFAU 2001) to collect baseline population characteristics.

On August 18th through August 25th, 2011, twelve FCIN nets were set for approximately 24-hours in duration. All fish captured were separated by species. Total length, weight sex, and state of maturity were recorded for all species. The gastrointestinal tracts of lake whitefish and cisco were removed and preserved in small glass 150 ml jars containing ~60 ml of 95% EtOH. All of those fish were captured in nets set between 20 to 30 m depth.

Before the nets were set, three vertical plankton tows taken with a 30 cm, 150 µm mesh net at each site. The three zooplankton tows were combined within a one liter plastic bottle. From the side of the boat furthest from the anchor, the net was lowered to the depth that the nets were set. Zooplankton samples and the nets were set simultaneously in order to estimate what prey items were available to the fish at each site. Moreover, the samples provided an opportunity to measure the proportional abundance of a prey type in fish diet relative to its proportional abundance at the site. A small amount (three capfuls) of carbonated water was added to the sample container, to narcotize zooplankton and reduce shrinkage and distortion. After 25 minutes, 95% ethanol was added to preserve the zooplankton. Bottles were stored at room temperature until analysis. Due to time constraints the zooplankton tows were not analyzed and may be used at a later date.

In the laboratory, fish stomachs were cut open with a razor blade. Gut fullness was observed and classified as a percentage of total potential value. The contents were flushed into a petri dish for examination, using a dissecting microscope. Identifiable organisms were counted in the petri dish. If possible, *Bythotrephes* were counted by the presence of whole organisms, and by estimating individuals based on the number of identifiable spine fragments, i.e. entire spines with and without barbs, broken spines with and without barbs, spine fragments or lateral barbs, and spine tips. Because spines were often tangled into large masses in cisco guts, it was difficult to separate and view individual spines and fragments without breaking them apart. The percentage of the stomach contents that were *Bythotrephes* was then estimated. The percentage of other and unidentifiable matter was also estimated as a percent. *Bythotrephes* resting eggs were also quantified.

RESULTS

Aquarium Experiments

Comparing the ability of pumpkinseeds from two different geographic areas to remove spines from *Bythotrephes*, the removal success was similar (Figure 1). A Wilcoxon Rank Sum test indicated that there was no significant difference in spine removal for pumpkinseeds from Michigan and Minnesota (Michigan mean=.89, SD=0.12; Minnesota mean=.86, SD=0.13; W = 38, p=0.8838). The pumpkinseed data were therefore further analyzed as one group.

Pumpkinseeds used in the experiment were observed to engulf the spiny water flea, separate the spine from the body, ingest the body and spit out the spine at least 57% of the time. The spine removal success of most pumpkinseeds was 80% or greater and four pumpkinseeds were 100% successful (Table 1, Figure 2). Rarely did any of the pumpkinseeds reject and recapture *Bythotrephes* more than once. In the majority of instances, *Bythotrephes* was ingested on the first encounter. Complete rejection of *Bythotrephes* never took place, unless fish reached a state of satiation.

Most of the time, pumpkinseeds attempted to attack *Bythotrephes* head-on. They would sometimes flare their operculi while trying to manipulate *Bythotrephes* in their mouth. Occasionally fish appeared to be moving their buccal cavity slightly or nibbling on *Bythotrephes,* before they would cough out the spine, which usually appeared fully intact.

There were also instances when little manipulation took place and the spine was almost instantly shucked.

When pumpkinseeds attacked *Bythotrephes* tail-on, the behavior was different. There was an increase in the amount of buccal and opercular cavity flaring. They often would appear to be coughing and either rejected and recaptured the *Bythotrephes* head-on, or just swallowed it whole without rejecting it.

The smallmouth bass used in the experiments were never successful in removing the spines from *Bythotrephes.* One perch successfully removed two spines (Table 1), but this was accompanied with violent flaring and repeated coughing. A Kruskal-Wallis rank sum test indicated that the spine removal success of pumpkinseeds, yellow perch, and smallmouth bass (pumpkinseed *mean*=0.87, *SD*=0.12; yellow perch *mean*=.01, *SD*=0.02; smallmouth bass *mean*=0.00, *SD*=0.00) were different, $\chi2(2, N=31)=22.73$, $p<0.0001$. The pumpkinseed had noticeably higher spine removal success than yellow perch and smallmouth bass (Figure 2).

Bass appeared to struggle the most while attempting to position the *Bythotrephes* for ingestion. *Bythotrephes* was often rejected and recaptured. Bass also flared their operculi excessively compared to the other fishes and shook violently while they jerked their head back and forth in a seizure-like behavior.

Twenty four hours after the experiment took place, the smallmouth bass were offered *Bythotrephes* again. Three of them attempted to engulf a *Bythotrephes* once, but aggressively flared their buccal and opercular cavities, twitched, coughed it out and avoided any additional *Bythotrephes* added to the aquarium. These fish all actively ate flake food when the flakes were offered to them. The four other bass attempted more than once to consume *Bythotrephes,* repeatedly rejecting and recapturing before they rejected the spiny waterflea altogether. The most *Bythotrephes* eaten by any bass, twenty four hours after feeding experiments, was six. All bass actively fed on flake food after avoiding *Bythotrephes*, showing that satiation was not a factor.

Holopedium was found mixed in with the *Bythotrephes* sample and was offered to a smallmouth bass. The bass attempted to engulf *Holopedium*, but shook violently and coughed until the *Holopedium* was removed. The bass avoided any further *Holopedium* dropped into the tank.

Yellow perch did not exhibit the dramatic aversion behavior of the bass twenty four hours after feeding experiments. They preyed on *Bythotrephes* in a similar manner as they did twenty four hours earlier. They flared their buccal and opercular cavities, coughed, and twitched, but never to the degree that the smallmouth bass did. Perch continued to attempt to eat *Bythotrephes*, with occasional rejection and recapture. One fish completely rejected *Bythotrephes* after eating eight individuals. That particular fish consumed flake food after the rejection bout, again suggesting that hunger and satiation were not factors.

Compton and Kerfoot (2004) described similar behaviors of bluegills in their experiments. Comparing three treatments (full, half, and spineless), the response of their bluegills to spine length appeared strongly non-linear, suggesting that long spines offer disproportionate benefits to *Bythotrephes*. When attempting to feed on a fully spined prey there was a dramatic difference in the buccal and operculi activity, and mouthing was often followed by rejection, as the fish spit out the *Bythotrephes* and attempted to recapture and re-ingest it. They did not document spine removal by their bluegills.

Comparing the three treatments (full spine, half spine, and spineless), the response of the pumpkinseeds appeared more linear than the response of Compton and Kerfoot's bluegills (Fig. 4). The pumpkinseed spent an average of 11.5±4.3 (grand mean±SD) seconds handling full-spined individuals, an average of 7.7±2.1 seconds handling half-spined individuals, and an average of 1.1±0.2 seconds handling spineless individuals, whereas the bluegill spent an average of 11.3±1.8 seconds handling spined individuals, 3.3±0.8 handling half-spined individuals, and an average of 0.8± 0.5 seconds handling spineless individuals. A one-way ANOVA revealed a significant difference in the mean handling times at the .05 alpha level between the individual handling times for *Bythotrephes* with differing spine length by pumpkinseed and bluegill [F(5, 42)=57.1, p<0.0001]. Post-hoc Tukey's HSD tests showed that the pumpkinseed spent more time handling a full-spined versus a half-spined, and a half-spined versus a spineless, as well as a full-spined versus spineless. The same was true for the bluegill. There was not a statistically significant difference in the bluegill and pumpkinseed mean handling times for the full-spined individuals, nor was there a difference in the mean handling times of the bluegill and pumpkinseed for the spineless individuals.

Post-hoc analysis did reveal that bluegill had significantly shorter mean handling times for half-spined individuals than pumpkinseeds. The pumpkinseed's responses to spine length suggested significant differences in handling times for spine-carrying *Bythotrephes*, but not the degree of disproportionate protection evident in bluegill responses (Fig. 4). The spine removal success by the pumpkinseed was not the same for the full-spined and half-spined *Bythotrephes* (Figure 5). A Wilcoxon Rank Sum test indicated that the pumpkinseeds were statistically more successful at removing the spine when it was fully intact rather than when the spine was cut in half (intact mean=.83, SD=0.20; half spine mean=.34, SD=0.22; W = 42.5, p= 0.01932).

One-way ANOVA revealed that there was a significant difference in the mean handling times for *Bythotrephes* with full spines at the .05 alpha level between the four species of fish used in analysis [F(3, 37) = 73.411, p <0.0001]. Post-hoc Tukey's HSD tests showed that pumpkinseed (*mean*= 9.001176, *SD*=1.83) and bluegill (*mean*=11.29, *SD*=1.52) had significantly shorter mean handling times than the yellow perch (*mean*=19.07, *SD*=2.59) and smallmouth bass (*mean*=21.04, *SD*=3.01). There was not a statistically significant difference between the handling times of the perch and bass. Although the handling times of the pumpkinseed and bluegill were closer to one another than they were to the other fishes (Figure 3), there was a slight statistically significant difference at p= 0.05.

However, this was not detected in the previous post-hoc analysis involving the handling time of pumpkinseed and bluegill for all spine lengths.

Enclosure Experiments

Stomach analysis of the perch from the preliminary enclosure experiment performed in Rainy Lake showed no signs of prey ingestion. There may have been stress induced on the perch by the large smallmouth bass staring at individuals through the transparent walls and ramming into the sides of the enclosures. The carboy zooplankton were not analyzed, however, many zooplankton within the carboy appeared alive when the carboy was removed from the water, before the contents were preserved.

Stomach analysis of pumpkinseed in the second enclosure experiment showed that the fish had eaten (Table 2). The enclosures had been placed in weeds allowing for more cover from predators. Many zooplankton within the carboys were still swimming around when the enclosures were removed from the water. It did not appear that many had died. The number of *Bythotrephes* eaten by fish was estimated mainly by counting how many full spines and spine fragments there were in stomachs and intestines, because other body parts were unidentifiable. The spines are relatively indigestible chitin and were clearly distinguishable. Because soft body parts could not be identified and tabulated, it was difficult to estimate how many instances that the pumpkinseeds removed the spine from the *Bythotrephes* before ingesting the body. *Bythotrephes* were the most numerous organisms tallied in the horizontal reference tow (Table 3).

Stomach analysis of the third enclosure revealed no sign of predation by the pumpkinseed on any organisms within the carboy. Most of the zooplankton appeared dead, either stirred up, or lying on the bottom of the carboy when it was removed from the water. The majority of the *Bythotrephes* appeared white, and starting to decompose or fall apart. The zooplankton were preserved but not analyzed.

Resting Eggs Experiments

There were only 3 out of 134 (2%) *Bythotrephes* resting eggs found in the feces of fish that were fed wax worms stuffed with resting eggs (Table 4). The 3 eggs came from an undigested head case of one wax worm. On one occasion, the resting eggs were accidentally spilled out of one wax worm before it could be fed to the fish. When poked with a forceps the eggs had become soft and no longer looked viable.

One hundred and four resting eggs were found in the fecal matter of fish that were fed live *Bythotrephes* (Table 4). During the egg hatching attempt, when temperature and light was increased for 1 week, 55 eggs appeared unhatched and undamaged, 10 appeared hatched, 14 appeared severely damaged, and 25 appeared to be in early stages of hatching (Table 5). Five first instar spines, three 3rd instar spines, one 2nd instar spine and three spine fragments were found in feces (Table 5). No *Bythotrephes* body parts were found. When the egg contents were reexamined a week later, there was no additional hatching.

Cisco/whitefish stomach analysis

Eleven of the 39 cisco stomachs were packed full with *Bythotrephes* and contained little to no other prey items (Table 7). Eight of the ciscos had nearly empty stomachs, but *Bythotrephes* made up 90% or more of what was eaten. The spines from individual stomachs that were packed with *Bythtorephes* were difficult to separate and count without breaking, because they were tangled together in large masses. On all those occasions, the spines and eggs were visible without a microscope, when stomachs were cut open. It was estimated that over 500 individual *Bythotrephes* were in some fish stomachs. Over 2000 *Bythotrephes* resting eggs were counted in one cisco stomach, and over 100 resting eggs in eight others. The majority of what was not *Bythotrephes* was usually unidentifiable, however some *Daphnia* resting eggs and combs were found in seven of the ciscos.

There was no evidence that whitefish in this study preyed directly on *Bythotrephes,* as this fish was usually a benthic feeder. Two of the whitefish had 1 *Bythotrephes* spine in them; no other *Bythotrephes* body parts were found in any of the whitefish. Whitefish stomach contents contained mainly small clams, bloodworms, *Pontoporeia*, and miscellaneous unidentifiable material.

DISCUSSION

Morphological and ecological diversity pertaining to feeding habits are entwined within the evolutionary history of fishes (Helfman 2009). Specialization of structures used in acquiring food, as well as prey manipulation techniques, enable some fish to successfully feed on a food source when others cannot. Understanding the connection between predator morphology, feeding mechanisms, diet, and habitat is critical when considering the establishment and distribution of prey items.

Bythotrephes possesses an enormously elongated caudal spine with barb-like projections. Learned aversion to *Bythotrephes* was documented in experiments with young bass and perch, as well as in other studies involving other young fishes such as lake trout (*Salvelinus namaycush*), rainbow trout (*Onchorhynchus mykiss*), fathead minnows (*Pimephales promelas*), and bluegill (Barnhisel 1991b, Barnhisel and Harvey 1995, Barnhisel and Kerfoot 2004, Compton and Kerfoot 2004). Bass and perch clearly displayed difficulty while attempting to ingest *Bythotrephes*. Their feeding behaviors were similar to how Barnhisel (1991a, b) and Compton and Kefoot (2004) described fish responses in *Bythotrephes* feeding experiments: repeated rejection and recapture, excessive flaring of operculi and buccal cavities, jerking, whole body convulsions, and avoidance.

Although there was no statistical difference between handling times for perch and bass (Fig. 3) during the feeding experiments, the bass completely rejected and avoided *Bythotrephes* twenty four hours after the experiment took place, whereas the perch responses were not as dramatic. The reason for the difference could be that the bass learned faster and retained the memory of the unpleasant experience longer than the perch. The ability to learn avoidance to *Bythotrephes*' spine seems critically important for survival of young fish. Compton and Kerfoot (2004) documented a high puncture rate of the stomach and lower digestive tract of spottail shiners (*Notropis hudsonius*) that didn't learn avoidance and were capable of ingesting large numbers of *Bythotrephes*.

The *Bythtorephes* handling times of bluegills and pumpkinseeds (Fig. 3) were comparable to each other; however, the pumpkinseeds did not display avoidance, whereas bluegills did (Compton and Kerfoot 2004). Bluegills and pumpkinseeds are very closely related, are capable of hybridizing, and have similar diets when they are younger. However, bluegills shift diets as they grow from feeding on littoral prey to feeding extensively on cladocerans that dwell in the open water (Mittelbach 1984). In water bodies in which pumpkinseeds coexist with bluegill, pumpkinseeds (~65 and 80mm SL) also shift, but to a diet primarily of gastropods (Mittelbach 1984, Huckins 1997). Because bluegill are adapted to consuming cladocerans in open water, it would seem that they would have a morphological advantage over pumpkinseed relative to *Bythotrephes* consumption. Bluegills were faster than pumpkinseeds at handling half-spined individuals, but surprisingly there was no statistical difference between the two for full-spined individuals (Fig. 4). The inconsistency may be explained by the ability of pumpkinseed to remove and eject fully developed spines and the possible decision not to remove and eject half-spines (Fig. 5). Half-spines may not have been ejected by pumpkinseeds as often because they are less of a digestive threat. Another possibility is that half-spines were more difficult to separate from the body whereas it is easier to manipulate the *Bythotrephes* when the spine was full. While bluegill may be faster at consuming half-spined *Bythotrephes*, it seems like full-spine removal was advantageous to the pumpkinseed in that they were as fast as bluegill at eating full-spined *Bythotrephes* Not only are pumpkinseeds' handling times equivalent to bluegills when the spine is removed, pumpkinseed also are not risking gut puncture; which could be why avoidance wasn't documented in pumpkinseeds. Without the spines, *Bythotrephes* became a high quality food source. A published study showed that *Bythotrephes* had higher essential fatty acid concentrations than *Daphnia* (Smyntek et al. 2008), a common food item for many small fish.

The feeding behavior that enables small pumpkinseeds to remove *Bythotrephes'* defensive spines might be explained by the species' very specialized ability to feed on snails as adults. Pumpkinseed engulf and manipulate snails between enlarged pharyngeal jaws that are, along with simultaneous contractions of a hypertrophied pharyngeal jaw musculature, effective in crushing shells (Wainwright *et al.* 1991, Lauder 1983). After crushing the shell, pumpkinseed use their oral and pharyngeal jaws to separate the body of the snail from the shell fragments, which are then ejected (Huckins 1997). Manipulating, separating, and ejecting shells from snail bodies could be analogous to manipulating, separating, and ejecting the spine from *Bythotrephes.* There was no statistical difference in the spine removal ability of pumpkinseeds caught from Pelican Lake and Portage Lake (Fig. 1). *Bythotrephes* had not yet been reported in Pelican Lake during the time fish were sampled, yet upon the first presentations of *Bythotrephes*, the pumpkinseed were successful at removing the spine. If the fish in our experiments were pre-adapted to handling *Bythotrephes* as prey, and the ability to separate shells is, in fact, analogous to removing spines, it makes sense why other fish species (even within the same family) were far less successful at removing the spines than were pumpkinseed (Fig. 2).

Instances when pumpkinseeds did not remove the spine from fully-spined *Bythotrephes* seemed associated with the orientation of the prey upon capture. The barbs on the spine are ventrally oriented, yet they point anterior at an angle of approximately 45°. If small fishes, with limited gape, attack *Bythotrephes* head on, it would seem as if the barbs would act as grappling hooks, reinforcing convulsions, violent shaking, and rejection. Fish rejection after capture due to handling difficulty increases the likelihood of *Bythotrephes* surviving predator attacks, indicating that not only may the tremendous length of the spine be a remarkable anti-predation adaptation, but the angle of the lateral barbs may also play such a role. It is possible that the pumpkinseed's behavior to remove the spine occurs when the spines make forward movement of the spine to the digestive tract difficult. The spine snagging in the fish's mouth could also be why handling times are so much longer for a spined *Bythotrephes* versus a spineless (Fig. 4). It would be interesting to determine how the presence and number of barbs effects handling times.

It is common for some populations of *Bythotrephes* to possess a red band near the end of the spine. The reason for this is not entirely clear. The red coloration could be a form of aposematitism that persuades fish to avoid attack per se, or to switch to an attack head-on. Water mites (Order Hydracarina), which are close to microcrustaceans in size and general habitat, are brilliantly colored red. Experiments have been carried out confirming the unpalatability of water mites, suggesting that the red color signals a particularly hazardous experience for fish (Kerfoot 1982). The metabolic cost to *Bythotrephes* in carrying the caudal spine remains largely unmeasured; however swimming speed seems forfeited by it. Relative spine weight (caudal spine/core body) could provide one proxy for estimating the cost of the spine (Branstrator 2005). If the red band is a form of aposematic coloration, *Bythotrephes* would short-circuit the predation cycle at an early stage, reduce risk by diminishing attack, and hence save energetic costs of flight.

Resting eggs are yet another remarkable adaptation that *Bythotrephes* possess, making fish not only predators but also vectors in their spread (Jarnagin et al. 2000). The hypothesis that fish aid in the dispersal of *Bythotrephes* is important when considering how many (1000+ in one individual) were found in the stomachs of the ciscos (Table 7*).* The results in the resting eggs hatching experiment of this study involving fish being fed live *Bythotrephes* indicated that some resting eggs did not pass through the guts of pumpkinseed intact and viable, as 144 out of the 244 (~60%) eggs from live individual *Bythotrephes* offered to the fish were unaccounted for(Table 4). It is unlikely that the large amount of missing eggs was an error in experimental design and therefore a possibility that the fish digested some eggs.

It may be worth noting that there seemed be an unexpected enzymatic reaction within manipulated wax worms that breaks down the otherwise resistant egg, as only 3 of 135 eggs within wax worm capsules survived gut passage (Table 4). The notion that there could be an enzymatic process that breaks down resting eggs could have potential applications in the realm of research examining ballast water treatment options and fishing equipment decontamination techniques. This could be the case if the reaction could be recreated on a large enough scale to be an effective decontamination technique in these situations. In one wax worm in particular, the resting eggs became soft and no longer viable about twenty minutes after they were loaded into the worm without being fed to the fish.

Jarnagin et al. (2000) found no evidence that gut passage reduced hatching success. It is unclear why the majority of the eggs defecated by pumpkinseed after being fed live *Bythotrephes* (not wax worm capsules) in this study didn't appear to have hatched (94/104) after temperature increased and daylight hours were simulated. Egg hatching was attempted in August 2012, due to scheduling constraints, and could be why hatching success was so poor. The experiment should be repeated with a timeline closer to what resting eggs encounter in natural settings, i.e. when spring really takes place. However, there is also the possibility that the reason why the eggs did not hatch is because passage through pumpkinseed digestive systems reduces the hatching success of *Bythotrephes* resting eggs.

Despite aversion behavior by small gape-limited fish, some late-season young of year (YOY) and larger fish switch diets and even prefer *Bythotrephes* as a food source. *Bythotrephes* invasions have provided a new and strong source of prey for fish such as alewife (Keilty 1990, Branstrator and Lehman 1996), yellow perch (personal communication with MNDNR staff), and *Coregonus* species (Coulas et al. 1998). Coregonids have a mouth and gillraker region adapted to small prey: long and numerous gillrakers without much space between them. It is not surprising that preliminary data clearly show that cisco from Rainy Lake will prey heavily upon *Bythotrephes* (Table 7). However, to determine the proportion of *Bythotrephes* removed by the cisco, future studies should include numerous additional seasonal zooplankton samples both in surrounding waters and cisco stomachs.

Rainy Lake is one of five major *Bythotrephes* infested large lakes contained within or bordering Voyageurs National Park in northern Minnesota. By preferentially feeding on small cladocerans like *Bosmina*, the spiny waterflea is shifting dominance towards copepods and larger bodied or gelatinous cladoceran species like *Holopedium gibberum* in Voyageurs National Park (Kerfoot et al. 2012, Unpublished, multipark spiny waterflea report). As described above, one smallmouth bass was observed having great difficulty attempting to ingest *Holopedium* (violent shaking, rejection and avoidance). Total zooplankton biomass has decreased in the presence of *Bythotrephes*, and the combination of the reduction and the shift in community composition to a less preferred or less consumable prey species may result in drastic reductions of energy transferred towards higher trophic levels in affected lakes (Hobmeier et al. 2011). This is a serious concern at Voyageurs National Park, where conserving the resources and wildlife for future generations is an ultimate goal.

The park, almost entirely surrounded by the infested water of the five large lakes, is dotted with numerous small interior lakes that have not been infested (Fig. 6, personal communication with Ryan Maki-Voyageurs National Park aquatic ecologist). In an attempt to prevent the spread of *Bythotrephes* and other exotic species, regulations were put in place disallowing privately owned water craft and float plane landings on any of the interior lakes and allowing the use of artificial bait only on these lakes. However, these lakes still seem highly at risk, especially since the primary method of transfer is assumed to be fisherman and lake recreationists and it has been observed that there is less than perfect compliance with the Voyageurs National Park interior lake regulations and Best Management Practices (personal communication with Ryan Maki-Voyageurs National Park). All of the lakes are less than five miles from infested water, while more than half of them are less than a mile and can be easily accessed on a maintained hiking trail (Fig. 6). Mukooda, a heavily fished lake, for instance is located approximately 100 meters from invaded water. However, fish communities haven't been considered and it is possible that consumption by large numbers of planktivorous fish could increase *Bythotrephes* mortality and make colonization in certain lakes more difficult.

Bythotrephes thrives in large cool oligotrophic to mesotrophic lakes in the Great Lakes Basin, however, because it also occurs in very shallow, fishless ponds, pools, and lakes throughout Europe and Asia, fish predation is a likely additional determinant of *Bythotrephes* occurrence (MacIsaac 2000). Because of this it is hypothesized here that lake limnological characteristics are not the only variables influencing the distribution of *Bythotrephes*. Fish community compositions have the potential to influence the distribution and establishment of *Bythotrephes* and complement the temperature limitation observed by Kerfoot et al. (2011). The following observations present interesting evidence:

1) Mukooda has cisco and pumpkinseed, as well as numerous other small planktivorous fish (MNDNR fish survey 2009, personal communication with

fisherman). It is possible that *Bythotrephes* is periodically introduced from nearby infested water, but has not been found because heavy fish predation causes death rates to exceed birth rates, disallowing establishment.

2) Lake Superior constitutes a continual source of *Bythotrephes* to the Keweenaw Waterway, but Portage Lake acts as a sink to *Bythotrephes,* even though its food resources are not considered a limiting factor (Compton and Kerfoot 2004). The temperature in Portage Lake is warmer than Lake Superior and has a variety of schooling planktivorous fishes, including pumpkinseed (Compton and Kerfoot 2004, personal observation and communication with local fisherman).

3) *Bythotrephes* disappeared from the Fish Lake Reservoir in Minnesota after water level changes paired with climatic conditions caused an increase in the abundance of centrarchid fish species (personal communication with Donn Branstrator, University of Minnesota-Duluth).

This research provides information in support of a broader goal of eventually developing a remedial treatment for aquatic systems that have been invaded by *Bythotrephes*. This study offers progress toward that goal in that we have found a species of juvenile fish that is able to break through *Bythotrephes'* defensive spine adaptation. The problem seems not to lie in the ability of large fishes to utilize *Bythotrephes* as a food source, but rather in the inability of small gape-limited fish to do so. Fish are assumed to be the most abundant in their small young of the year stages. Taylor Jarnagin et al. (2004) concluded that because of aversion behavior to the defensive spine, it is rare for perch < ~60mm to utilize *Bythotrephes* as a food source. Concurrently, they predicted that perch > ~100mm would choose alternative larger prey items. Kerfoot et al. (2011) also documented a narrow size "window" for yellow perch to consume *Bythotrephes* in Lake Michigamme and several lakes in Voyageurs National Park. Small perch fail to consume *Bythotrephes*, whereas larger year-1 fish switch to larger benthic species. It is possible that because there is evidence of young pumpkinseeds successfully removing the spine and not learning aversion, that they have the ability to influence the distribution of *Bythotrephes* through predation by filling in for the size class of other fish species that isn't able to utilize *Bythotrephes* as a food source because of its spine. Fish clearly put significant pressure on *Bythotrephes*. Late-season young of year and larger fish and can be responsible for approximately 62-71% of *Bythotrephes'* mortality (Jarnagin et al. 2004). Young pumpkinseed may add significantly enough pressure on top of what is already coming from larger planktivorous fish to actively exclude *Bythotrephes* from lakes.

Whether pumpkinseeds possess the ability to serve as biological control agents depends upon several critical variables: 1) Over *Bythotrephes* geographic range, how often would it encounter a pumpkinseed-occupied lake, 2) within a lake, do the habitat preferences of *Bythotrephes* and pumpkinseeds lead to spatial or temporal overlap, and 3) in the demographic diet progression, is *Bythotrephes* a preferred food item?

Pumpkinseeds are native to and most abundant in the Great Lakes Drainage and throughout the northeastern quarter of the United States and are rarely found in the southcentral or southwestern region of the continent (Huckins 1997). The native geographic range of pumpkinseeds overlaps the North American distribution of *Bythotrephes* that ranges from New England and Quebec to Minnesota and Manitoba (Kerfoot et al. 2011).

Bythotrephes is thought to be limited by limnological characteristics, such as lake area, depth, transparency, chlorophyll concentrations and temperature (MacIsaac et al. 2000, Branstrator 2006, Kerfoot et al. 2011). A study carried out in Voyageurs National Park examining the vulnerability of its interior lakes to *Bythotrephes* (Maki et al. 2009) revealed that seven lakes (Mukooda, Cruiser, Quill, Loiten, Beast, Little Trout, O'Leary) had morphometric characteristics and water chemistry along with zooplankton communities that could easily support *Bythotrephes* if invaded. Of those lakes, three of them (Mukooda, Quill, Loiten) have known populations of pumpkinseeds (Voyageurs National Park fish survey), suggesting that pumpkinseeds are present in lakes that can support *Bythotrephes* limnologically.

Of the introduced fishes in Europe, pumpkinseeds are thought to be one of the most successful colonizers of new environments due to the flexibility and plasticity existing in several aspects of their life history (Tomeĉek et al. 2007). Nevertheless, their presence in the Great Lakes region seems somewhat dependent on vegetation cover. Juveniles and adults are common in shallow areas with heavy macrophytes, and often swim in schools. A variety of factors influence the habitat use of pumpkinseeds. One factor in particular is the presence of other fish species. For instance, habitat use of pumpkinseed can be predisposed by the abundance of predator smallmouth bass that restrain them to shallow, potentially safer habitats (Godinhog 1997). Ecological segregation occurs when bluegill (a competing congener) feed on zooplankton from the open water and drive pumpkinseed to mainly feed in shallow littoral habitats (Werner and Hall 1979, Osenberg et al. 1998). In lakes without bluegill however, there can be two forms of pumpkinseeds: a "limnetic" form and a "littoral" form and they differ in their body size and shape, trophic structure and diet (Robinson 1996, Robinson 2000, McCairns and Fox 2004,). The limnetic form is more streamlined and has more closely spaced gill rakers. While the littoral form may stick to feeding on benthic prey (such as snails), the limnetic form strays from macrophytes to forage extensively on zooplankton (Robinson 2000). It seems likely that a limnetic form, while searching for zooplankton, would encounter and possibly select *Bythotrephes* as a prey item if it was present.

Bythotrephes occurs in various parts of a lake, but seems to prefer temperatures between 10 and 24 °C, and is typically limited to 4 and 30°C (Grigorovisch et al. 1998). Although pumpkinseed are tolerant to colder temperatures, and are present in water bodies devoid of bluegill due to winter hypoxia (Fox 1994), they prefer temperatures between 22–31 °C (Müller and Fry 1976), which overlaps the upper quarter portion of *Bythotrephes* temperature preference.

Bythotrephes maximum density is often observed in the cooler layers of epilimnetic waters, below the photic zone but above the thermocline in thermally stratified lakes. Yan et al. (2001) theorized that *Bythotrephes* seeks refuge from fish (in particular cisco) predation by exhibiting vertical migration of embryo-carrying adults to a refuge layer below the photic zone, but above the hypolimnion, during the day. Hartleb and Haney (1998) studied the effects of thermal stratification and light gradients on *Daphnia* feeding behavior of pumpkinseeds. They found that thermal stratification restricted the depth at which pumpkinseeds fed. The pumpkinseeds fed almost exclusively in the epilimnion, under thermal stratification. Without thermal stratification, the pumpkinseeds foraged throughout the water column, but were less effective in areas with low light. They concluded that thermal stratification and decreased light creates a refuge for *Daphnia*, thus negatively affects the ability of pumpkinseed to search and feed on zooplankton. In theory, because pumpkinseeds apparently avoid areas of sudden temperature changes and are less effective at foraging below the photic zone, the refuge tactic *Bythotrephes*' uses that is effective against cisco predation, would also be effective against pumpkinseed predation in thermally stratified lakes.

A scenario where pumpkinseed could eliminate *Bythotrephes* is as follows: A shallow lake that contains pumpkinseeds and not bluegills, because the bluegills cannot tolerate winter hypoxia. Without bluegills, the pumpkinseeds are not driven to feed mainly in littoral habitats, instead the limnetic form of pumpkinseed is present feeding extensively on zooplankton in open water. Because the lake is shallow, it stays mixed, pumpkinseeds aren't restricted thermally, and there is no thermal or photic refuge for zooplankton. An angler transfers *Bythotrephes* into the lake on fishing gear previously used in infested water. The limnologic characteristics of the lake alone do not limit the survival of *Bythotrephes* (*Bythotrephes* typically thrives in large, cool, deep lakes, but it also occurs in very shallow, fishless ponds, pools, and lakes throughout Europe and Asia; MacIsaac

2000). Successful at removing *Bythotrephes'* defensive spine, pumpkinseeds prey on *Bythotrephes*. The elimination of early season adult *Bythotrephes* and the failure of late season *Bythotrephes* juveniles to reach adulthood and produce resting eggs before being eaten by the pumpkinseeds results in the eventual elimination of *Bythotrephes.*

Information from the literature regarding whether or not *Bythotrephes* is a preferred food item in the demographic diet progression of pumpkinseeds was unable to be found. If the spine removal behavior of the pumpkinseeds in this study is a general trait of all pumpkinseeds, *Bythotrephes* spines would not show up in large numbers in pumpkinseed gut analysis and *Bythotrephes* presence in pumpkinseed diets could be overlooked.

Enclosure experiments were attempted in an effort to answer the question or whether or not pumpkinseed would actively select *Bythotrephes* as a food source. Large smallmouth bass were seen hovering around and ramming into the first enclosures that were set. The perch within the enclosures were very exposed and visible to predators through the transparent carboys. While swimming frantically to escape the predatory bass they likely bumped into the sides of the enclosure while trying to find shelter. This could be why stomach analysis didn't reveal any signs that those fish had eaten and why one died during the experiment. If these enclosure experiments are repeated it will be important to keep the enclosures in locations offering cover. It seems critically important to place the carboys in a habitat that has plants or other structures that can aid in reducing the amount of stress in response to predators. The enclosure experiments emphasize why small fish have difficulty foraging away from littoral cover in open water with large predators. This could also suggest niche differences between *Bythotrephes* and small fish.

The enclosures in the second experiment were anchored in macrophytes. Gut analysis revealed that pumpkinseed in those enclosures had eaten (Table 2). The fish in the second experiment likely had less stress, because plants kept them more hidden from predators and, therefore, they had more energy for foraging. The number of *Bythotrephes* eaten by pumpkinseeds in the second enclosure experiment was estimated by quantifying how many full spines and spine fragments were in each pumpkinseed stomach. It was difficult to determine how many instances that the pumpkinseed removed the spine from the *Bythotrephes* and ingested the body because many body parts were beginning to be digested and were unidentifiable whereas the spines are indigestible and were clearly distinguishable. Undoubtedly this could have caused an error in the estimation of how many *Bythotrephes* were actually eaten. The spine removal success of the pumpkinseed in the aquarium experiments was at least 57% and 80% or greater for most individual fish. If the feeding behavior of the fish in the aquariums is analogous to the fish in the enclosures our estimates for how many *Bythotrephes* were actually consumed would have been much greater.

The third enclosure experiment took place in mid-October. Stomach analysis did not reveal that the pumpkinseed fed on any type of prey in those enclosures. The majority of the zooplankton was seen lying on the bottom, when the carboy was removed. It is possible that extreme temperature fluctuations killed the zooplankton. The cool air temperatures could have caused enough of a difference in the water temperature that when the zooplankton were transferred to the enclosures they didn't have enough time to acclimate to the cold ambient temperatures. The water in Portage Lake also could have become too cold and it could have been so late in the year that the zooplankton were already close to dying and dropping their resting eggs to overwinter. These enclosure experiments could probably be successfully carried out in less drastic temperature conditions. *Bythotrephes* is typically to limited water temperatures that range between 4 and 30°C (Grigorovisch et al. 1998). More thorough pumpkinseed stomach analysis should be attempted, looking in particular for *Bythotrephes* body parts, like mandible pairs, rather than spines. Underwater cameras could also be set up to document and quantify the spine removal success of the pumpkinseed in the enclosures or in the wild.

In conclusion, lake limnological characteristics may not be the only variables influencing whether or not *Bythotrephes* can survive in lakes. It is possible that *Bythotrephes* is found more often in large, cool lakes because fish predation removes them from warm, shallow lakes. There may be an important predator-prey interaction involving certain fish species, like pumpkinseed, with specialized morphology for prey manipulation. Fish community compositions have the potential to influence the distribution and establishment of *Bythotrephes* in time and space. Further research could determine if pumpkinseeds possess the ability to serve as biological control agents for *Bythotrephes* invasions.

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TABLES

Table 1. Processing time and *Bythotrephes* spine removal success (proportion of spines spit out) for pumpkinseed (*Lepomis gibbosus*), yellow perch (*Perca flavescens*), and smallmouth bass (*Micropterus dolomieu*). Additional published bluegill (*Lepomis macrochirus*) data (Compton and Kerfoot 2004) included.

Species	Location	Total Length(mm)	Total Presentations	Spine Removal Success	Mean Handling Time ± SE
Lepomis gibbosus	Portage Lake, MI	56	12	0.92	10±1.8
Lepomis gibbosus	Portage Lake, MI	59	13	1	6.8±0.8
Lepomis gibbosus	Portage Lake, MI	65	14	0.78	11.6±2.2
Lepomis gibbosus	Portage Lake, MI	68	12	0.92	7.6±1.0
Lepomis gibbosus	Portage Lake, MI	70	13	0.69	11.6±1.7
Lepomis gibbosus	Portage Lake, MI	67	18	0.8	8.3±1.3
Lepomis gibbosus	Portage Lake, MI	45	15	1	7.5±0.4
Lepomis gibbosus	Portage Lake, MI	47	12	1	8.0±0.7
Lepomis gibbosus	Pelican Lake, MN	49	13	1	11.3±2.1
Lepomis gibbosus	Pelican Lake, MN	55	17	0.82	6.8±0.9
Lepomis gibbosus	Pelican Lake, MN	55	17	0.94	7.0±0.6
Lepomis gibbosus	Pelican Lake, MN	55	16	0.94	11.0±1.2
Lepomis gibbosus	Pelican Lake, MN	48	15	0.8	8.9±0.7
Lepomis gibbosus	Pelican Lake, MN	65	15	1	7.3±0.6

Table 1. Continued

Lepomis gibbosus	Pelican Lake, MN	48	15	0.8	8.7±1.2
Lepomis gibbosus	Pelican Lake, MN	59	14	0.57	11.4±1.2
Lepomis gibbosus	Pelican Lake, MN	50	14	0.85	9.1±1.4
Perca flavescens	Rainy Lake, MN	49	16	0	21.3±1.5
Perca flavescens	Rainy Lake, MN	52	16	0	19.1±1.3
Perca flavescens	Rainy Lake, MN	50	16	0	18.3±1.7
Perca flavescens	Rainy Lake, MN	57	18	0	18.0±0.9
Perca flavescens	Rainy Lake, MN	48	24	0.04	15.0±0.8
Perca flavescens	Rainy Lake, MN	56	16	0	18.6±1.7
Perca flavescens	Rainy Lake, MN	53	21	0	23.2±2.5
Micropterus dolomieu	Portage Lake, MI	57	15	0	26.5±1.0
Micropterus dolomieu	Portage Lake, MI	53	35	0	23.2±0.8
Micropterus dolomieu	Portage Lake, MI	50	40	0	18.2±0.5
Micropterus dolomieu	Portage Lake, MI	57	12	0	20.2±1.2
Micropterus dolomieu	Portage Lake, MI	53	15	0	18.1±0.9
Micropterus dolomieu	Portage Lake, MI	52	32	0	19.5±0.7
Micropterus dolomieu	Portage Lake, MI	51	26	0	21.7±0.7
Lepomis macrochirus	Portage Lake, MI	49	32		14.4±0.9
Lepomis macrochirus	Portage Lake, MI	52	29		10.5±0.8

Table 1. Continued

Lepomis macrochirus	Portage Lake, MI	53	30	12.0±0.9
Lepomis macrochirus	Portage Lake, MI	60	31	11.5±1.4
Lepomis macrochirus	Portage Lake, MI	61	30	10.4±1.0
Lepomis macrochirus	Portage Lake, MI	65	28	10.2±1.0
Lepomis macrochirus	Portage Lake, MI	67	30	10.1±1.2

Table 2. Summary of number of food items found in yellow perch and pumpkinseed stomachs for enclosure experiments.

Species	Total Length(mm)	Date (mm/dd/yyyy)	Food Items	
			Bythotrephes	Other Zooplankton
Perca flavescens	49	08/05/2011	0	0
Perca flavescens	52	08/05/2011	0	0
Perca flavescens	50	08/05/2011	0	0
Lepomis gibbosus	56	09/23/2011	10	3
Lepomis gibbosus	48	09/23/2011	6	2
Lepomis gibbosus	55	09/23/2011	4	0
Lepomis gibbosus	59	10/19/2011	0	0
Lepomis gibbosus	50	10/19/2011	0	0
Lepomis gibbosus	55	10/19/2011	0	0

Table 3. Zooplankton tallies of horizontal reference tow taken for enclosure experiments September 23, 2011.

Cladocerans	
Bythotrephes longimanus	351
Holopedium gibberum	30
Daphnia galeata mendotae	178
Bosmina longirostris	5
Copepods	
Nauplii	12
Epischura lacustris	1
Leptodiaptomus spp.	178
Acanthocyclops vernalis	35

Table 4. Number of *Bythotrephes* resting eggs ingested and found in fecal matter during pumpkinseed feeding trials. Pumpkinseed diet included live *Bythotrephes* carrying resting eggs and wax worms with inserted *Bythotrephes* resting eggs.

Length of Fish (mm)	Type of prey offered to fish	Number of eggs ingested	Number of eggs found in fecal matter
60	Resting eggs inside wax worm	6	0
60	Resting eggs inside wax worm	12	0
75	Resting eggs inside wax worm	12	0
62	Resting eggs inside wax worm	11	0
60	Resting eggs inside wax worm	11	0
55	Resting eggs inside wax worm	10	0
60	Resting eggs inside wax worm	12	0
75	Resting eggs inside wax worm	8	0
62	Resting eggs inside wax worm	30	0
60	Resting eggs inside wax worm	22	3
60	Live *Bythotrephes*	27	10
48	Live *Bythotrephes*	8	4
45	Live *Bythotrephes*	15	5
41	Live *Bythotrephes*	41	18
51	Live *Bythotrephes*	52	20
53	Live *Bythotrephes*	101	47

Table 5. Resting eggs and spines detected in *Bythotrephes* hatching experiment. All eggs used in experiment were found in fecal matter of pumpkinseeds after they had been fed live *Bythotrephes* that carrying resting eggs during an aquarium experiment.

Eggs that appeared hatched	10
Eggs that appeared severely damaged	14
Unhatched and undamaged eggs	55
Egg appearing to be in the early stages of hatching	25
1rst instar spines	5
2nd instar spines	1
3rd instar spines	3
Spine fragments	3

Table 6. Processing time and spine rejection rate by pumpkinseed (*Lepomis gibbosus*) for *Bythotrephes* with varying spine lengths: Full spine, half spine and no spine.

			Mean Handling Time(s) ± SE(N)			Spine Removal Success	
Species	Location	TL(mm)	Full Spine	Half Spine	No Spine	Full Spine	Half Spine
Lepomis gibbosus	Portage Lake,MI	56	10.0±1.8 (12)	5.8±0.9(12)	1.4±.3(10)	1.00	0.45
Lepomis gibbosus	Portage Lake,MI	59	6.7±0.8(13)	8.3±1.5(12)	1.0 ±0.0(9)	1.00	0.58
Lepomis gibbosus	Portage Lake,MI	65	11.7±2.9(10)	10.0±3.3(8)	1.0±0.0(9)	0.70	0.34
Lepomis gibbosus	Portage Lake,MI	68	7.6±1.2(10)	5.6±1.5(8)	1.1±0.1(9)	0.90	0.50
Lepomis gibbosus	Portage Lake,MI	87	17.1±5.9(13)	4.0 ±0.6(10)	1.2±0.2(9)	0.90	0.14
Lepomis gibbosus	Portage Lake,MI	49	16.0±2.9(6)	6.5± .2(6)	1.0±0.0(6)	0.50	0

Table 7. Gut analysis of cisco and whitefish caught from Rainy Lake in FCIN nets set between 20 to 30 meters in August 2012. Contents identified as prey items other than *Bythotrephes* are listed as % other.

			GUT ANALYSIS				
Species	**Length (mm)**	**Weight (g)**	**% fullness**	**% *Bythotrephes***	**% unidentifiable**	**% other**	***Bythotrephes* resting eggs**
Cisco	185	50	80	50	50		10
Cisco	141	22	0				
Cisco	162	36	50	40	60		29
Cisco	208	80	100	10	87	3 (*Daphnia*)	1
Cisco	282	210	50		98	2 (*Daphnia*)	
Cisco	160	36	100	100			325+
Cisco	222	104	5			5 (*Daphnia* , Glycera)	
Cisco	352	506	90		90	8 (*Daphnia*)	
Cisco	152	32	30	1	90	20 (Misc. zooplankton)	
Cisco	162	40	50	100			76
Cisco	170	40	100	100			260+
Cisco	203	84	100	100			2000+
Cisco	184	52	50	100			44
Cisco	160	36	100	100			360
Cisco	192	60	100	95	3	2 (*Daphnia*)	113
Cisco	196	60	50	100			40
Cisco	143	28	100	100			200+
Cisco	134	22	0				
Cisco	185	50	0				
Cisco	169	32	100	100			420+

Table 7. Continued.

Cisco	216	100	50	100			103
Cisco	223	102	10		100		
Cisco	119	10	50	90	10		6
Cisco	118	16	0				
Cisco	341	408	5		100		
Cisco	124	10	50	100			70
Cisco	216	88	50	100			
Cisco	120	14	100	100			38
Cisco	363	596	100		95	*5 (Daphnia , Mysis)*	
Cisco	115	8	100	100			26
Cisco	403	712	100		100		
Cisco	157	28	100	100			178
Cisco	117	10	90	100			35
Cisco	176	44	25	100			20
Cisco	284	226	100		100		
Cisco	302	292	0				
Cisco	131	20	20	100			
Whitefish	483	1100	50		50	50 (Pelecypoda, Glycera, *Diporeia*)	
Whitefish	543	1550	50		10	90 (*Diporeia,* Pelecypoda, Glycera)	
Whitefish	543	1550	50		10	90 (*Diporeia,* Pelecypoda, Glycera)	
Whitefish	510	1850	0				
Whitefish	325	332	10		5	5 (Pelecypoda, Glycera)	
Whitefish	390	520	0				
Whitefish	510	1450	0				
Whitefish	492	1150	5			*5 (Diporeia)*	

Table 7. Continued.

Whitefish	457	1000	5	1		*4 (Daphnia)*
Whitefish	484	1250	0			
Whitefish	465	1150	50			100 (*Daphnia,* Pelecypoda)
Whitefish	406	580	1			100 (*Diporeia)*
Whitefish	467	1050	5	1		4 (*Diporeia* , Pelecypoda, Glycera)
Whitefish	505	1300	50		20	80 (*Diporeia* , Pelecypoda, Glycera)
Whitefish	425	720	0			
Whitefish	436	848	0			
Whitefish	489	1300	50		10	90 (*Diporeia* , Pelecypoda, Glycera)
Whitefish	329	300	0			
Whitefish	171	38	50		30	70 (*Diporeia* , Pelecypoda, Glycera)

FIGURES

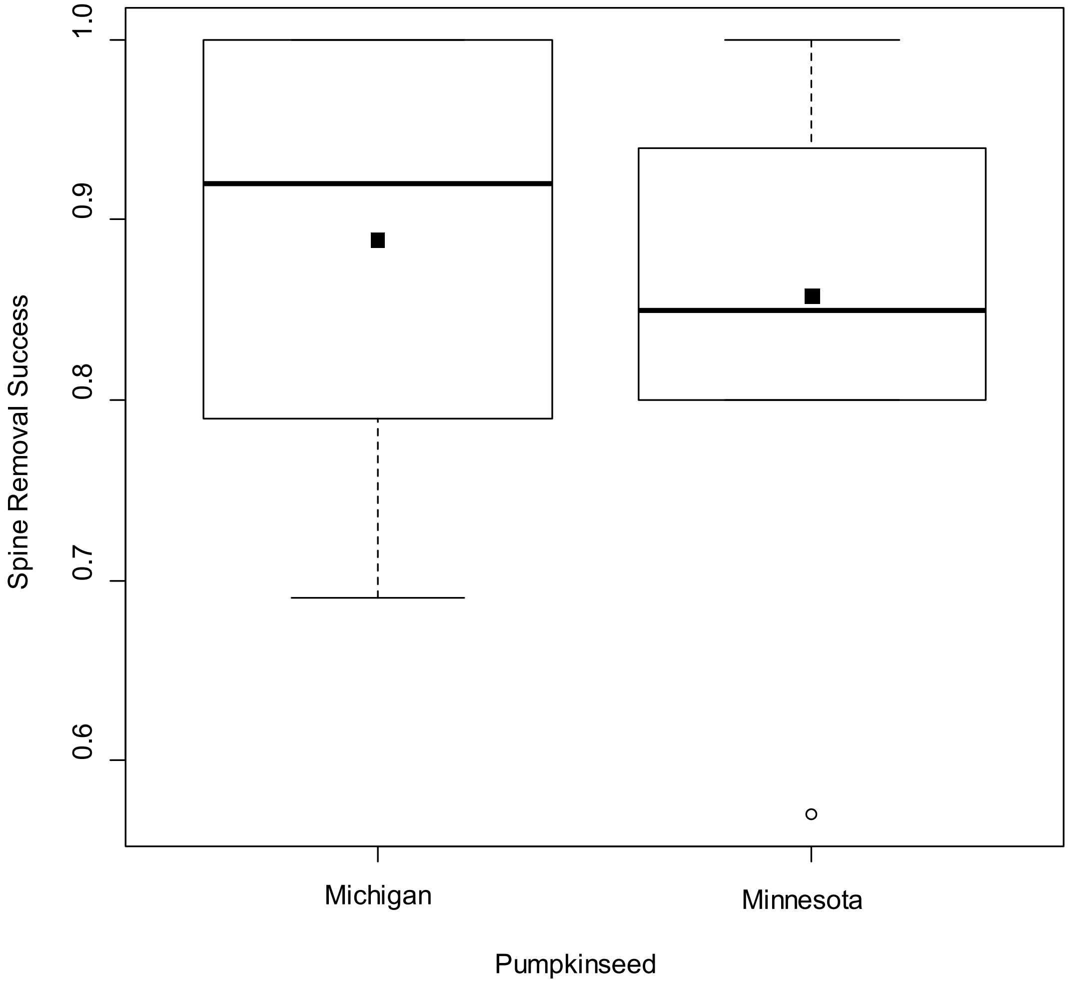


Figure 1. Ability of individual pumpkinseeds from Michigan and Minnesota to remove *Bythotrephes*' spines. The box plots show the median (horizontal line), mean (dark square), first and third quartile (ends of the box), range (vertical lines), and outliers (hollow circles).

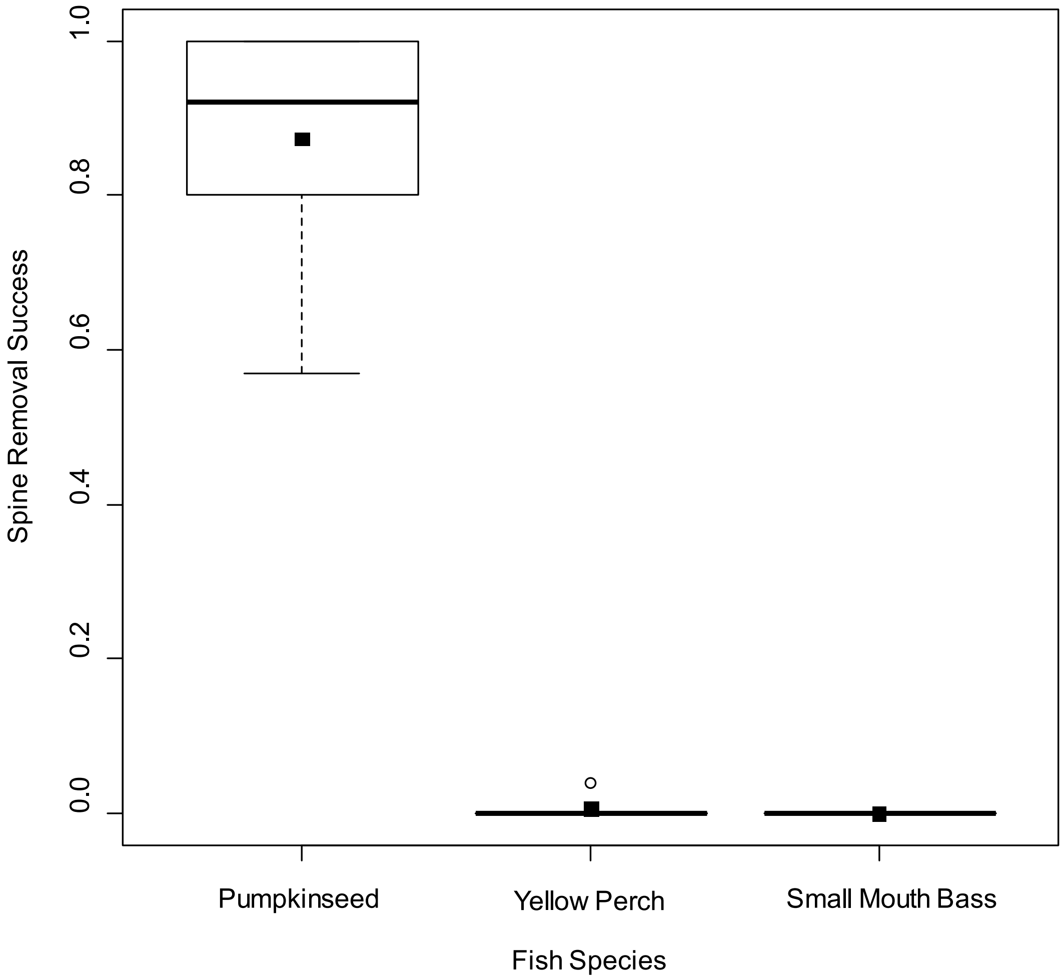


Figure 2. Spine removal success rate by individual pumpkinseed, yellow perch, and smallmouth bass (1.0 = 100% success). The box plots present the median (horizontal line), mean (dark square), first and third quartile (ends of the box), range (vertical lines), and outliers (hollow circles).

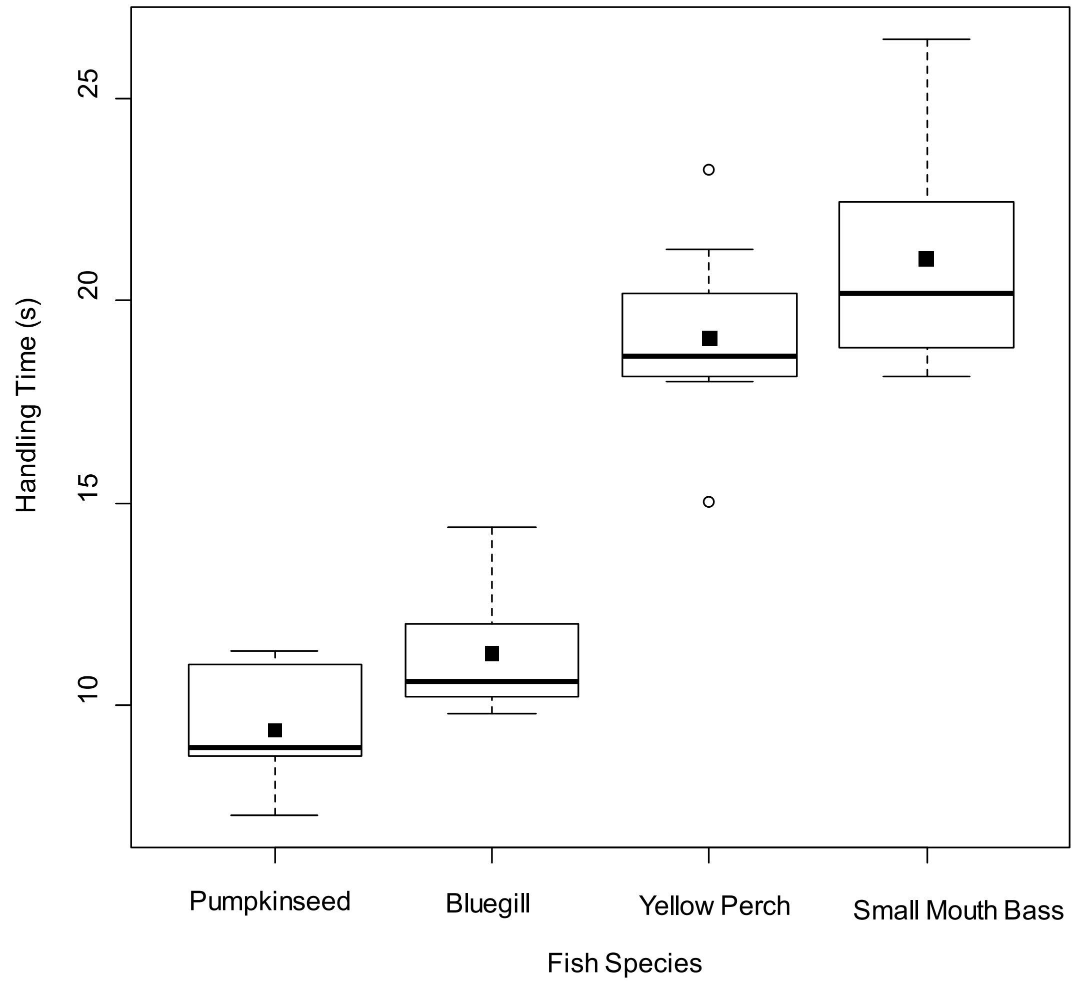


Figure 3. Average handling times for *Bythotrephes* by individual pumpkinseed, bluegill, yellow perch, and smallmouth bass. The box plots compare median (horizontal line), mean (dark square), first and third quartile (ends of the box), range (vertical lines), and outlier values (hollow circles). Bluegill data is from Compton and Kerfoot (2004).

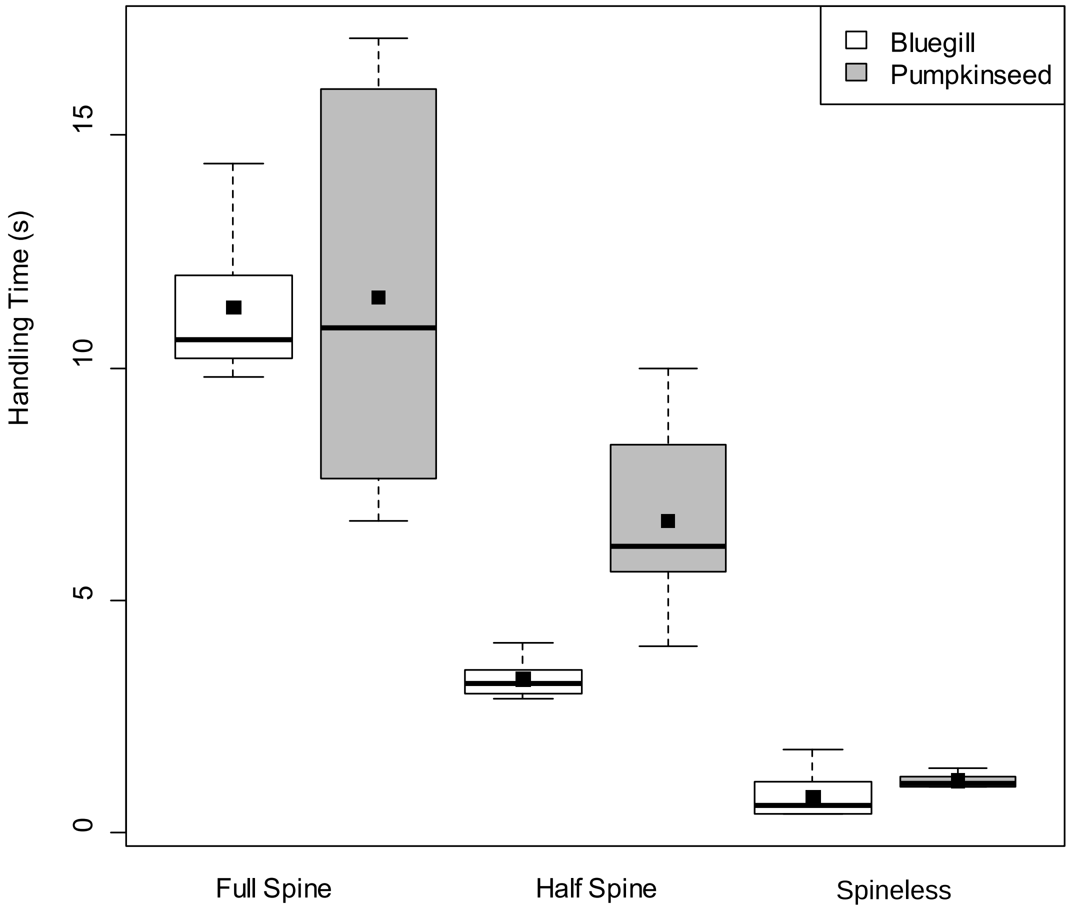


Figure 4. Comparison of individual pumpkinseed and bluegill mean handling times for *Bythotrephes* with varying spine lengths: full-spine, half-spine, and spineless. Bluegill data is from Compton and Kerfoot (2005).

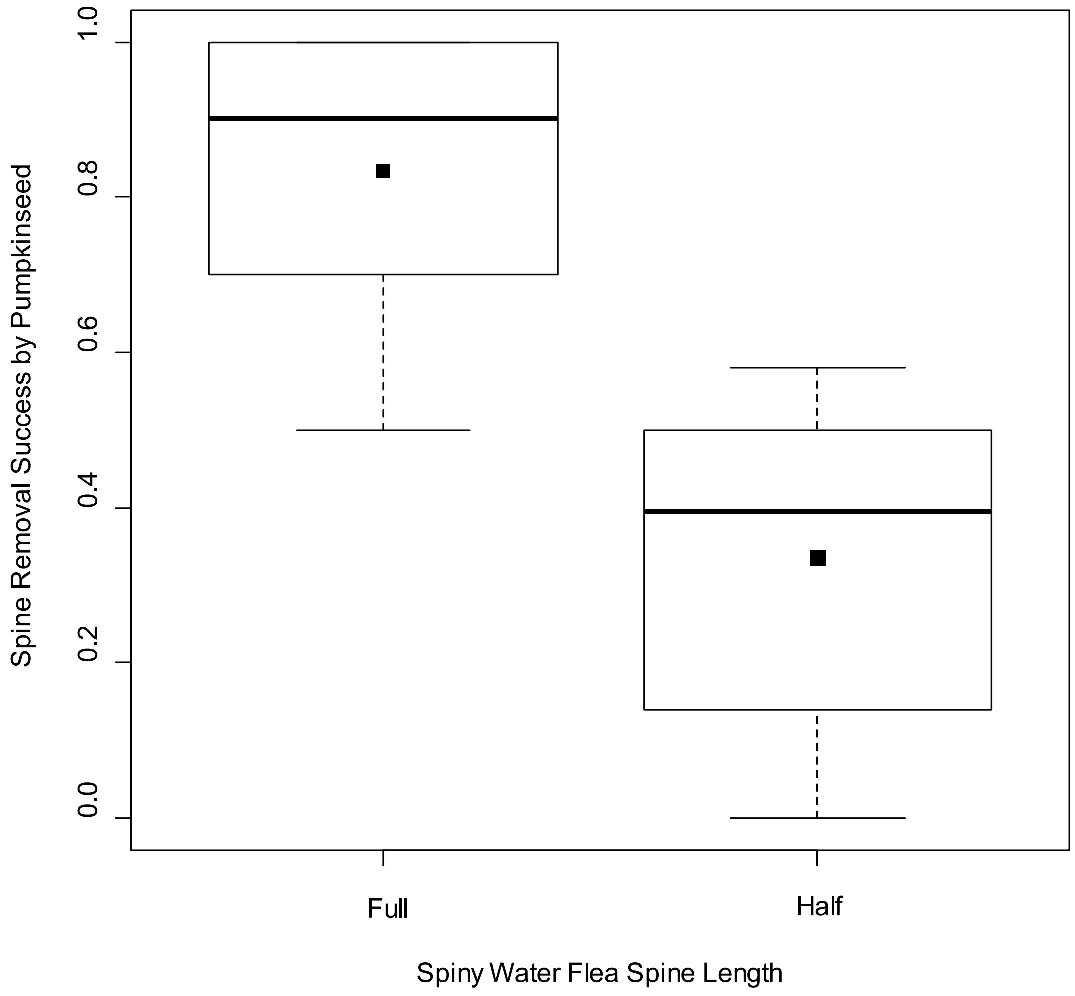
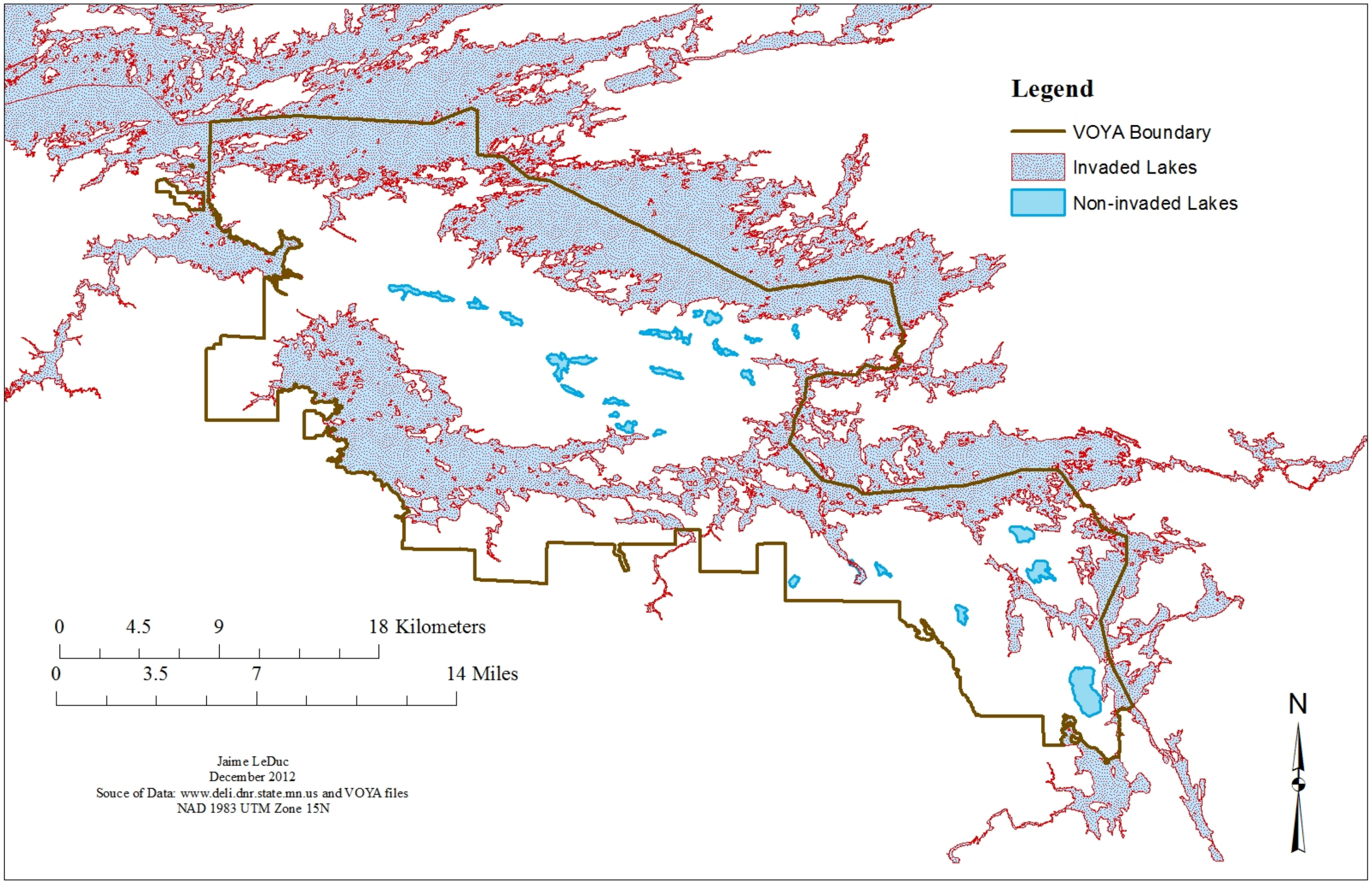


Figure 5. Spine removal success by pumpkinseeds for *Bythotrephes* with varying spine lengths: full-spine and half-spine.



Jaime LeDuc
December 2012
Souce of Data: www.deli.dnr.state.mn.us and VOYA files
NAD 1983 UTM Zone 15N

Figure 6. Map showing invaded and non-invaded lakes in Voyageurs National Park