ADDRESSING ANTHROPOGENIC AND ENVIRONMENTAL PERTURBATIONS IN AQUATIC SYSTEMS

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ADDRESSING ANTHROPOGENIC AND ENVIRONMENTAL PERTURBATIONS IN AQUATIC SYSTEMS

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The overall goal of the set of experiments described in this dissertation was to better understand the influence of a variety of perturbations in aquatic ecosystems using multiple analytical and methodological approaches. I used these techniques to address three important challenges currently facing aquatic systems managers, including: 1) alewife die-offs in the Great Lakes and other freshwater systems 2) elevated thiaminase activity in clupeid fish and 3) mercury contamination in aquatic food webs. My objectives were to better understand and in some cases explore potential means to remediate negative impacts associated with these issues by using a variety of metrics as indicators of anthropogenic and environmental perturbation.

BIOGRAPHICAL SKETCH

Jesse Michael Lepak was born in Wausau, Wisconsin on the 23rd of May, 1978 to Michael and Dianne Lepak. He grew up there and attended Wausau East High School where he graduated salutatorian and accepted a scholarship to attend an in-state University. Jesse began his collegiate academic career at the University of Wisconsin-Madison. He completed various courses in chemistry and biology, originally focusing on the dentistry profession. His interest shifted to the environment after completing a directed study with Dr. John J. Magnusson for a 1999 biology course. The project was a comparison of minnow populations in three Madison area ponds. Jesse's new-found desire to work with the environment motivated him to seek courses in ecology and environmental studies. Jesse declared biology, zoology and biological aspects of conservation as his majors after being exposed to and enjoying an ecological curriculum. Jesse's specific interest in aquatic ecology and fisheries was solidified in the summer of 2000 while working as an intern for Dr. James F. Kitchell at the University of Notre Dame Environmental Research Center. His experience there and encouragement from Dr. Kitchell motivated Jesse to seek a Master's degree in Natural Resources under the advisement of Dr. Clifford Kraft at Cornell University. Jesse completed this task in January 2004 and has since moved on to become a Ph.D. candidate. This dissertation will complete his requirements to earn a Ph.D.

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CHAPTER ONE
ADDRESSING ANTHROPOGENIC AND ENVIRONMENTAL PERTURBATIONS IN AQUATIC SYSTEMS

Abstract:

The overall goal of the set of experiments described in this dissertation was to better understand the influence of a variety of perturbations in aquatic ecosystems using multiple analytical and methodological approaches. I used these techniques to address three important challenges currently facing aquatic systems managers, including: 1) alewife die-offs in the Great Lakes and other freshwater systems, 2) elevated thiaminase activity in clupeid fish and 3) mercury contamination in aquatic food webs. My objectives were to better understand and in some cases explore potential means to remediate negative impacts associated with these issues by using a variety of metrics as indicators of anthropogenic and environmental perturbation.

Introduction:

This introductory chapter is designed as a brief overview and background of the context and major findings of the research presented in this dissertation. The four other chapters of this dissertation address: 1) alewife mortality, condition, and immune response to prolonged cold temperatures, 2) the effect of stressors on thiaminase activity in alewife, 3) alewife and gizzard shad thiaminase response to stressors and 4) changes in methylmercury bioaccumulation in an apex predator in response to removal of an introduced piscivore.

Nearly every ecosystem has been impacted by anthropogenic and environmental perturbations. Understanding and quantifying perturbations is one method to help address ecological problems. Combining various techniques allowed me to approach three important and disparate problems from several perspectives (e.g., predator, prey, nutrients, enzymes, hormones, contaminants) and at multiple scales (e.g., temporal scales from minutes to hours, to days, to seasons, to years; spatial scales from microcosms to whole-system manipulations). I chose metrics of anthropogenic and environmental perturbation that manifested themselves in organisms. These included measures of: thiaminase activity, total and methylmercury concentrations in fish and invertebrates, stable isotope ratios, white blood cell counts, plasma cortisol and glucose levels, water content, Fulton's K, age and growth, diet composition combined with several routine biotic and abiotic measures. Many of these techniques are flexible and can be used to varying degrees across species in a variety of ecosystems to address environmental problems stemming from ecological interactions and to conduct informative ecological research. When applied and interpreted correctly, these metrics serve as indicators of anthropogenic and environmental perturbation and can be used to help address and understand environmental responses to a wide variety of perturbations in aquatic, marine and terrestrial systems.

The results from this dissertation provide information that can be used directly to better understand and study systems that are, or could potentially be influenced by alewife die-offs, early mortality syndrome and mercury contamination. Understanding the causes and impacts of these problems is useful so that managers can implement strategies and better communicate with stakeholders in order to address these and other fishery issues. The major findings of this dissertation were that: 1) alewife mortality may be related to alterations in alewife immune systems and further

information regarding the immune response of alewife to cold temperatures and possibly exposure to *Saprolegnia sp*. under severe conditions may help predict when alewife are expected to experience mortality events, 2) thiaminase activity in clupeids is not related to stress as it was measured in the set of experiments reported in this dissertation and these results – in conjunction with other recent evidence – suggest that thiaminase activity is likely related to changes in gastrointestinal microbial communities, and 3) empirical data showed that a predator removal resulted in an increase in methylmercury (MeHg) concentrations in another top predator. This provides evidence that changes in food web structure and dynamics have the potential to alter MeHg concentrations in fish.

My interests in aquatic ecology are focused on the dynamics of food web interactions in response to perturbation. This dissertation addresses three disparate topics including: 1) alewife die-offs in the Great Lakes and other freshwater systems, 2) elevated thiaminase activity in clupeid fish and 3) mercury contamination in aquatic food webs. A background description of these three issues follows. My objectives were to better understand and in some cases explore potential means to remediate negative impacts associated with these issues by using a variety of metrics as indicators of anthropogenic and environmental perturbation.

Alewife die-offs in the Great Lakes and other freshwater systems:

Alewife (*Alosa pseudoharengus*) have been recognized for more than three decades as one of the most important forage fish in the Laurentian Great Lakes (Smith 1970), as evidenced by the heavy reliance of salmonine predators on alewife as prey (Jude *et al.* 1987; Lantry 2001; Madenjian *et al.* 2002). Fluctuations in alewife abundance and condition can affect salmonine growth and survival (Stewart et al. 1981; Rand and Stewart 1998). As a result, salmonine communities in the Great

Lakes are closely linked to the success of alewife populations upon which they rely as forage.

Massive alewife die-offs have been observed throughout the Great Lakes and in other inland lakes since the late 1800s (Goode 1884; Pritchard 1929; Colby 1973). Researchers have suggested that several factors (e.g., temperature induced spawning stress, low food availability resulting in poor condition) could cause mortality (Flath and Diana 1985; O'Gorman and Schneider 1986; Bergstedt and O'Gorman 1989), and cold temperatures are known to challenge the osmoregulatory capabilities of alewife (Stanley and Colby 1971; Snyder and Hennessey 2003). Temperature changes can alter the permeability of cell membranes in teleost fishes, leading to impaired ion transport and membrane-associated enzyme function (Hazel and Williams 1990; Hazel 1993). As such, severe winter temperatures were considered as a possible contributor to alewife mortality (Eck and Brown 1985; O'Gorman and Schneider 1986; Bergstedt and O'Gorman 1989). Fungal infections of *Saprolegnia sp*. were documented in field-collected and laboratory-reared alewife that had been exposed to cold winter temperatures (Graham 1956; Brown 1968; Colby 1973), but the causal nature of these infections has not been evaluated.

Although several studies have been conducted to investigate the effects of cold temperatures on the immune response of teleost fishes, factors influencing the immune response of alewife have not been previously evaluated. It has been suggested that exposure to cold temperatures may produce immunosuppression, as measured by a decrease in lymphocyte function and number of circulating lymphocytes (lymphopenia) in bluegill (*Lepomis macrochirus*) (Cuchens and Clem 1977), channel catfish (*Ictalarus punctatus*) (Clem et al. 1984; Miller and Clem 1984), hybrid striped bass (*Morone chrysops* x *Morone saxatilis*) (Hrubec et al. 1997) and pinfish (*Lagodon rhomboids*) (Abruzzini et al. 1982). Lymphopenia has also been induced in brown

trout (*Salmo trutta*) (Pickering 1984) and rainbow trout (*Oncorhyncus mykiss*) (Barton et al. 1987) by feeding cortisol, a stress hormone, directly to fish. Furthermore, previous investigations have shown that immune system dysfunction in fish increases their susceptibility to antigens and subsequent mortality (Arkoosh 1998a; Arkoosh 1998b; Arkoosh and Collier 2002).

Based on previous observations, two lines of evidence prompted me to evaluate whether the immune response of alewife changed in response to contrasting winter temperature conditions representative of mild and severe winters: 1) the observed immune response to cold temperatures in other fishes, and 2) observations that colder temperatures increased the susceptibility of alewife to *Saprolegnia sp.* infection and subsequent mortality (Graham 1956; Brown 1968; Colby 1973). In this study I evaluated adult alewife mortality, condition and immune system response to different thermal conditions in replicated pond experiments. I hypothesized that alewife exposed to mild winter temperatures would experience reduced mortality, lower water content, and greater levels of immune system activity as compared to alewife exposed to colder temperatures, and that alewife mortality would be near 100% in ponds with severe winter temperatures.

Elevated thiaminase activity in clupeid fish:

Thiamine (vitamin B_1) is an essential vitamin necessary for the conversion of carbohydrates and fats into energy and has been found to act at some level across all kingdoms of life. Thiamine is vital to processes associated with normal functioning of the nervous and digestive systems, as well as the heart. Because of the ubiquitous need for thiamine, thiamine deficiency has the potential to influence a wide variety of organisms including fish, birds, reptiles and mammals – even humans.

Since the mid-1800's, millions of cases of thiamine deficiency in humans have been confirmed, many resulting in death. Up to 10,000,000 reported cases are estimated to have occurred in China alone (Williams 1961). In the Philippines from 1954 to 1958, thiamine deficiency was the fourth leading cause of death (over 20,000 deaths), exceeded only by pneumonia, tuberculosis and bronchitis (Williams 1961). Members of the Japanese and African military and African law enforcement have also suffered casualties numbering in the thousands from thiamine deficiency (Williams 1961; Aykroyd 1970). Countries that have reported serious human thiamine deficiencies include Canada, Ghana, Ivory Coast, Japan, Malaysia, Myanmar, Singapore, Thailand and the United States (Williams 1961; Aykroyd 1970; Pongpanich et al. 1974; Neumann et al. 1979; Lee 1994).

Combined with an improved knowledge of dietary requirements and use of appropriate supplements, thiamine deficiency has been less of a problem since the 1950's, particularly for humans and agricultural animals. However, thiamine deficiency remains a concern when it has been caused by the thiamine-destroying enzyme thiaminase rather than a lack of dietary thiamine. Thiaminase is a destructive enzyme that has been detected in a variety of organisms including plants, bacteria, multiple salt and freshwater prey and sport fish species, lobster, shrimp and shellfish such as clams, mussels and scallops (as compiled in: Harris 1951; Fujita 1954). Thiaminase has been linked to thiamine deficiency in fox, mink, ruminants, fish and humans and is likely responsible for thiamine deficiency in other organisms (Green et al. 1937; Stout et al. 1963; Edwin and Jackman 1970; Earl and McCleary 1994; Fitzsimons and Brown 1998; Honeyfield et al. 2005). The consumption of uncooked fish, shellfish and other sources of dietary thiaminase by humans has resulted in beriberi (thiamine deficiency) and death (Vimokesant et al. 1982; Earl and McCleary 1994). Fox and mink populations were found to exhibit reproductive failure as a result

of consuming raw fish tissue containing thiaminase (Green et al. 1937; Stout et al. 1963). The thiamine deficiency resulting in the deaths of thousands of Japanese soldiers was linked directly to the consumption of uncooked fish containing thiaminase (Williams 1961; Aykroyd 1970). Humans are now aware that heating thiaminase denatures the enzyme, therefore cooking food is sufficient to avoid consuming dietary thiaminase. However, wild organisms that consume dietary items high in thiaminase (especially raw fish which are used in feeds and consumed by predators) have the potential to become thiamine deficient, especially when exposed to thiaminase in natural environments where thiamine supplements and treatments are not always feasible.

Reproductive failure was observed in the brood stocks of salmonine fish in North American hatcheries around the 1960's. Although researchers investigated the possibility that toxins like DDT and PCB's were the cause of the reproductive failure (Burdick et al. 1964; Johnson and Pecor 1969; Stauffer 1979), the true cause remained unknown. Offspring from the affected fish experienced high levels of mortality several weeks before yolk absorption; lesions, hemorrhaging, convulsive and erratic swimming and other ailments were experienced prior to death (Fisher et al. 1995b). A coho salmon (*Oncorhynchus kisutch*) fry mortality in the wild with similar symptoms was first observed in 1967 (Johnson and Pecor 1969). During the next several decades managers and researchers working in the Laurentian Great Lakes and Finger Lakes of New York (USA) increasingly recognized that other salmonine fishes – including lake trout (*Salvelinus namaycush*), Atlantic salmon (*Salmo salar*), Chinook salmon (*Oncorhynchus tshawytscha*), rainbow trout (*Oncorhynchus mykiss*), and brown trout (*Salmo trutta*) – suffered from a similar reproductive failure in natural systems (Fisher et al. 1995b; Marcquenski and Brown 1997; McDonald 1998). Baltic Sea observations of sac-fry mortality in Atlantic salmon (Norrgren et al. 1993; Bengtsson

et al. 1994; Karlsson et al. 1996) and sea-run brown trout (*Salmo trutta*) (Soivio 1996) were also attributed to consumption of clupeid prey containing thiaminase. These occurrences of mortality were eventually related to thiamine deficiency when it was found that treatment of affected fish with a 48-hour thiamine bath eliminated symptoms in natural populations of Finger Lakes Atlantic salmon and Great Lakes lake trout (Fisher et al. 1995a; Fitzsimons et al. 1995). The ailments were coined "Cayuga Lake Syndrome" in the United States as it was first found in Cayuga Lake (New York, U.S.A.) and "M74" in the Baltic because it was first described in 1974 – the "M" signifying the word miljörelaterad, meaning environmentally-caused thiamine deficiency (Norrgren et al. 1993; Fisher et al. 1995b; Ikonen 2006). Early Mortality Syndrome (or EMS) is now recognized as a thiamine deficiency in salmonine fish in systems throughout North America and Europe.

A large body of field and laboratory work conducted during the past decade supports a plausible hypothesis for an association between a prey base comprised of a proportion of nonnative alewives (*Alosa pseudoharengus*) and Baltic herring (*Clupea harengus*) and the recruitment difficulties currently experienced by salmonines. Alewife and other clupeids (for example Baltic herring) contain some of the highest levels of thiaminase activity ever measured in fish and have been linked to persistent mortality in the offspring of their salmonine predators in the Laurentian Great Lakes, Finger Lakes (New York), the Baltic Sea and other freshwater systems (Fitzsimons et al. 2005; Tillitt et al. 2005; Ikonen 2006). Investigations into thiaminase activity as a possible cause of EMS have shown that thiaminase activity in alewife can be highly variable (Fitzsimons et al. 2005; Tillitt et al. 2005). In these studies, thiaminase activity within alewife tissue samples varied from lake to lake and within lakes depending on sampling season and location. The source of this variation is unknown; however, EMS has been found to be prevalent in salmonines with diets dominated by

clupeids high in thiaminase – this is believed to be the primary factor responsible for EMS in alewife predators (Fitzsimons et al. 1999; Honeyfield et al. 2002; Honeyfield et al. 2005a).

Thiaminase in alewife has been attributed to thiaminase positive bacteria (*Paenibacillus thiaminolyticus*) isolated from alewife viscera (Honeyfield et al. 2002). Alterations in dietary levels of thiaminase (in the form of feral alewife containing thiaminase and bacterial thiaminase sources) have been used to induce EMS in laboratory experiments with lake trout (Honeyfield et al. 2005b). Although the ultimate source or sources of thiaminase contributing to EMS are unknown in natural systems, this evidence suggests that bacterial thiaminase may play a role in salmonine thiamine deficiency. Although research has focused mainly on alewife and Baltic herring, other clupeid fish have been found to express thiaminase in high concentrations. For example, gizzard shad have very high levels of thiaminase activity (means from 15,000 to 30,000 pmol thiamine$\cdot g^{-1}\cdot min^{-1}$; Tillitt et al. 2005; Honeyfield et al. *in press*; J. Ross et al. unpublished data; S. Marquenski, unpublished data) relative to alewife (on the order of 5,000 pmol thiamine$\cdot g^{-1}\cdot min^{-1}$, Tillitt et al. 2005). Given their tendency to dominate biomass and their wide distribution in U.S. waters (Timmons et al. 1978; Johnson et al. 1988), thiaminase expression in gizzard shad has the potential to negatively affect many apex predator populations whose diets are comprised of large quantities of gizzard shad. Interestingly, no evidence exists to support that EMS occurs within alewife, Baltic herring, gizzard shad or other fish containing high thiaminase activity. The mechanism preventing these species from suffering thiamine deficiency remains unknown.

Thus far the sole management action in response to the presence of thiaminase in Great Lakes and Finger Lakes fishes has been to stock hatchery-reared salmonids, and to treat eggs and fry with thiamine to maintain the viability of hatchery-raised

salmonids. Unfortunately these practices can alter natural reproduction and increase competition in both salmonids and other apex predators. Although this preemptive treatment has protected valuable sport fisheries, such efforts are time consuming and expensive, and have obscured the underlying problem and reduced incentives to characterize the true cause of reproductive problems to effectively manage these fisheries. Understanding the source of reproductive failure and how interactions occur between primary producers, forage fish and predators to manifest EMS is imperative for resolving this threat to valuable fisheries.

Mercury contamination in aquatic food webs:

Mercury contamination in fish is a serious issue affecting fisheries in the northeastern United States and is of concern worldwide to human and ecosystem health (Bodaly et al. 1993; Johnston et al. 2003; Kamman et al. 2005). The northeastern U.S. receives high levels of atmospheric mercury deposition (Chen et al. 2005; Kamman et al. 2005) and mercury levels in aquatic ecosystems are unlikely to change in the near future (Hunter et al. 2003). Due to bioaccumulation, MeHg concentrations are particularly high in long-lived, slow-growing fish that feed at the top of the aquatic food web – i.e., large piscivores. Unfortunately, these large piscivores with the highest concentrations of MeHg are also the primary targets for harvest and consumption by anglers.

Methylmercury, the primary form of mercury found in sport fish, is toxic to humans and is readily absorbed by the blood stream before being distributed to the brain and body tissues. Although MeHg is eliminated from the body naturally, it can accumulate in the blood stream over time if consumption levels exceed the body's capacity for excretion (USEPA 2001a; USEPA and USFDA 2004). Elevated blood MeHg concentrations have been linked to neurological damage leading to impaired

vision and loss of motor coordination and feeling; at high levels, seizures, severe neurological impairment, and death may result (National Research Council 2000; USEPA 2001a). Further complications may include damage to the lungs, kidneys, cardiovascular system, gastrointestinal tract, and immune system (National Research Council 2000; USEPA 2001a; Institute of Medicine 2007; Mergler et al. 2007). Methylmercury presents a significant threat to the developing central nervous systems of unborn babies and children and can impact cognition, memory, attention, language, and fine motor and visual spatial skills (National Research Council 2000; USEPA 2001a; Institute of Medicine 2007; Mergler et al. 2007). The current EPA reference dose for MeHg (0.1 μg/kg/day; USEPA 2001b) corresponds to the maximum level of exposure recorded without deleterious effects on human fetuses. Methylmercury intake below this level is therefore not expected to cause health effects during a person's lifetime (National Research Council 2000; USEPA 2001b; Rice et al. 2003).

An important distinction between MeHg and other forms of mercury is that MeHg is the form that bioaccumulates most readily because it is absorbed by living tissue. Thus, MeHg poses a direct threat to humans when it is consumed. In most cases the form of mercury in sport fish is assumed to be >95% MeHg (Bloom 1992). This assumption is widely accepted and due to prohibitive costs associated with MeHg analyses, total mercury (T-Hg) is often measured as a substitute for MeHg. For the purposes of this chapter, MeHg will refer only to the methylated form of mercury, T-Hg refers to measures of total mercury and the term "mercury" will refer to mercury in the environment in any form or when groups of studies are referenced together when the forms of mercury being discussed were not consistent across studies.

Mercury bioaccumulation in fish is dependant upon many biological factors, including fish diet (Harris and Bodaly 1998; Johnston et al. 2003; Swanson et al. 2003), fish trophic position (Power et al. 2002), food web structure (Johnston et al.

2003; Swanson et al. 2003) and energy sources (Power et al. 2002). Several methods have been attempted to remediate the effects of mercury in organisms consumed by humans. Research efforts to reduce T-Hg accumulation in fish have included additions of selenium (Turner and Rudd 1983; Turner and Swick 1983; Björnberg et al. 1988) and limestone (Håkanson et al. 1988) to systems, which resulted in slight decreases in T-Hg concentrations in fish. The removal of certain fish (predominantly top predators) from lakes has also been suggested as a means of reducing T-Hg concentrations in fish (Gothberg 1983; Verta 1990; Rask 1996). Although fish removals have been shown to be much more effective than chemical measures, to date this method has only been successful in small lake systems (< 25 ha), and the mechanisms behind subsequent decreases in T-Hg accumulation are not well understood (Gothberg 1983; Verta 1990; Rask 1996). Verta (1990) suggested that "growth dilution" was the primary factor leading to decreased T-Hg concentrations in the fish remaining after removal. Growth dilution is a process whereby a reduction in competition leads to higher growth rates (due in combination to improved growth efficiency and increased energy content of available prey items) and subsequently lower concentrations of MeHg in fish at a given length. Growth dilution may occur if the MeHg concentration in a fish's diet remains relatively constant while the energy content of the diet increases and/or the activity required for consumption decreases due to increased food availability –thereby increasing growth while MeHg input remains constant. These circumstances result in lower concentrations of MeHg in the tissues of faster-growing individuals; however, this mechanism was not verified in the cases of decreased fish densities described above.

Shifts in the structure and composition of aquatic communities can influence MeHg bioaccumulation rates. Stable isotope studies have established that increased trophic position and enriched $\delta^{13}C$ signatures in predators are correlated with

increased concentrations of T-Hg in fish (Power et al. 2002; Swanson et al. 2003). Bioenergetics models have shown that the mercury content in a predator's diet is largely responsible for the mercury accumulated within the tissue and that subsequent changes in diet, metabolism, and growth rate can effectively alter mercury concentrations in fish (Harris and Bodaly 1998; MacRury et al. 2002; Trudel and Rasmussen 2006). These model findings are compelling in that they identify potential sources of variability in mercury bioaccumulation.

Approach and findings:

A brief outline of each of the subsequent chapters in this dissertation follows. The specific fishery issues the experiments were designed to address are shown in *italics*. The design and metrics of response for each experimental perturbation that was conducted are described briefly, followed by the major findings of each chapter.

Alewife die-offs in the Great Lakes and other freshwater systems:

In Chapter Two I evaluated adult alewife mortality, condition and immune system response to different thermal conditions in replicated pond experiments. I hypothesized that alewife exposed to mild winter temperatures would experience reduced mortality, lower water content, and greater levels of immune system activity than alewife exposed to colder temperatures, and that alewife mortality would be near 100% in ponds with severe winter temperatures.

- Alewife mortality did not differ between fish experiencing severe winter temperatures and mild winter temperatures;
- Alewife circulating lymphocyte counts were significantly lower in fish experiencing severe winter temperatures relative to those experiencing mild winter temperatures.

Elevated thiaminase activity in clupeid fish:

In Chapter Three I discuss experiments to evaluate the effect of two stressors, reduced water salt content and food limitation, on alewife thiaminase activity. Alewife were subjected to treatments in replicated tanks in which conductivity was lowered (< 100 μS/cm) for 8 days and feeding was limited for 39 days. Alewife circulating white blood cells, plasma cortisol, plasma glucose and whole body thiaminase were measured in individual alewife to assess their response to these experimental treatments.

- Alewife thiaminase activities were not related to the salt or food limitation treatments;
- Alewife thiaminase activities were not related to circulating white blood cell counts, plasma cortisol or glucose levels;
- Alewife circulating white blood cell counts were significantly lower in fish that experienced stressful conditions relative to control alewife;
- Alewife held in experimental tanks had mean thiaminase activity more than double that of alewife originally collected from Cayuga Lake (source population) at the completion of the experiment.

In Chapter Four I discuss a set of experiments in replicated pond systems to evaluate the influence of stressful conditions on alewife and gizzard shad thiaminase. Severe winter temperatures, and low food availability were selected as treatments in this set of experiments. I hypothesized that these treatments would result in stressful conditions, ultimately leading to an increase in clupeid thiaminase activity.

- Alewife and gizzard shad held in ponds had mean thiaminase activity comparable to those measured in natural systems;

- Alewife and gizzard shad had measures of condition (water content and Fulton's K) comparable to those measured in natural systems (exception: controls);
- The only significant treatment effect was on gizzard shad mean thiaminase activity in the nutrient addition ponds.

Mercury contamination in aquatic food webs:

In Chapter Five I evaluated (MeHg) concentrations in a native top predator, lake trout, before and after the large-scale removal of an introduced predator, smallmouth bass. I used metrics of stable isotopes, diet and growth to better understand the mechanisms behind the changes in empirical lake trout MeHg concentrations. I used a bioenergetics modeling approach to compare empirical findings with theoretical estimates of lake trout MeHg concentrations when simulating lake trout growth and diet conditions of the pre- and post-removal periods.

Empirical findings:

- Lake trout MeHg concentrations have significantly increased from 2000 to 2007, and empirical evidence negates the importance of growth dilution;
- Variation in lake trout MeHg concentration was best explained by a principal component including length, weight and age. Year was the next most significant factor explaining variation in lake trout MeHg;
- Lake trout $\delta^{13}C$ and trophic position did not explain a significant amount of variation in lake trout MeHg concentration;
- Lake trout age at length has significantly decreased from 1996 to 2007.

Bioenergetics simulations results:

- Higher MeHg in prey fish (post-removal diet) relative to invertebrates (pre-removal diet) was the most important factor influencing lake trout MeHg;

- Higher energy content of prey fish (post-removal diet) relative to invertebrates (pre-removal diet) did not result in significant growth dilution in lake trout;
- Accounting for the proportion of MeHg relative to T-Hg in diet items and predators is important for obtaining appropriate empirical and model results.

Discussion and implications for future research:

Alewife die-offs in the Great Lakes and other freshwater systems:

My results indicate that six weeks of exposure to temperatures <2°C was not sufficient to cause an increase in alewife mortality when compared with alewife exposed to less severe temperatures. Additionally, alewife condition – measured as the proportion of water in fish tissue – did not differ between fish subjected to mild and severe temperatures. Diet analyses of alewife collected in January from a single mild temperature pond indicated that these fish were feeding at low levels, but I was unable to capture fish from the other ponds for comparison. The most distinct difference between alewife exposed to the mild and severe temperature treatments was the presence of lower circulating lymphocyte counts in alewife subjected to severe winter conditions.

By evaluating the alewife response to cold temperatures in replicated pond systems, Chapter Two expands upon what was learned about alewife thermal tolerance in laboratory studies conducted three decades ago (Colby 1973). Alewife collected for the experiments conducted by Colby (1973) were young-of-year alewife from Lake Michigan that never experienced winter conditions outside of the laboratory. When these fish were acclimated to low temperatures for prolonged periods of time in the laboratory they experienced mortality once temperatures were reduced to 4.0ºC and lower.

Alewife white cell counts were significantly lower in fish exposed to cold temperatures. Although the procedure used for counting cells is limited to making comparisons between fish within the same study, white cell counts may present a useful and underutilized measure of the status of immune system integrity in other fishes captured in the wild. White cell counts have been used extensively in laboratory and clinical trials involving salmonid fishes, but they have not been used widely in natural systems (Anderson 1996). Basic measures of white cell differentials and counts, when combined with appropriate study design, have provided useful insights in natural systems (e.g., Barker et al. 1994; Arkoosh 1998b; Arkoosh and Collier 2002). White cell differentiation and enumeration are techniques that can be employed without harming individual fish, thereby facilitating the evaluation of fish immune system and stress responses to contrasting environmental conditions. I believe that the potential utility of these techniques has not been fully realized in natural aquatic systems.

Surface and nearshore temperatures in the Laurentian Great Lakes approach 0ºC during most winters. Although alewife often move to deeper, warmer (i.e., 3.0 to 4.0ºC) water after fall turnover (Wells 1968), Great Lakes alewife can be exposed to temperatures <3.0ºC for prolonged periods (Schroeder 1963). Recent climate circulation models project future changes in global climate, and it is likely that Great Lakes ice conditions and nearshore temperatures will be affected (Hodgkins et al. 2002). These results suggest that severe winter temperatures have the potential to induce changes in alewife immune response which could lead to increased mortality. Graham (1956) suggested that alewife experiencing stress from rapid temperature changes were more susceptible to infection by *Saprolegnia sp.* than other alewife. Brown (1968) reported a 20% incidence of *Saprolegnia sp.* infection in alewife visually examined after the 1967 die-off in Lake Michigan. Colby (1973) observed

that 100% of adult alewife exposed to cold temperatures contracted "*Saprolegnia*-like" fungal infections while control alewife experienced only 30% mortality. Despite these observations – and despite concerns about large alewife die-offs in the Great Lakes – little emphasis has been placed on studying the response of alewife to disease. My results expand upon previously available knowledge regarding the temperature tolerance and immune response of alewife in field conditions and will hopefully lead to further studies expanding the current understanding of factors influencing winter alewife mortality.

Elevated thiaminase activity in clupeid fish:

My data suggest that variability in clupeid thiaminase is not related to stress as it was evaluated in this set of experiments, but rather that there could be some relationship between clupeid condition and/or diet composition and thiaminase activity. The only trend that was consistent across experiments was that alewife and gizzard shad that came from groups of fish that were in better condition (as indicated by water content and Fulton's K) and consumed high quality diets were higher in thiaminase relative to those in poorer condition that were not consuming high quality diets. The mean thiaminase activities, water contents and Fulton's K for the various groups of clupeids evaluated in this study are shown in Table 1.1.

Table 1.1 Experimental results of alewife and gizzard shad, tank and pond experiments. Group mean values for thiaminase and condition (as measured by water content and Fulton's K) are shown.

System	Species	Treatment	Thiaminase (pmol/g/min)	Condition
Cayuga Lake	Alewife	Wild	6,900	72% water
Tanks	Alewife	Salt limitation and controls	15,000	56% water
Tanks	Alewife	Food limitation and controls	17,000	51% water
Waneta Lake	Alewife	Wild	3,700	69% water
Ponds	Alewife	Severe and control temperatures	5,200	71% water
Acton Lake	Gizzard shad	Wild	25,000	0.99 (Fulton's K)
Ponds	Gizzard shad	Nutrients	30,000	0.81 (Fulton's K)
Ponds	Gizzard shad	Sediments	24,000	0.82 (Fulton's K)
Ponds	Gizzard shad	Nutrients and sediments	22,000	0.85 (Fulton's K)
Ponds	Gizzard shad	No nutrients or sediments	22,000	0.74 (Fulton's K)

Alewife from the laboratory experiments had mean water contents lower than any published value for alewife, while their thiaminase activity was higher than any group of alewife collected from natural freshwater systems (Hartman and Brandt 1995; Tillitt et al. 2005). Alewife and gizzard shad from the pond experiments that were held under more natural conditions had mean water contents, Fulton's K and thiaminase activities that were comparable to those of the source population and other published values following the completion of the experiments (Hartman and Brandt 1995; Tillitt et al. 2005; Vanni unpublished data). The alewife held in tanks were not exposed to any external sources of thiaminase found in natural aquatic systems (e.g., cyanobacteria, zooplankton or other dietary sources) and their food was heat treated, denaturing any thiaminase found in the feed. These data suggest that laboratory conditions altered alewife internal conditions in such a way resulting in a significant increase in thiaminase activity.

The results found in this set of experiments were unexpected because it was thought that alewife and gizzard shad exposed to stressful conditions would exhibit higher levels of thiaminase activity relative to control fish based on previous findings.

Large differences in clupeid thiaminase in this set of experiments were associated with large differences in clupeid condition. It is possible that smaller-scale effects could have occurred that were not detectable because of the limited sample size, experimental design, treatments and sample populations used in these experiments. It should be noted that gizzard shad in the ponds where nutrients were added (high N and P content) were the only shad to show significantly elevated thiaminase activities relative to other fish despite their being in good condition (as measured by Fulton's K) in the case of the ponds with nutrients and sediments added and those with sediments alone added. This suggests that although gizzard shad mean Fulton's K was positively correlated with thiaminase activity, factors other than, though perhaps related to, condition – such as diet composition – may influence the expression of thiaminase. It is possible that the makeup of clupeid diets (i.e., the proportion of proteins, lipids, complex and simple carbohydrates, or other components) may differentially impact the resulting thiaminase activity.

The isolation of thiaminase-positive bacteria from alewife viscera represents an intriguing potential source of alewife thiaminase (Honeyfield et al. 2002). The composition of bacterial communities and resulting gene expression within fish viscera are altered by different feeding regimes and fish condition (Syvokienë and Mickënienë 1999). Additionally, thiaminolytic bacteria have been shown to out-compete other types of bacteria in the gastrointestinal tracts of ruminants under certain feeding regimes (e.g., increased carbohydrate consumption), ultimately resulting in thiamine deficiency (Brent 1976). The results of this set of experiments suggest that clupeids that are feeding on high quality energy sources with specific compositions have the potential to harbor high levels of thiaminase activity. I suggest that this phenomenon could be related to internal conditions in clupeids affecting bacterially produced thiaminase. Thus, clupeid feeding conditions may result in the proliferation

of thiaminolytic bacterial populations and/or altered expression of thiaminase by these bacteria. Although this is speculation, the presence of thiaminolytic bacteria within alewife as a primary source of thiaminase should be explored.

The characterization of internal clupeid microbial communities and the expression of thiaminase in response to experimental treatments may provide important insights into thiaminase research. Thiaminase is primarily found in the visceral tissues of alewife and gizzard shad; only small amounts of thiaminase have been detected in muscle tissue (D. Honeyfield unpublished data; S. Marquenski unpublished data). Evaluations of clupeid thiaminase might benefit greatly from the inclusion of components to identify and quantify the microbial species present in these visceral tissues, and to characterize microbial expression of thiaminase. I suggest that future experiments be designed that vary clupeid feeding rates and diet composition (especially with respect to N and thiamine content) while characterizing changes in clupeid internal microbial communities and microbial expression of thiaminase.

Mercury contamination in aquatic food webs:

The empirical and theoretical findings of this study were unexpected. Growth dilution for T-Hg has been shown to occur in the field and the laboratory and has been modeled using bioenergetics (Göthberg 1983; Verta 1990; Rask et al. 1996; Borgmann and Whittle 1992; Simoneau et al. 2005). I hypothsized that the increase in growth (indicated by the decrease in age at length) that was observed in the lake trout following the smallmouth bass removal would result in lower MeHg concentrations in the population. However, others have found that growth dilution is not as important as changes in the mercury content of fish diets in some cases and my results are analogous to those studies (Harris and Bodaly 1998; Stafford and Haines 2001; MacRury et al. 2002). The reason growth dilution was not an important factor in my

study was because of the low concentrations of MeHg in invertebrate diet items relative to prey fish.

The bioenergetics simulation did indicate that growth dilution could potentially reduce MeHg concentrations, corroborating empirical data from lake trout collected in 2006. This group of lake trout had the fastest growth of any group evaluated in this study in conjunction with concentrations of MeHg comparable to values found in lake trout prior to and immediately following the initiation of the smallmouth bass removal. However, this effect (the combination of increased lake trout growth efficiency and prey item energy content) was minimal and the high lake trout growth rates observed in 2006 in lake trout consuming prey fish did not reduce concentrations of MeHg below those found in slower growing lake trout – before the smallmouth bass removal began – that primarily consumed invertebrate prey.

Most of the T-Hg in fish is generally assumed to be primarily (~95%) in the form of MeHg (Bloom 1992). However, this is not the case with invertebrates and prey fish (Huckabee et al. 1979; Hildebrand et al. 1980; J. Loukmas pers comm.; Lepak unpublished data) and even in some fish predators (Lepak unpublished data). Due to the prohibitively high costs of MeHg analyses, previous model predictions of T-Hg accumulation do not always account for the fact that in certain cases, T-Hg concentration may not be representative of MeHg concentration in top predators and more importantly, in their prey (Borgmann and Whittle 1992; Trudel and Rasmussen 2006). Although these studies used measurements of T-Hg without applying a correction factor accounting for the proportion of MeHg accumulated, others have used literature or empirical values to account for MeHg (Harris and Bodaly 1998; MacRury et al. 2002). In this study I found that diet items varied widely in their proportion of MeHg relative to T-Hg, especially when comparing invertebrate and fish prey. The ability to interpret empirical observations of lake trout MeHg concentration

would have been confounded without taking into account differences in prey MeHg concentration. For example, if T-Hg measurements were used for invertebrate diet items the bioenergetics model would have predicted that lake trout collected during the pre-removal period – consuming invertebrates with T-Hg concentrations similar to prey fish but with less MeHg – were higher in T-Hg concentrations than those collected during the post-removal period. The conclusions drawn from the simulations neglecting to account for percent MeHg would be that growth dilution was very important and accounts for large differences in lake trout MeHg concentration in the study system.

The results from this Chapter suggest that changes in food web structure and dynamics can have significant influences on MeHg concentrations in top predators such as lake trout. Specifically, alterations in fish density can influence the individuals remaining within a system. This mechanism could potentially be used to increase growth rates while maintaining or reducing levels of T-Hg intake, resulting in "growth dilution" observed previously (Göthberg 1983; Verta 1990; Rask 1996). Importantly, several food web components must be characterized before the resulting MeHg concentrations in fish within a given system can be understood. For example, the Little Moose Lake manipulation showed that T-Hg content alone of species (predator and prey) is not necessarily important in determining the effects of food web changes on MeHg concentrations in lake trout. As such, one must identify and understand the characteristics (e.g., energy and MeHg content) of the resources that may become more or less available as a result of changes in food web dynamics. By understanding these and other characteristics, it may be possible to evaluate how alterations in food webs could influence the MeHg concentrations in sport fish which has the potential to minimize human health risk.

Conclusion:

Nearly every aquatic ecosystem experiences environmental and anthropogenic perturbations. The results from this dissertation provide information that can be used to better understand and manage systems that are or could potentially be influenced by perturbations. The variety of metrics used in the set of experiments described in this dissertation were successful to varying degrees for providing a means of investigating the mechanisms behind three important challenges currently facing aquatic systems managers, including: 1) alewife die-offs in the Great Lakes and other freshwater systems, 2) elevated thiaminase activity in clupeid fish and 3) mercury contamination in aquatic food webs. I have achieved my objective of gaining a better understanding and exploring potential means to remediate negative impacts associated with these issues.

Most of the techniques I used to conduct this set of experiments are flexible research tools that can be used to varying degrees across species in a variety of ecosystems, including aquatic, marine and terrestrial systems. By applying these techniques, I was able to evaluate ecosystem characteristics responding to perturbation at the level of the individual through the community level. I was also able to monitor biotic and abiotic factors that had the potential to be influenced by perturbation. When used correctly, these metrics serve as indicators of perturbation at multiple scales and can be used to help address and understand environmental responses to a wide variety of perturbations in aquatic, marine and terrestrial systems.

Understanding the sources and impacts of: 1) alewife die-offs in the Great Lakes and other freshwater systems, 2) elevated thiaminase activity in clupeid fish and 3) mercury contamination in aquatic food webs is useful because this information may be applied to management strategies to reduce negative impacts associated with these environmental issues. Aquatic systems are influenced by stochastic events, variation

in aquatic community composition and other various perturbations that can act relatively rapidly in certain cases (e.g., fish die-offs or temperature fluctuations). Additionally, strategies that change fish community structure are frequently used in managing sport fisheries. Methods including fish stocking, setting regulations on fish harvest by anglers, and the removal and introduction of species can modify aquatic ecosystems. All of these factors have the potential to alter fish density, community composition, prey type and availability, as well as fish size and age structure through changes in predation and competition for resources. Quantifying and monitoring the impacts of environmental and anthropogenic perturbation with respect to key characteristics (e.g., alewife mortality, increased thiaminase activity in clupeids or MeHg concentration in sport fish) is important because these characteristics have the potential to change rapidly in response to perturbation, often with far-reaching impacts.

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CHAPTER TWO
ALEWIFE MORTALITY, CONDITION, AND IMMUNE RESPONSE TO PROLONGED COLD TEMPERATURES

Abstract:

Alewife, *Alosa pseudoharengus* have been recognized for several decades as one of the most important forage fish in the Laurentian Great Lakes. Although massive alewife die-offs have regularly been observed throughout the Great Lakes and other inland lakes, little substantive information is available regarding physiological mechanisms associated with adult alewife mortality. Long-term field surveys have shown a correlation between cold winter temperatures, poor condition and adult alewife mortality. In this study, adult alewife were held in replicate pond systems and subjected to contrasting cold temperatures (4 and < 2°C) representing mild and severe winter conditions. We evaluated alewife mortality, condition and immune response to these temperatures. In contrast to our expectations, alewife exposed to mild and severe winter temperatures showed no difference in mortality or condition (measured as the ratio of dry to wet weight). Survival of alewife held in ponds with mild winter conditions (~ 4°C) was similar to that of alewife exposed to prolonged periods (more than six weeks) of temperatures < 2°C. This result contrasts with previous observations indicating that alewife cannot tolerate temperatures < 3°C. Circulating lymphocytes from alewife exposed to severe winter temperatures were significantly lower in number (~40%) compared to fish experiencing milder winter conditions, suggesting sub-lethal immunosuppression in response to the colder winter temperatures. Although colder winter temperatures did not directly induce alewife

mortality, these results suggest that winter conditions that result in colder water temperatures can produce immunosuppression, thereby increasing alewife susceptibility to disease and mortality.

Introduction:

Alewife (*Alosa pseudoharengus*) have been recognized for more than three decades as one of the most important forage fish in the Laurentian Great Lakes (Smith 1970), as evidenced by the heavy reliance of salmonine predators on alewife as prey (Jude *et al.* 1987; Lantry 2001; Madenjian *et al.* 2002). Fluctuations in alewife abundance and condition can affect salmonine growth and survival (Stewart *et al.* 1981; Rand and Stewart 1998). As a result, salmonine communities in the Great Lakes are closely linked to the success of alewife populations upon which they rely as forage.

Massive alewife die-offs have been observed throughout the Great Lakes and in other inland lakes since the late 1800s (Goode 1884; Pritchard 1929; Colby 1973). Researchers have suggested that several factors (e.g., temperature induced spawning stress, low food availability resulting in poor condition) could cause mortality (Flath and Diana 1985; O'Gorman and Schneider 1986; Bergstedt and O'Gorman 1989), and cold temperatures are known to challenge the osmoregulatory capabilities of alewife (Stanley and Colby 1971; Snyder and Hennessey 2003). Temperature changes can alter the permeability of cell membranes in teleost fishes, leading to impaired ion transport and membrane-associated enzyme function (Hazel and Williams 1990; Hazel 1993). As such, severe winter temperatures were considered as a possible contributor to alewife mortality (Eck and Brown 1985; O'Gorman and Schneider 1986; Bergstedt and O'Gorman 1989). Fungal infections of *Saprolegnia sp*. were documented in field-collected and laboratory-reared alewife that had been exposed to cold winter

temperatures (Graham 1956; Brown 1968; Colby 1973), but the causal nature of these infections has not been evaluated.

Although several studies have been conducted to investigate the effects of cold temperatures on the immune response of teleost fishes, factors influencing the immune response of alewife have not been previously evaluated. Exposure to cold temperatures has been suggested to produce immunosuppression, as measured by a decrease in lymphocyte function and number of circulating lymphocytes (lymphopenia) in bluegill (*Lepomis macrochirus*) (Cuchens and Clem 1977), channel catfish (*Ictalarus punctatus*) (Clem *et al.* 1984; Miller and Clem 1984), hybrid striped bass (*Morone chrysops* x *Morone saxatilis*) (Hrubec *et al.* 1997) and pinfish (*Lagodon rhomboids*) (Abruzzini *et al.* 1982). Lymphopenia has also been induced in brown trout (*Salmo trutta*) (Pickering 1984) and rainbow trout (*Oncorhyncus mykiss*) (Barton *et al.* 1987) by feeding cortisol, a stress hormone, directly to fish. Several cell types serve as indicators of an immune response to stimuli (Iwama and Nakanishi 1996): lymphocytes (comprised of T and B-cells, which are the primary cell types generally responsible for activating macrophages and producing antibodies respectively), neutrophils (short-lived, granulated phagocytic cells that combat microorganisms), monocytes (longer-lived phagocytic cells that combat microorganisms) and eosinophils and basophils (both involved in inflammatory and allergic responses associated with viral infections and parasites). Previous investigations have shown that immune system dysfunction in fish increases their susceptibility to antigens and subsequent mortality (Arkoosh 1998b; Arkoosh and Collier 2002).

Based on previous observations, two lines of evidence prompted us to evaluate whether the immune response of alewife changed in response to contrasting winter temperature conditions representative of mild and severe winters: (1) the observed immune response to cold temperatures in other fishes, and (2) observations that colder

temperatures increased the susceptibility of alewife to *Saprolegnia sp.* infection and subsequent mortality (Graham 1956; Brown 1968; Colby 1973). In this study we evaluated adult alewife mortality, condition and immune system response to different thermal conditions in replicated pond experiments. We hypothesized that alewife exposed to mild winter temperatures would experience reduced mortality, lower water content, and greater levels of immune system activity than alewife exposed to colder temperatures, and that alewife mortality would be near 100% in ponds with severe winter temperatures.

Methods:

This experiment was conducted in replicate 0.1 ha ponds (2.5 m maximum depth) at the Cornell Experimental Pond Facility. Each pond is lined with clay, limiting water exchange between ponds and other inputs such as groundwater that might provide thermal refuge during winter conditions. Anoxic conditions were not observed within the study ponds, and water conductivity was ≈ 300 μS/cm. throughout the experiment.

Alewife were collected from Waneta Lake (Schuyler County, NY) on 26 Oct 04 from a single site with a water temperature of approximately 10º C. Based on previous experience, alewife were kept at approximately 10ºC during transport and water temperature in the experimental ponds was also 10ºC at the time of stocking. Thirty alewife were dried for water content analysis prior to stocking, and 160 alewife were stocked into each of four fishless ponds (640 alewife total) on 27 Oct 04. Only mature, adult alewife between 120 and 140 mm were used in this experiment. Contrasting winter temperatures were produced by maintaining ice-free conditions using aeration in two of the study ponds, beginning at the time of ice formation (early December). Ponds were aerated using a small land-based air compressor with four

hoses contained in individual, large (0.5 m diameter) weighted flower pots (two in each pond) from 11 Dec 04 to 5 Jan 05 to generate cold pond temperatures. Hoses were held in place through the bottom of the upright flower pots and were never in contact with pond sediment, thereby minimizing resuspension and circulation of substrate material but allowing thorough thermal mixing. Temperature loggers were deployed at a depth of 1 m in each of the four ponds from December 04 to April 05.

We attempted to collect alewife from the frozen ponds to evaluate alewife feeding activity during the winter months using 30-m long, 25.4 mm stretch mesh, monofilament gill nets during mid-January 05. Weighted ropes were placed on the bottom of each pond prior to ice formation and then used to pull gillnets across the ponds.

On 21 Apr 05 water was pumped from each pond to achieve a depth of approximately 1.2 m. A bag seine approximately 40-m long and 1.2-m tall was used to collect 25 live alewife from each pond. Alewife were sacrificed using MS-222 (125 mg/L) and blood samples were drawn using a 28-gauge needle and 0.5 ml non-heparinized syringe from the caudal vessels in the hemal arch of ten individual alewife from each of the four ponds. For white cell differential counts (the process of identifying and counting different types of white blood cells), blood smears were prepared immediately on glass slides that were dried and subsequently fixed and stained using a three step Diff-Quik kit (Sigma-Aldrich Chemical; St. Louis, MO).

We performed white cell differential counts by locating monolayered cell regions on each slide (using 1,000x magnification), then identifying the first 200 white cells that were observed within each blood smear. These analyses were conducted by a single, trained individual for standardization. The resulting data was used to generate a ratio of lymphocytes, neutrophils, monocytes and other granulocytes to total white blood cells that was subsequently used to estimate differential white cell

counts. Due to their relative scarcity, eosinophils and basophils were combined into a single category, called "other granulocytes". A total white blood cell count was determined by averaging the number of white blood cells within ten high-power fields (400x magnification) in a monolayer portion of each slide, then multiplying this value by 2,000 to arrive at a cell count quantified as number per microliter (Campbell 1994a; Campbell 1994b). The differential ratios and leukocyte count data were combined to obtain a total count for each cell type. These estimated values for each cell type assumed that each slide was prepared in a similar fashion (i.e., similar pressure was used for each smear) and that the blood was of similar viscosity. Cells were not counted at slide locations that included clumped regions of cells. In order to maintain consistency in the white cell count estimates, these counts were conducted by the same trained individual who processed the differential counts.

Fifteen alewife were sacrificed (see Methods above) from each of the four ponds at the end of the experiment to compare the ratio of dry/wet weight in fish from each experimental treatment. Alewife were kept on ice until they could be accurately weighed (to the nearest 0.5 g) and measured in the lab, then were subsequently reweighed after being placed in a drying oven at 60º C for 168 hours. The proportion of water in each individual was used as an indicator of condition, following the approach described by Flath and Diana (1985). After alewife were collected for these analyses, the ponds were drained completely and all remaining alewife were collected and counted.

Mean total mortality for alewife in each pond as well as alewife initial (Waneta Lake) versus final (post-pond experiment) water content were compared with an analysis of variance (ANOVA) using SPlus (Insightful Corporation, Seattle, Washington). Mixed-model analyses (pond as a random effect and treatment as a fixed effect using the PROC_MIX procedure) were conducted using SAS (SAS

Institute Inc.) to test for treatment effects on alewife water content and white blood cell counts.

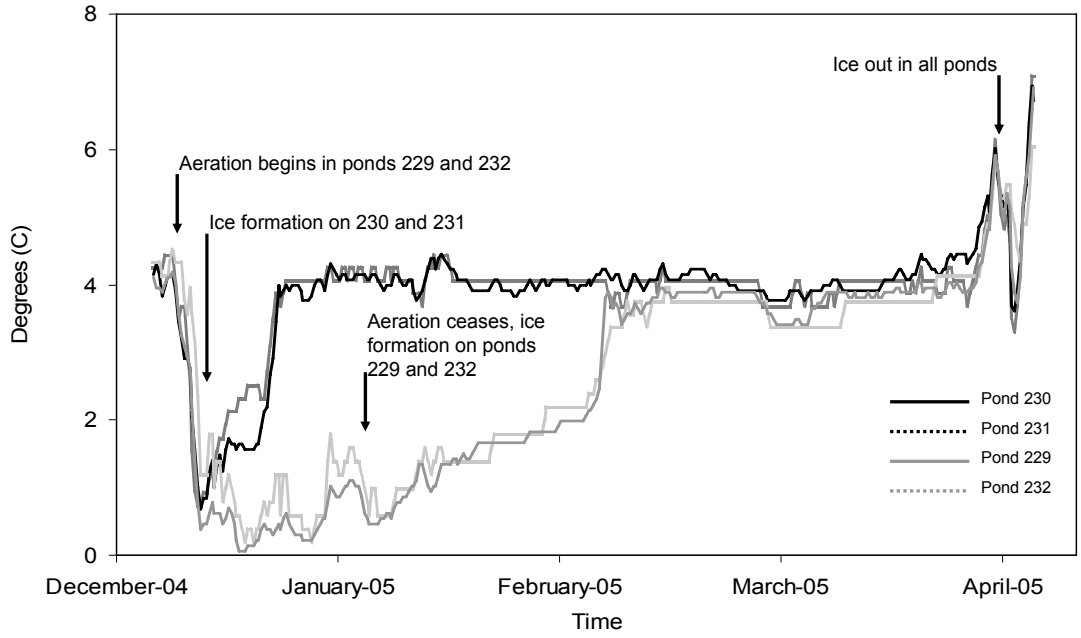

Figure 2.1. Temperatures throughout the experimental period for each experimental pond. Status of the aeration system and ice conditions are included in the figure. Ponds #230 and 231 experienced mild winter temperatures while ponds #229 and 232 experienced more severe winter temperatures.

Survival in both temperature treatments (mild temperature ponds: 28% and 27.5%; severe temperature ponds: 30% and 23%) was not significantly different (one-way ANOVA; $F = 0.13$, $p = 0.75$, n's = 2). Eleven alewife were caught in gill nets set in a mild temperature pond (#231) on January 11, however, additional attempts to capture alewife in the other ponds were unsuccessful. Diet items found within stomachs of the 11 alewives included chironomid larvae, cladocerans and immature ephemeropterans, along with sediment and small pieces of macrophytes. A total of 36 individual diet items weighing approximately 1 g (total wet weight) were present in all stomachs analyzed yielding an average ration of < 0.01 g/g body weight. The alewife

that were removed in mid-January were excluded from the survival analysis, however, their inclusion did not change the significance of the comparison (results not shown).

No difference in the dry/wet weight ratio was observed between alewife maintained in ponds with mild and severe winter temperatures, (Mixed-model; $F < 0.01$, $p < 0.99$, n's = 30) suggesting alewife condition was similar (*cf* Hartman and Brandt 1995). The mean percent water (± 1 SD) of alewife collected prior to stocking (69.2 ± 2.9%) was slightly lower than for fish at the end of the experiment (70.8 ± 2.3%) (one-way ANOVA; $F = 3.81$, $p = 0.03$, n's = 30). A Tukey's pairwise comparison confirmed that the percent water content of alewife at the time of stocking was lower than that of fish removed from ponds at the end of the experiment.

Circulating lymphocyte, neutrophil, monocyte and other granulocyte counts varied between alewife subjected to mild and severe temperatures (Figure 2.2). The lymphocyte counts of the alewife subjected to mild temperatures was 60% higher than those for fish subjected to severe temperatures (see Table 2.1). Neutrophil, monocyte, and other granulocyte counts were not significantly different between the alewife from mild temperature ponds and severe temperature ponds respectively.

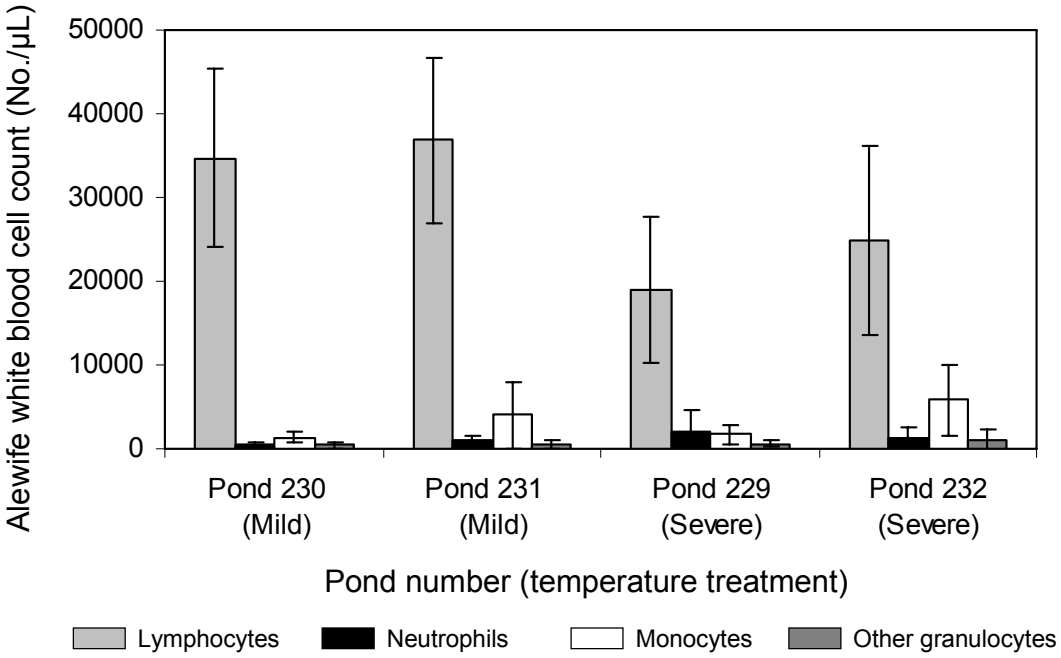

Figure 2.2 Mean estimated cell counts from alewife collected from ponds in April 2005. Indices were defined as the mean white cell count per field at 1000x magnification multiplied by the white cell differentials determined from the first 200 white cells identified. Error bars represent one standard deviation from the mean.

Table 2.1. Mean (± 1 std. dev.) white cell differential counts (No./µl) by treatment. The *F*-statistic and *p* value for Mixed-model analyses are listed. N=20 for every treatment in each comparison.

Cell type	Mild temperature	Severe temperature	*F*	*p*
Lymphocyte	36,000 ± 10,000	22,000 ± 10,000	18.37	< 0.01
Neutrophil	720 ± 570	1,800 ± 1,900	5.24	0.15
Monocyte	3,800 ± 3,700	2,700 ± 3,000	0.21	0.69
Other granulocyte	770 ± 1,000	480 ± 460	1.39	0.25

Discussion:

Our results indicate that six weeks of exposure to temperatures < 2°C was not sufficient to cause an increase in alewife mortality when compared with alewife exposed to less severe temperatures. Additionally, alewife condition – measured as the proportion of water in fish tissue – did not differ between fish subjected to mild and severe temperatures. Alewife water content was slightly greater when the fish were collected in the spring relative to when they were stocked, but this was likely due to lower feeding rates during the winter months (Flath and Diana 1985). Diet analysis of alewife collected in January from a single mild temperature pond (#231) indicated that these fish were feeding at low levels, but we were unable to capture fish from the other ponds for comparison. The most distinct difference between alewife exposed to the mild and severe temperature treatments was the presence of lower circulating lymphocyte counts in alewife subjected to severe winter conditions.

By evaluating the alewife response to cold temperatures in replicated pond systems, this study expands upon what was learned about alewife thermal tolerance in laboratory studies conducted three decades ago (Colby 1973). Alewife collected for the experiments conducted by Colby (1973) were young-of-year alewife from Lake Michigan that never experienced winter conditions outside of the laboratory. When these fish were acclimated to low temperatures for prolonged periods of time in the laboratory they experienced mortality once temperatures were reduced to 4.0º C and lower. Colby (1973) also collected adult alewife that had experienced winter conditions in Lake Michigan and subjected them to decreased water temperatures. 100% of the alewife contracted "*Saprolegnia*-like" fungal infections prior to death when held at temperatures approximately 5.6ºC and cooler (Colby 1973). Alewife in those experiments were reported to have appeared healthy prior to the initiation of cold temperature stress. Wells (1968), however, collected thousands of alewives in

Lake Michigan at temperatures < 3.0ºC in February and March, and smaller numbers of alewife were captured at shallow depths in even colder temperatures approaching 2.0º C throughout the sampled water column. With these observations in mind, it is worth noting that we observed similar high mortality in both ~4ºC and <2ºC pond treatments.

Although considerable mortality was observed in our study, these types of field experiments remain challenging to conduct. Stress associated with transporting and maintaining alewife in relatively small ponds might have contributed to the observed mortality, however, these conditions were similar for all alewife used in the study (aside from the temperature manipulation). We also recognize that high overall levels of mortality in this study may have limited our ability to detect small differences in mortality resulting from severe winter temperatures. However, we expected differences in mortality to be large. Nevertheless, we have demonstrated that some alewife can survive extended periods of time in water less than 2.0°C under certain conditions. These results were unexpected.

The lower counts of lymphocytes observed in alewife from ponds with severe winter temperature conditions was similar to that reported from other fish species exposed to altered thermal conditions. For example, striped bass were found to have significantly lower white cell counts (~20,000 total white cells versus ~60,000 per microliter respectively) after a six week exposure to cold (10ºC) versus warm (18ºC, 24ºC and 29ºC) water temperatures in the laboratory (Hrubec et al. 1997). Differences in lymphocyte counts of alewife were also similar to those observed in induced stress trials using brown trout (Pickering 1984) and rainbow trout (Barton et al. 1987). Although we observed small, non-significant differences in the counts of other cell types in response to experimental temperature treatments, the response in lymphocyte counts (the dominant cell type) was larger, and significant differences between

treatments were consistently observed. The lower lymphocyte counts likely play an important role in alewife health and susceptibility to antigens. Although the cell counts do not provide direct evidence of pathological challenges, previous studies have suggested that changes within fish immune systems similar to those observed in our study have increased the susceptibility of fish to disease (Barker et al. 1994; Arkoosh et al. 1998b; Arkoosh and Collier 2002). Limited blood work was conducted on alewife in the late 1970's in a study of a red cell infection called piscine erythrocytic necrosis (PEN) (Sherburne 1977). Since that time hematological characteristics of alewife have remained largely unexplored, and immunosuppression induced by cold temperatures has never been investigated. White cell data are not available for alewife from other ecosystems, thus comparisons between alewife white blood cell counts at the end of this experiment relative to alewife collected from natural settings remain speculative.

Substantial evidence linking massive alewife mortalities to disease has been found in the past. Several studies have shown that a range of factors – such as low temperatures, anthropogenic pollutants and general stress – can increase the incidence of disease and parasitism in a variety of fish species (Bly et al. 1997; Arkoosh et al. 1998a; Harris et al. 2000). These studies have confirmed that immunosuppression limits the ability of fishes to fend off disease and parasites and decreases their ability to survive when exposed to such challenges. The lower lymphocyte counts at colder temperatures indicates that alewife experiencing winter conditions of similar duration and intensity might be susceptible to disease and subsequent mortality resulting from a reduced white cell count. Graham (1956) suggested that alewife experiencing stress from rapid temperature changes were more susceptible to infection by *Saprolegnia sp.* than other alewife. Brown (1968) reported a 20% incidence of *Saprolegnia sp.* infection in alewife visually examined after the 1967 die-off in Lake Michigan. Colby

(1973) observed that 100% of adult alewife exposed to cold temperatures contracted "*Saprolegnia*-like" fungal infections while control alewife experienced only 30% mortality. Despite these observations – and despite concerns about large alewife die-offs in the Great Lakes – little emphasis has been placed on studying the response of alewife to disease.

Age and maturity has been shown to affect the composition of white cell types in fish (Blaxhall 1972; McCarthy et al. 1975; Hrubec et al. 2001). The alewife used in this study were mature, adult fish, and only fish between 120 and 140 mm total length were evaluated for blood cell composition analyses. Thus, alewife age and maturity were not factors in the results of this study. Aeration was used to cool two of the four ponds, and the physical disturbance caused by aeration had the potential to alter other conditions such as dissolved oxygen, suspended solids and water movement. An effort was made to minimize the aeration disturbance of pond substrates by using flower pots to minimize interactions between bubbles and pond substrate, yet at the same time water movement was sufficient to ensure that the cold ponds were well-mixed throughout the water column with respect to temperature and dissolved oxygen.

Alewife white cell counts were significantly lower in fish exposed to cold temperatures, and though the procedure used for counting cells is limited to making comparisons between fish within the same study, white cell counts may present a useful and underutilized measure of the status of immune system integrity in other fishes captured in the wild. White cell counts have been used extensively in laboratory and clinical trials involving salmonid fishes, but they have not been used widely in natural systems (Barton and Iwama 1991; Anderson 1996). Basic measures of white cell differentials and counts, when combined with appropriate study design, have provided useful insights in natural systems (e.g., Barker et al. 1994; Arkoosh 1998b; Arkoosh and Collier 2002). White cell differentiation and enumeration are

techniques that can be employed without harming individual fish, thereby facilitating the evaluation of fish immune system and stress responses to contrasting environmental conditions. We believe that the potential utility of these techniques has not been fully realized in natural aquatic systems.

Speculative explanations regarding the cause of massive alewife die-offs in the Great Lakes have focused on attempts to find correlations between environmental or biological factors – such as temperature, food availability and spawning stress – and long-term estimates of alewife abundance and condition (Flath and Diana 1985; O'Gorman and Schneider 1986; Bergstedt and O'Gorman 1989). Our experimental results suggested that poor alewife immune system integrity may also be related to severe winter temperatures. Severely cold temperatures could be responsible for producing sub-lethal effects causing alewife to be more susceptible to mortality from disease, parasitism, and bacterial infection. It is likely that under our study conditions – i.e. these were closed systems with low alewife density, the fish were relatively small, and the fish community was exposed to the same pathogens – all alewife were at a lower risk of disease and parasitism than under ambient Great Lakes conditions (Bagge and Valtonen 1999; Valtonen et al. 2003; Bagge et al. 2004). Although high mortality in this experiment may have limited our ability to detect slight differences in mortality if the alewife in the ponds had been exposed to high levels of pathogens, it is possible that alewife experiencing severe winter temperatures would have responded differently (e.g. higher mortality) relative to alewife in warmer ponds.

Alewife water content was not related to the cold temperature treatments in this experiment. This suggests that factors other than severe over-winter temperatures (e.g. alewife condition going into winter or spawning or some other stress) play a more important role in determining alewife mortality and condition in spring than severe temperatures themselves. Interactions between water temperature, winter

duration, food availability, and alewife feeding efficiency could also affect alewife mortality and condition, but information regarding food availability, daily ration, and activity were not available to directly evaluate these factors. Nevertheless, because our experimental treatments did not influence fish condition, our results suggest that severe winter temperatures of the duration and intensity simulated in this study by themselves have little direct effect on alewife condition.

Surface and nearshore temperatures in the Laurentian Great Lakes approach 0ºC during most winters. Although alewife often move to deeper, warmer (i.e., 3.0 to 4.0ºC) water after fall turnover (Wells 1968), Great Lakes alewife can be exposed to temperatures < 3.0ºC for prolonged periods (Schroeder 1963). Recent climate circulation models project future changes in global climate, and it is likely that Great Lakes ice conditions and nearshore temperatures will be affected (Hodgkins et al. 2002). Our results suggest that severe winter temperatures have the potential to induce changes in alewife immune response which could lead to increased mortality. Our results expand upon previously available knowledge regarding the temperature tolerance and immune response of alewife in field conditions and will hopefully lead to further studies expanding our understanding of factors influencing winter alewife mortality.

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CHAPTER THREE
EVALUATING THE EFFECT OF STRESSORS ON THIAMINASE ACTIVITY IN ALEWIFE

Abstract:

No consistent explanation has been found for variability in alewife *Alosa pseudoharengus* thiaminase activity, despite the influence of alewife thiaminase on large-scale salmonine mortality in the Laurentian Great Lakes. We conducted experiments to evaluate the effect of two stressors, reduced water salt content and food limitation, on alewife thiaminase activity. Alewife were subjected to treatments in replicated tanks in which conductivity was lowered (< 100 μS/cm) for 8 days and feeding was limited for 39 days. Alewife circulating white blood cells, plasma cortisol, plasma glucose and whole body thiaminase were measured in individual alewife to assess their response to these experimental treatments. Alewife from salt and food-limited controls had significantly larger numbers of circulating white blood cell counts relative to salt and food-limited treatments (means = 24,000 and 19,000 to 11,000 and 9,000 cells/μL for alewife from the two control and salt-limited treatment tanks respectively; 34,000 and 30,000 to 21,000 and 16,000 cells/μL for alewife from the two control and food-limited treatment tanks respectively). No significant differences were found between alewife thiaminase activities in treatments versus their respective controls. Mean thiaminase activity in study alewife increased from 6,900 to 16,000 $pmol \cdot g^{-1} \cdot min^{-1}$ from the time of their collection in Cayuga Lake to the start of lab experiments 1 ½ to 2 ½ years later; the latter value was more than twice that of previously reported levels of thiaminase activity from alewife collected in the wild.

These data suggest that variability in alewife thiaminase is not related to stress from salt or food limitation, but laboratory holding conditions significantly increased thiaminase through a mechanism not evaluated by our experimental treatments.

Introduction:

Alewife *Alosa pseudoharengus* have been the most important forage fish in the Great Lakes for over 40 years (Madenjian et al. 2002; Mills et al. 2003; Dobiesz et al. 2005). Following alewife introductions and subsequent proliferation, Great Lakes food webs have been completely altered such that salmonine predators rely primarily on alewife as prey (Jude et al. 1987; Lantry 2001; Madenjian et al. 2002; Mills et al. 2003). Over the past three decades, a thiamin-deficiency-related reproductive failure (early mortality syndrome: EMS) has been observed in valuable Great Lakes fishes, including lake trout *Salvelinus namaycush* and Atlantic salmon *Salmo salar* (McDonald et al. 1998; Honeyfield et al. 2005a). High levels of a thiamin-destroying enzyme, thiaminase, have been found in alewife (Fitzsimons et al. 2005; Tillitt et al. 2005), and thiaminase is responsible for EMS in salmon and trout that typically prey upon alewife (Fitzsimons et al. 1999). Characteristically, offspring of salmonine fishes susceptible to EMS die shortly after hatching, but fry from identical egg sources survive and exhibit normal behavior when treated with thiamin (Fitzsimons and Brown 1998). The mechanism behind the expression of thiaminase in alewife is unknown, and no single factor has consistently explained observed variability in thiaminase activity – though high levels of thiaminase have been associated with algae blooms, (Burkholder 1998; McDonald et al. 1998; Fitzsimons et al. 1999) alewife condition, season and location (Fitzsimons et al. 2005; Tillitt et al. 2005).

Honeyfield et al. (2002) isolated thiaminase positive bacteria (*Paenibacillus thiaminolyticus* and other closely related bacteria) from alewife viscera, and alterations

in diet levels of bacterially produced thiaminase have been used to demonstrate EMS in laboratory experiments with lake trout (Honeyfield et al. 2005b). In situations in which thiaminolytic bacteria are the source of thiaminase activity in alewife, changes in physiological conditions – resulting from environmental or other sources of stress – that influence the growth characteristics of these bacteria can be expected to affect thiaminase activity (Tillitt et al. 2005). Laboratory studies have shown that one response of many fish to chronic stress is a reduction in number of circulating white blood cells, which play a role in suppressing bacteria (Pickering 1984; Barton et al. 1987). The interaction between the clupeid immune system and internal bacterial communities is not understood, though changes in circulating white blood cells have been shown to serve as indicators of an immune response to stressful stimuli in other fish (Iwama and Nakanishi 1996). Limited blood work was conducted on alewife in the late 1970's in a study of a red blood cell infection called piscine erythrocytic necrosis (PEN) (Sherburne 1977). Since that time hematological characteristics of alewife have remained largely unexplored.

The influence of stress upon alewife has been of interest since massive alewife die-offs were observed throughout the Great Lakes in the 1960s, and a limited number of early research efforts evaluated physiological changes related to alewife mortality (Stanley and Colby 1971; Colby 1973). Subsequent studies suggested that several stressful factors (e.g., freshwater altering osmoregulation and low food availability) could contribute to alewife mortality (Mills et al. 2003; Snyder and Hennessey 2003). Stress response in fish is linked to increased circulating plasma cortisol concentrations (Barton and Iwama 1991; Gamperl et al.1994; Mommsen et al. 1999), subsequently leading to immunosuppression when stressful conditions are chronic, i.e., occurring on the order of days to weeks or more (Mommsen et al. 1999). Intensive performance and mortality studies on fish have been conducted in order to examine the influence of

various environmental conditions – including salinity and food availability – upon cortisol secretion (Barton and Iwama 1991; Mommsen et al. 1999). Most of these studies were conducted on salmonine fishes, and only two studies have evaluated cortisol levels in clupeid fishes, none of which included alewife (Davis and Parker 1986; Shrimpton et al. 2001). Thus no information regarding alewife plasma cortisol or glucose levels is available from previous studies, and the effect of salt and food limitation on alewife circulating white blood cells and thiaminase has not been evaluated.

Given knowledge of the association between thiaminolytic bacteria and thiaminase in alewife, we expected that environmental stressors capable of influencing alewife mortality and other physiological characteristics would also influence alewife thiaminase activity. Indirect effects associated with alewife stress could alter thiaminase activity, possibly through a mechanism whereby thiaminase is produced more efficiently and/or thiaminolytic bacteria proliferate. Appropriate genetic tools have not yet been applied to evaluate thiaminolytic bacterial communities or the expression of the thiaminase gene.

Low salinity and food limitation were selected as treatments in this experiment to examine their effects on thiaminase in alewife. We hypothesized that these treatments would result in lower circulating white blood cells, ultimately leading to an increase in thiaminase activity. Alewife plasma cortisol and glucose levels were also measured to evaluate if acute factors could contribute to differences in thiaminase activity.

Methods:

Alewife were collected in Spring 2003 (food-limited trials) and 2004 (salt-limited trials) from Cayuga Lake, then were transported by truck to the USGS

Northern Appalachian Research Facility (Wellsboro, PA). Whole alewife (N = 10) from the 2004 sampling period were flash frozen on dry ice and sent for thiaminase analysis as described below to evaluate initial thiaminase activity. An additional 30 alewife collected in 2004 were weighed and then dried for a minimum of 120 hours to evaluate the water content of alewife as an indicator of condition. Prior to the start of experiments alewife were held in 2 m diameter circular flow-through tanks (4 m^3 volume) fed by well-water at densities of approximately 50 to 100 fish m^{-3}. Commercial trout feed was provided to alewife once each hour from 0800 to 1600 daily with automated feeders until experimental trials began in Spring 2005. Alewife were transferred from holding tanks to identical experimental tanks in groups of 22 (12 fish for analysis, along with an additional 10 fish to be used in the event of mortality). Alewife from the same holding tank were used for each treatment and associated control to ensure that fish within all comparisons were held and transferred under similar conditions prior to experiments. Conductivity within holding tanks was maintained above 300 μS/cm by adding artificially produced saltwater using sodium chloride to incoming well-water, and these salinity levels were regulated individually for each tank by a metered pump. Water temperatures were maintained at approximately 13.5°C throughout the experiment with the use of heaters in each tank. All holding tanks were slowly adjusted to these conditions to ensure that fish experienced as little stress as possible prior to experiments. A minimum of 72 hours was allowed for acclimation after the transfer process, which is a typical amount of time required for physiological "resting" plasma cortisol levels to return to normal after tank transfer and handling stress (Pickering 1984). Fish in each tank were randomly assigned to a control or treatment group. Each treatment and control group were replicated twice, resulting in a total of four experimental tanks containing 22 alewife apiece for both the salt and food limitation trials (176 alewife total).

At the completion of each trial, 12 alewife were removed individually from each tank using a small dip net with as little disturbance as possible to the remaining fish. Alewife were collected by allowing a single fish to swim into the dip net under its own power, after which it was slowly removed from the tank. The netting procedure was conducted under low light conditions to further minimize stress to alewife during sampling. Each alewife was heavily anaesthetized (MS-222, 125 mg/L) and subsequently killed. Blood (~650 µL) was extracted from the caudal vessel in the hemal arch of each captured alewife using heparinized syringes, after which blood was centrifuged at 20,800x gravity for five min. Plasma was decanted from the blood samples and stored at -20ºC until plasma cortisol and glucose analyses were conducted. A solid phase RIA (radioimmunoassay) was used to measure plasma cortisol (Davis and Parker 1986) and a double enzymatic procedure was used to measure glucose (Sigma Diagnostic, St Louis, Missouri) at the College of Veterinary Medicine, Cornell University. Duplicate measurements were taken for each individual sample. If the duplicate measurements did not correspond or the means of these measurements were outside of the acceptable range relative to other samples in the experiment (based on the discretion of laboratory personnel experienced in measuring cortisol and glucose), the sample was run in triplicate. All blood samples were collected within 4 min of netting each individual fish.

An additional droplet of blood from each alewife netted was collected for white blood cell differential counts. The droplet was used to immediately prepare blood smears on glass slides that were dried and subsequently fixed and stained using a three step Diff-Quik kit (Sigma-Aldrich Chemical; St. Louis, MO). We performed white blood cell differential counts by locating monolayered cell regions on each slide (using 1,000x magnification), then identifying the first 200 white blood cells that were observed within each blood smear. These analyses were conducted by a single,

trained individual for standardization. The resulting data were used to generate a ratio of lymphocytes, neutrophils, monocytes and other granulocytes. Due to their relative scarcity, eosinophils and basophils were combined into a single category, referred to as "other granulocytes". A white blood cell count was determined by averaging the number of white blood cells within 10 high-power fields (400x magnification) in a monolayer portion of each slide, then multiplying this value by 2,000 to arrive at a cell count quantified as number per microliter (Campbell 1994a; Campbell 1994b). The differential ratios and white blood cell count data were combined to obtain a total count for each cell type. These estimated values for each cell type assumed that each slide was prepared in a similar fashion (i.e., similar pressure was used for each smear) and that the blood was of similar viscosity. Cells were not counted at slide locations that included clumped regions of cells. In order to maintain consistency in the white blood cell count estimates, these counts were also conducted by a single, trained individual.

Thiaminase analyses were conducted on each of the 12 captured fish per tank. Thiaminase activity ($pmol \cdot g^{-1} \cdot min^{-1}$) was measured on individual whole, homogenized fish at the Canadian Center for Inland Waters, Burlington, Ontario using the radiometric procedure described by Zajicek et al. (2005). All 48 alewife sent for thiaminase analysis were flash frozen on dry ice immediately after collection and blood sampling prior to shipping. Remaining alewife (i.e. those not evaluated for thiaminase) from the original 22 individuals in each tank were weighed, and whole body water content (an indicator of condition, see Flath and Diana 1985) was measured by drying each fish in a drying oven for a minimum of 120 hours at 60ºC.

Experimental treatment conditions:

Salinity treatments were initiated March 21, 2005 and ended on March 28, 2005. Salinity levels in the two treatment tanks were lowered to less than 100 $\mu S \cdot cm^{-1}$ in an attempt to induce osmoregulatory stress, and all other conditions within treatment and control tanks remained as stated above. Food limitation treatments began April 8, 2005 and ended on May 16, 2005. Provision of food was ceased in the two treatment tanks to represent low food availability as a form of stress, and all other conditions within treatment and control tanks remained as stated above. Both of these treatments were chosen because they had been shown previously to produce mortality in alewife held in captivity at the USGS Northern Appalachian Research Laboratory (Honeyfield, unpublished data).

Netting stress trial:

A brief experiment was conducted to evaluate the assumption that the process of netting individual alewife did not influence plasma cortisol levels in other fish in the tank. Alewife were transferred from a holding tank to three additional tanks with similar conductivity, temperature and feeding conditions in groups of 15 (45 fish total), after which these alewife were subsequently re-captured 72 hours later. During this and other study experiments, fish were not chased and were only netted from tanks when they swam into the capture net on their own accord. After netting, alewife were sampled for plasma cortisol as described above. The only exception to this procedure was the 14th fish from tank 3 (see Results) which was inadvertently chased vigorously for over a minute and processed after a period of approximately 10 min. Aside from this exception, all other fish were captured and blood collection was completed in less than four minutes.

General stress trial:

In an attempt to assess ambient levels of alewife plasma cortisol and glucose in a hatchery setting we conducted an exploratory experiment to determine plasma cortisol and glucose in stressed and unstressed fish. Alewife collected from Cayuga Lake in Spring 2003 and held in captivity until April 2005 were used to evaluate whether stress in the hatchery had limited their ability to respond to stressful conditions. Fifteen alewife were transferred to a holding tank. Following a 72-hour acclimation period, five alewife were taken directly from the tank and blood was immediately collected for plasma cortisol and glucose analysis as described above. The remaining alewife (N = 10) were also captured, placed in a bucket with about 2 L of water and chased vigorously with a net for 5 min. These alewife were left in the bucket for an additional 10 min after which sampling for plasma cortisol and glucose was conducted as described above.

Statistical analyses:

Relationships between alewife thiaminase and circulating white blood cells, plasma cortisol and glucose were evaluated with a linear regression using SAS (SAS Institute Inc.). Mixed-model analyses (tank as a random effect – due to random assignments of fish and treatments to individual tanks – and treatment as a fixed effect) were conducted with the PROC_MIXED procedure using SAS to test for treatment effects on alewife thiaminase, white blood cell counts, plasma cortisol and glucose. The PROC MIX procedure in SAS is a generalization of the standard linear model procedure that specifically addresses individual measurements in experimental units that can be grouped or clustered (in this case, tanks) to account for random variability in these experimental units. The Satterthwaite method was used to calculate degrees of freedom (reported in tables summarizing these analyses). Simple

linear regression was conducted using SAS to examine the relationship between alewife plasma cortisol and glucose as a function of the time after the first alewife was sampled in the netting stress trials. Two-sample *t*-tests were conducted using SAS to test for the effect of experimental treatments on plasma cortisol and glucose levels within alewife from the general stress trial.

Results:

The initial thiaminase activity of the ten alewife collected from Cayuga Lake in Spring 2004 was 6,900 ± 2,800 $pmol \cdot g^{-1} \cdot min^{-1}$ (mean ± SD). The overall value of alewife thiaminase activity for all 96 study fish at the completion of our experiments (ranging from 1 ½ to 2 ½ years after fish were captured in the wild) was 16,000 ± 5,900 $pmol \cdot g^{-1} \cdot min^{-1}$ (mean ± SD), which was a substantial increase relative to the initial activity measured when alewife were first brought to the laboratory. No significant difference was found between mean thiaminase levels in alewife from salt-limited and food-limited treatment tanks versus their respective control tanks (Table 3.1). Regression analyses showed no significant relationship between alewife circulating white blood cells, plasma cortisol and glucose and thiaminase activity within all treatment and control fish (Table 3.2). Alewife water content of the 30 fish sampled from Cayuga Lake in Spring 2004 was 72.0% ± 1.6 (mean ± SD); the water content of alewife at the completion of the experiments was 56.3% ± 3.5% (mean ± SD) for control and treatment fish in the salt-limited experiment (N = 40) and 51.1% ± 6.9% (mean ± SD) for control and treatment fish in the food-limited experiment (N = 37).

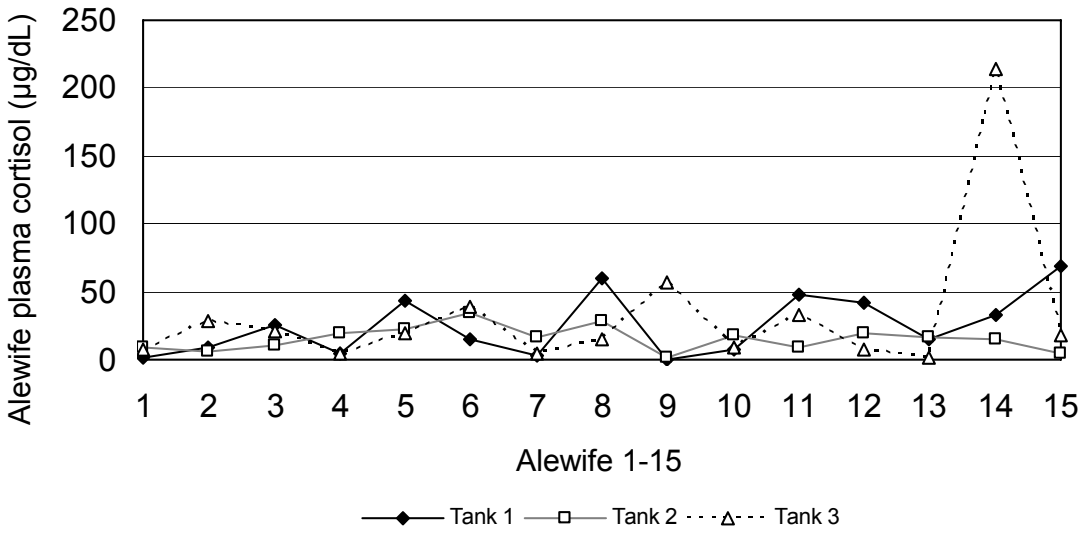

Figure 3.1. Alewife plasma cortisol levels (µg/dL) from the netting stress trial. Replicate tanks 1 through 3 were sampled at random in that order (and subsequently named accordingly) and fish 1 through 15 from each tank were sampled and named similarly for ease of presentation. No significant trends were shown in alewife plasma cortisol as a function of sampling time, including and excluding the data associated with the 14th fish from tank 3 which was chased for an extended period due to poor netting and then processed approximately 10 min after the initial netting attempt.

Total white blood cell counts were lower in both treatments (salt and food-limited) relative to their associated controls (Table 3.1). Alewife plasma cortisol was significantly higher in fish from control tanks relative to fish from salt-limited

Table 3.1. Alewife thiaminase ($pmol \cdot g^{-1} \cdot min^{-1}$), total white blood cell count (cells/µL) and plasma cortisol (µg/dL) and glucose (mg/dL) data showing mixed-model comparisons between salt-limited and food-limited treatments and controls. Sample means (± SD), (number of fish tested), *F*-statistic and *p*-values are listed. Numerator degrees of freedom for all comparisons = 1. Denominator degrees of freedom for the plasma cortisol comparison for the salt-limited treatment = 45 and 46 for the thiaminase comparison for the salt-limited treatment and the total white blood cell comparison for the food-limited treatment. Denominator degrees of freedom for all other comparisons = 2.

Treatment	Thiaminase (pmol·g-1·min-1)	Total white blood cell count (#/µL)	Plasma cortisol (µg/dL)	Plasma glucose (mg/dL)
Salt control 1	18,000 ± 6,000 (12)	24,000 ± 9,400 (12)	18.7 ± 14.9 (12)	109.0 ± 58.3 (12)
Salt control 2	16,000 ± 3,900 (12)	19,000 ± 6,200 (12)	15.1± 8.7 (12)	53.4 ± 15.8 (12)
Salt limited 1	14,000 ± 5,900 (12)	11,000 ± 3,800 (12)	8.1 ± 3.5 (11)	116.4 ± 35.7 (12)
Salt limited 2	15,000 ± 3,500 (12)	9,000 ± 4,600 (12)	7.8 ± 8.1 (12)	115.6 ± 44.8 (12)
(*F*, *P*)	3.79, 0.06	**17.34, 0.05**	**10.20, <0.01**	1.57, 0.34

Treatment	Thiaminase (pmol·g-1·min-1)	Total white blood cell count (#/µL)	Plasma cortisol (µg/dL)	Plasma glucose (mg/dL)
Food control 1	14,000 ± 4,500 (12)	34,000 ± 12,000 (12)	8.2 ± 6.2 (12)	91 ± 31.7 (12)
Food control 2	17,000 ± 7,300 (12)	30,000 ± 15,000 (12)	21.4 ± 14.3 (11)	126.3 ± 49.5 (12)
Food limited 1	21,000 ± 8,200 (12)	21,000 ± 11,000 (12)	6.7 ± 10.7 (12)	56.7 ± 27.3 (12)
Food limited 2	17,000 ± 4,400 (12)	16,000 ± 12,000 (12)	9.0 ± 13.9 (11)	108.4 ± 72.7 (11)
(*F*, *P*)	1.53, 0.34	**14.68, <0.01**	1.05, 0.41	0.71, 0.49

Table 3.2. Linear regression statistics for the relationship between alewife total white blood cell counts (cells/µL) and plasma cortisol (µg/dL) and glucose (mg/dL) and thiaminase levels ($pmol \cdot g^{-1} \cdot min^{-1}$) in salt and food-limited treatments and controls. R^2, *p*-values and (N's) associated with the comparisons are listed.

Treatment	Total white blood cell count (#/µL)			Plasma cortisol (µg/dL)			Plasma glucose (mg/dL)		
	r^2	*P*	N	r^2	*P*	N	r^2	*P*	N
Salinity treatment and control	0.01	0.46	48	0.02	0.29	47	0.04	0.18	48
Feeding treatment and control	< 0.01	0.82	48	0.01	0.49	46	0.05	0.12	47

Table 3.3. Alewife lymphocyte, neutrophil, monocyte and other granulocyte count (cells/µL) data showing mixed-model comparisons between salinity and food-limited treatments and controls. Sample means, (± SD), (number of fish tested), *F*-statistic and *p*-values are listed. Numerator degrees of freedom for all comparisons = 1. Denominator degrees of freedom for the monocyte comparison for the food-limited treatment and for the other granulocyte comparison for the salt and food-limited treatments = 46. Denominator degrees of freedom for all other comparisons = 2.

Treatment	Lymphocyte count (#/µL)	Neutrophil count (#/µL)	Monocyte count (#/µL)	Other granulocyte count (#/µL)
Salt control 1	17,000 ± 7,400 (12)	5,300 ± 6,000 (12)	2,000 ± 1,400 (12)	150 ± 200 (12)
Salt control 2	16,000 ± 4,900 (12)	2,200 ± 3,500 (12)	1,200 ± 530 (12)	67 ± 100 (12)
Salt limited 1	7,100 ± 3,900 (12)	2,600 ± 2,400 (12)	1,400 ± 840 (12)	130 ± 230 (12)
Salt limited 2	6,300 ± 3,400 (12)	1,700 ± 1,500 (12)	860 ± 520 (12)	120 ± 120 (12)
(*F*, *P*)	**42.76, <0.01**	1.00, 0.42	0.89, 0.44	0.05, 0.82

Treatment	Lymphocyte count (#/µL)	Neutrophil count (#/µL)	Monocyte count (#/µL)	Other granulocyte count (#/µL)
Food control 1	29,000 ± 10,000 (12)	2,500 ± 1,500 (12)	1,300 ± 660 (12)	320 ± 580 (12)
Food control 2	22,000 ± 13,000 (12)	7,200 ± 6,800 (12)	1,500 ± 1,100 (12)	230 ± 230 (12)
Food limited 1	18,000 ± 11,000 (12)	2,200 ± 2,000 (12)	650 ± 610 (12)	76 ± 120 (12)
Food limited 2	13,000 ± 12,000 (12)	2,800 ± 2,300 (12)	890 ± 620 (12)	82 ± 120 (12)
(*F*, *P*)	4.88, 0.16	0.98, 0.43	**7.24, <0.01**	**4.45, 0.04**

treatment tanks; alewife plasma cortisol was not significantly different in food control tanks relative to food-limited tanks (Table 3.1). Alewife plasma glucose levels in control tanks were not significantly different from their respective salt and food-limited treatments (Table 3.2). Seven measurements of plasma cortisol and glucose were tested in triplicate based on disparity in duplicate measurements or atypical values based on the discretion of laboratory personnel. Three of these repeat analyses where similar to initial results despite exceptionally high mean values, therefore these values were included in subsequent statistical analyses. Four of these analyses conflicted with the initial results, i.e. the third value was not comparable to either of the first two measurements. Therefore, the values from these fish were excluded from further analyses (one measurement of plasma cortisol was excluded from one salt-

limited and one food-limited treatment and one food-control group, and one measurement of plasma glucose was excluded from one food-limited treatment).

Alewife lymphocyte counts were significantly greater in control tanks relative to salt-limited treatment tanks. Monocyte and other granulocyte counts were higher in control tanks relative to food-limited tanks. All other comparisons of differential white blood cell counts were not significant (Table 3.3).

No evidence was found for an influence of the process of netting fish on plasma cortisol in other alewife within the same tank, with the exception of a single alewife that was inadvertently chased during capture (Figure 3.1). Results from this trial are shown in Figure 3.1 in the order in which alewife were captured and processed. The fourteenth fish from tank 3 – which was exposed to substantial handling stress and was not processed until approximately 10 min after its first exposure to being chased by a net – had elevated plasma cortisol. No significant increases or decreases ($p > 0.05$ in all linear regression analyses) in alewife plasma cortisol or glucose within any of the replicate tanks were found in analyses that both included and excluded this fish from the regression analyses. Based on this evidence that plasma cortisol did not respond to the netting procedure, we consider longer-term indicators of stress (e.g., white blood cell counts) to have also been unaffected by the netting procedure.

Fish that were vigorously chased showed significantly greater plasma cortisol levels relative to controls (Table 3.4). Although plasma cortisol levels in these alewife were significantly greater than levels measured in fish that did not experience the vigorous chasing, plasma glucose levels in these alewife were unaffected (Table 3.4).

Table 3.4. Alewife plasma cortisol (µg/dL) and glucose (mg/dL) data showing results from two-sample *t*-tests comparing control versus deliberately stressed fish. Sample means (± SD), (number of fish tested), *t*-statistic and *p*-values are listed. Degrees of freedom for both tests is 13.

Treatment	Plasma cortisol (µg/dL)	Plasma glucose (mg/dL)
Control	2.0 ± 3.5 (5)	85.6 ± 25.1 (5)
Vigorous chasing	43.9 ± 12.9 (10)	68.1 ± 3.6 (10)
(t, P)	**9,58, <0.01**	1.55, 0.20

Discussion:

Experimental treatment conditions – i.e. reduced food and reduced salinity – in this study resulted in lower circulating total white blood cell counts in alewife exposed to the treatments relative to their respective controls. This response has been shown in previous studies of fishes to be an indicator of an altered immune response (Pickering 1984; Barton et al. 1987). However, alewife thiaminase levels did not increase in response to these experimental treatments. In addition, no significant relationship was found between alewife plasma cortisol and glucose levels – both of which have been reported as primary and secondary stress indicators, (Barton 1997) – and alewife thiaminase activity. The overall alewife response to experimental treatments in our study suggests that alewife circulating white blood cells and plasma cortisol and glucose levels were not associated with alewife thiaminase activity. However, mean alewife thiaminase activities observed in this experiment were found to be more than twice as high (mean = 16,000 $pmol \cdot g^{-1} \cdot min^{-1}$) as levels observed in alewife collected from other lakes in the Great Lakes basin (mean values ranging from 1,700 to ~6,000 $pmol \cdot g^{-1} \cdot min^{-1}$), which have shown substantial variation both seasonally and across systems (Fitzsimons et al. 2005; Tillitt et al. 2005).

Total white blood cell counts of fish within control tanks were higher relative to treatment tanks in salt and food-limited trials, and alewife lymphocytes were

significantly greater in number in the salt-limited controls relative to their respective treatments. The lower counts of white blood cells observed in alewife from the salt-limited treatments were similar to those observed in other fish species exposed to chronic stress. For example, striped bass were found to have significantly lower white blood cell counts (~20,000 total white blood cells versus ~60,000 total white blood cells/μL respectively) in laboratory experiments after a 6 week exposure to cold (10ºC) versus warm (18º, 24º and 29º) water temperatures that were intended to produce chronic stress (Hrubec et al. 1997). In our study alewife monocyte and other granulocyte counts were significantly higher in control tanks relative to treatment tanks in the food-limited trial. Our results indicate that salt and food limitation resulted, to different degrees, in lower circulating white blood cell counts. However, the absence of a concurrent thiaminase response provides no support for the hypothesis that thiaminolytic bacteria or any other internal processes responsible for producing thiaminase were influenced by the alewife immune system response observed in these experiments.

The significantly lower alewife plasma cortisol levels observed in the salt-limited experimental treatment were unexpected. This observed response may have resulted from an extended exposure to stressful conditions, e.g. an initial cortisol increase in response to the salt limitation treatment might have been subsequently followed by a decline in cortisol. In a previous study, fish exposed to prolonged stressful conditions from a polluted environment exhibited an impaired response to immediate stress and cortisol levels within these fish did not increase in response to stress from capture and handling (Hontela et al. 1992). These investigators also observed that the pituitary corticotropes (cells in the anterior pituitary that produce the adrenocorticotropic hormone responsible for stimulating the adrenal gland and thereby increasing cortisol production) were atrophied in their study fish. A similar level of

impairment in alewife within our salt-limited treatment may have influenced the observed alewife plasma cortisol response. It is also possible that the exposure to low salt levels may have influenced unknown alewife physiological processes not evaluated in this study that also influenced the plasma cortisol response to stress. However, plasma cortisol levels observed in alewife within the salt-limited treatment tanks remained within a similar range of values as were measured in other groups of alewife in our experiments.

Plasma glucose levels were not significantly different in alewife from control and treatment tanks in the salt and food-limited trials. Previous studies have shown that environmental stressors – including osmoregulatory challenges – produced increases in plasma glucose levels in several species of fish (Barton and Iwama 1991; Mommsen et al. 1999), whereas starvation has been shown to decrease levels of plasma glucose in fish as a result of decreased concentrations of blood metabolites from the lack of diet-supplied glucose (Zammit and Newsholme 1979; Black and Love 1986). Overall mean concentrations of alewife plasma glucose in the salinity and food-limited trials relative to their respective controls showed trends similar to these previous studies, but these trends were not significant. Additional data regarding the short-term response of alewife plasma glucose levels to experimental treatments were not collected during the course of our experiments, which lasted from seven (salt-limited treatment experiment) to 38 (food-limited treatment experiment) days.

It is important to note that the timing of the rapid increase in alewife plasma cortisol (approximately 15 minutes) observed in response to vigorous chasing within the general stress trial corresponded with expectations based on previous studies, as did the lack of a rapid plasma glucose response. The significant observed increase in plasma cortisol levels following induced stress from chasing fish with a net confirms that alewife used in this experiment were capable of responding to stress. We also

consider it important to note that alewife densities in tanks during experimental trials were maintained at a level approximately one order of magnitude lower than densities maintained in holding tanks prior to the start of the experiment, in an attempt to ensure that any induced stress resulted from treatment effects. The data presented within this Chapter is the first published information regarding alewife white blood cells, plasma cortisol and glucose levels and thereby represents a contribution to current knowledge of such factors in clupeid fishes.

Although significant differences were found in alewife circulating total white blood cell counts in response to experimental treatments, our results suggest that these treatments (i.e., low salinity and low food availability) did not influence alewife thiaminase activity in our study fish. It is possible that thiaminolytic bacteria associated with alewife viscera (Honeyfield et al. 2002) might respond to changes in internal physiological conditions in alewife that were not associated with factors evaluated in this study or that thiaminase activity in alewife could be controlled by some other factor or combination of factors. Furthermore, alewife in this study were not exposed to any known external sources of thiaminase found in natural aquatic systems (e.g., cyanobacteria, zooplankton), and their artificial feed was thermally treated in order to denature any dietary sources of thiaminase, thereby eliminating food as a thiaminase source while these fish were maintained in the laboratory. We therefore consider the following observations in our study to be of particular importance: (1) the mean value of thiaminase activity within our study alewife, (16,000 $pmol \cdot g^{-1} \cdot min^{-1}$) was more than two times greater than previously published mean values for alewife collected from other North American lakes (Fitzsimons et al. 2005), and (2) the mean thiaminase levels for alewife in our experiments – conducted after 1 ½ to 2 ½ years of laboratory rearing after the study alewife were collected from Cayuga Lake in Spring 2004 – had increased from 6,900 to 16,000 $pmol \cdot g^{-1} \cdot min^{-1}$.

This suggests that additional research is warranted to investigate changes in physiological responses and thiaminase activity within captive alewife, beginning at the point of capture and continuing through an extended period in captivity.

We discount the possibility that stress during captivity resulted in the observed two-fold increase in thiaminase activity based on two considerations. First, fewer than ten alewife died during the 12 months prior to the initiation of the experiments; lack of mortality generally provides an indication of satisfactory rearing conditions. Second, alewife grew rapidly during captivity and exhibited exceptional body condition – measured as percent water content – at the start of the experiments (51-56%) versus at the time of capture in the wild (72%). Whole body lipid content has been shown to be inversely related to water content, and the water content of captive alewife in these experiments was lower than that of any fish previously reported (Hartman and Brandt 1995). It is not credible to suggest that stressed fish could improve body condition and sustain low mortality for more than a year in captivity, particularly a species such as alewife that is not readily maintained in laboratory conditions (Colby 1973).

In summary, the following key observations resulted from our experiments:

1. We found no evidence linking stress to thiaminase activity in alewife subject to our experimental treatments.
2. We found no evidence that alewife in our experiments were chronically stressed by laboratory conditions, based on the fact that plasma cortisol levels increased as expected when netting stress was applied.
3. We observed lower white blood cell counts in alewife subject to experimental treatments designed to produce stress (low salinity and low food availability) relative to control alewife, and similar white blood cell responses have been reported in response to similar stresses in other studies.

4. Thiaminase levels in alewife within our study increased in response to laboratory holding conditions, but through a mechanism that was not evaluated by the experimental treatments.

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CHAPTER FOUR
ALEWIFE AND GIZZARD SHAD THIAMINASE RESPONSE TO STRESSORS

Abstract:

Over the past three decades, a thiamin-deficiency-related reproductive failure (early mortality syndrome: EMS) has been observed in valuable salmonine fishes in the Great Lakes, Finger Lakes, and the Baltic Sea. The cause of EMS has been linked to the consumption of clupeid fish, which contain high levels of a thiamine-destroying enzyme called thiaminase. High activities of thiaminase have been reported from clupeids such as alewife *Alosa pseudoharengus*, gizzard shad *Dorosoma cepedianum* and Baltic herring *Clupea harengus*, but no consistent explanation has accounted for the wide range of observed variation in clupeid thiaminase. Chronic stress has been shown to suppress the immune systems of alewife and other fish, reducing the number of circulating white blood cells available to suppress bacteria. Because the presence of thiaminase has been associated with thiaminolytic bacteria isolated from alewife viscera, we hypothesized that stressful conditions – potentially limiting clupeid immune response or altering internal physiological conditions – would allow for bacterial proliferation and a subsequent increase in thiaminolytic activity. In this study, alewife and gizzard shad were exposed to severe winter temperatures and low food availability respectively in replicated pond experiments to evaluate the influence of stressful conditions on clupeid thiaminase activity. Though responses in circulating white blood cell counts and metrics of fish condition indicated that experimental treatments affected clupeids, these effects were not related to increased thiaminase activity. The only significant treatment effect on clupeid thiaminase was the increase

in mean thiaminase activity in gizzard shad from ponds where only high quality energy sources were available. These data indicate that variability in clupeid thiaminase may be related to diet composition and/or feeding rates.

Introduction:

Clupeid fishes – including alewife *Alosa pseudoharengus*, gizzard shad *Dorosoma cepedianum* and Baltic herring *Clupea harengus* – have been regularly observed to contain high activities of thiaminase, a thiamine-destroying enzyme. A large body of field and lab work conducted during the past decade support a plausible hypothesis for an association between a prey base comprised of a large biomass of thiaminase-containing clupeids and the recruitment failures often experienced by Great Lakes, Finger Lakes (NY, U.S.A.) and Baltic Sea salmonine predators. Reproductive failure was first observed in the brood stocks of salmonine fish in North American hatcheries during the 1960's. A coho salmon (*Oncorhynchus kisutch*) fry mortality with similar symptoms was first observed in wild fish in 1967 (Johnson and Pecor 1969). During the next several decades managers and researchers working in the Laurentian Great Lakes and Finger Lakes increasingly recognized that other salmonine fishes – including lake trout (*Salvelinus namaycush*), Atlantic salmon (*Salmo salar*), Chinook salmon (*Oncorhynchus tshawytscha*), rainbow trout (*Oncorhynchus mykiss*), and brown trout (*Salmo trutta*) – suffered from a similar reproductive failure (Fisher et al. 1995; Marcquenski and Brown 1997; McDonald 1998). Baltic Sea observations of sac-fry mortality in Atlantic salmon (Norrgren et al. 1993; Bengtsson et al. 1994; Karlsson et al. 1996) and sea-run brown trout (*Salmo trutta*) (Soivio 1996) were also attributed to consumption of clupeid prey containing thiaminase.

In North America the syndrome associated with these mortalities has been referred to as "early mortality syndrome" (EMS) and is caused by thiamine deficiency. During the mid-1990s it became increasingly evident that thiamine-deficient salmonids were susceptible to EMS (Fisher et al. 1996; Fitzsimons et al. 1999). Thiamine (vitamin B_1) is an essential vitamin necessary for the conversion of carbohydrates and lipids into energy and is ubiquitous across all kingdoms of life. Offspring of salmonine fishes susceptible to EMS die shortly after hatching, but fry from identical egg sources survive and exhibit normal behavior when treated with thiamine (Fitzsimons 1995; Fisher et al. 1996).

Thiaminase in alewife has been attributed to thiaminase positive bacteria (*Paenibacillus thiaminolyticus*) isolated from alewife viscera and the presence of thiaminase in alewife is believed to be the primary factor responsible for EMS in alewife predators (Fitzsimons et al. 1999; Honeyfield et al. 2002; Honeyfield et al. 2005a) – though thiamine deficiency has not been observed within clupeids containing high thiaminase activity. Laboratory experiments have induced EMS in lake trout by altering dietary levels of thiaminase, using feral alewife containing thiaminase and bacterial sources of thiaminase (Honeyfield et al. 2005b). Although the ultimate source or sources of thiaminase contributing to EMS are unknown in natural systems, this evidence suggests that clupeid and/or bacterial thiaminase play a key role in salmonine thiamine deficiency.

Both EMS in salmonines and thiaminase activity in clupeids fluctuate widely, and factors affecting levels of thiaminase activity in clupeids are poorly understood (Wistbacka et al. 2002; Brown et al. 2005; Fitzsimons et al. 2005; Tillitt et al. 2005; Ikonen 2006). No factor has consistently explained the observed variability in clupeid thiaminase activity, and a mechanistic understanding of the processes influencing thiaminase activity remains unknown. However, if internal microbial populations are

the source of thiaminase activity in alewife and other clupeids, changes in physiological conditions – resulting from environmental or other postulated sources of stress – that influence the growth characteristics of bacteria can be expected to affect thiaminase activity (Tillitt et al. 2005). For example, increases in alewife thiaminase have been observed in association with decreases in water temperature in natural systems and transport by truck and subsequent holding in captivity (Tillitt et al. 2005; Lepak et al. 2008; J. Fitzsimons, personal comm.). These increases in thiaminase may be related to physiological stress, though the mechanism behind the observed increases remains unknown.

The interaction between the clupeid immune system and internal bacterial communities is poorly characterized, though one response of many fish to chronic stress is a reduction in the number of circulating white blood cells that play a role in suppressing bacteria (Pickering 1984; Barton et al. 1987; Mommsen et al. 1999). Performance and mortality studies have been conducted in order to examine the influence of various environmental conditions – including severe winter temperatures, salinity and food availability – on fish (Barton and Iwama 1991; Hrubec et al. 1997; Mommsen et al. 1999). Most of these studies were conducted using salmonine fishes, however, given the knowledge of the association between thiaminolytic bacteria and thiaminase in alewife, we expected that environmental stressors capable of influencing clupeid mortality and other physiological characteristics could also influence clupeid thiaminase activity. For example, thiaminase could be produced more efficiently by bacteria and/or thiaminolytic bacteria could proliferate as a result of indirect physiological effects associated with stressful conditions.

Despite the fundamental role of clupeids in producing EMS in valuable apex predators including sport fish, little is known about factors responsible for inducing thiaminase production in clupeids. Although research has focused mainly on alewife

and Baltic herring, other clupeid fish have been found to contain thiaminase in high concentrations. For example, gizzard shad have high levels of thiaminase activity (means from 15,000 to 30,000 pmol thiamine·g^{-1}·min^{-1}; Tillitt et al. 2005; Honeyfield et al. 2008; J. Ross et al. unpublished data; S. Marquenski, unpublished data) relative to alewife (on the order of 5,000 pmol thiamine·g^{-1}·min^{-1}; Tillitt et al. 2005). Given the tendency of gizzard shad to dominate biomass and their wide distribution in U.S. waters (Timmons et al. 1978; Johnson et al. 1988), thiaminase in gizzard shad has the potential to negatively affect apex predator populations whose diets are dominated by these prey fish. Therefore, we designed a set of experiments in replicated pond systems to evaluate the influence of stressful conditions on alewife and gizzard shad thiaminase. Severe winter temperatures, and low food availability were selected as treatments in this set of experiments. We hypothesized that these treatments would result in stressful conditions, ultimately leading to an increase in clupeid thiaminase activity.

Methods:

Alewife pond experiment:

Alewife were collected from Waneta Lake (Schuyler County, NY) on the evening of 26 Oct 04 and transported by truck to the Cornell Experimental Pond Facility in Ithaca, New York. Alewife were stocked at a density of 160 individuals into each of four fishless ponds (640 alewife total) on the morning of 27 Oct 04. Each individual pond was approximately 1,800 m^3 with a maximum depth of approximately 2 m. Contrasting winter temperatures were produced by maintaining ice-free conditions (using an aeration system described in Lepak and Kraft 2008) on two of the four study ponds from 11 Dec 04 to 5 Jan 05. On 21 Apr 05 each pond was drawn down to a depth of approximately 1.0 m. A bag seine approximately 40-m long and

1.2-m tall was used to collect the remaining alewife from the ponds. Alewife (10 from each pond) were collected for thiaminase and circulating white blood cell analyses. The remaining surviving alewife from each pond were used for wet-dry weight analysis as an indicator of fish condition. Thiaminase analyses were conducted at the Canadian Center for Inland Waters, Burlington, Ontario using the procedure described by Zajicek et al. (2005). Mixed-model analyses (pond as a random effect and treatment as a fixed effect using the PROC MIX procedure) were conducted using SAS (SAS Institute Inc.) to test for the effect of treatment on alewife thiaminase and circulating white blood cell counts.

Gizzard shad pond experiment:

This experiment was conducted in collaboration with Dr. Michael Vanni at Miami University's Ecology Research Center experimental pond facility. Replicate ponds (approximately 800 m^3 with a maximum depth of approximately 2.5 m) lined with heavy duty plastic were used to evaluate the influence of nutrient and sediment additions on pond chemistry and gizzard shad from Acton Lake, Ohio (source population) were stocked within the ponds. The treatments, each with three replicates were: no nutrient or sediment addition (Control), nutrient addition (+N), sediment addition (+S) and addition of nutrients and sediment (+N+S). The experiment was initially designed to evaluate the influence of agricultural inputs on gizzard shad in ponds, however, a thiaminase component was added to evaluate the influence of withholding nutrients and sediments in control ponds relative to the treatment ponds with nutrients and/or sediments added.

All nutrient treatments (+N and +N+S) received weekly additions of ammonium nitrate (NH_4NO_3) and sodium phosphate ($NaPO_4\,H_2O$) at loading rates of 150 µg N/L pond water and 15 µg P/L pond water per week. These loading rates

represent typical inputs in Acton Lake, a nearby hypereutrophic reservoir and the source for the gizzard shad in this experiment (M. Vanni unpublished data). All treatments with sediment additions (+S and +N+S) first received a layer (approximately 2 cm) of Acton Lake sediments and then received weekly additions of Acton Lake sediments (0.06 m^3; ~70 kg) as a slurry that was sprayed with a pump. This weekly input rate mimicked the long term sedimentation rate in Acton Lake (M. Vanni unpublished data).

Ponds were filled by gravity on 29 May 04 with water from an oligotrophic supply pond (6.8 μg chlorophyll/L) and each subsequently inoculated with 400 L of Acton Lake water to provide an inoculum of species typical of productive environments. On 10 June 04, all ponds were stocked with gizzard shad at a biomass of ~175 kg wet mass/ha. The experiment lasted 11 weeks, and on 8 Sept 04, the ponds were drained and all gizzard shad were collected, weighed and measured. M. Vanni provided gizzard shad for thiaminase analysis as well as data regarding water quality and gizzard shad Fulton's K (an indicator of condition; $K = 100\ W/L^3$, where W is the mass in grams and L is the total length in cm; Williams 2000). At the completion of the experiment, gizzard shad were collected by electrofishing to evaluate P and N excretion rates (M. Vanni personal comm).

Gizzard shad (three from each individual pond, nine in total for each treatment) were sent for thiaminase analysis in two different batches. Thiaminase analyses were conducted at the Canadian Center for Inland Waters, Burlington, Ontario using the procedure described by Zajicek et al. (2005). Gizzard shad from the second batch of samples lost a predictable amount of thiaminase while in storage (at -20 ºC). These significant differences (ANCOVA; $F_1 = 28.42$, $p < 0.01$) were accounted for during analysis of covariance evaluating gizzard shad thiaminase. ANOVA and ANCOVA analyses were conducted using SAS (SAS Institute Inc.) to test for the effect of

treatment on gizzard shad thiaminase and condition (Fulton's K) as well as the relationship between gizzard shad thiaminase and condition (Fulton's K). However, due to the limited amount of data available for gizzard shad thiaminase, a Monte Carlo randomization simulation using 10,000 iterations was conducted using SAS (SAS Institute Inc.) to assess the validity of the relationship between gizzard shad thiaminase and condition. Gizzard shad Fulton's K was based on mean values for adult and juvenile gizzard shad (young-of-year fish were excluded as they were not tested for thiaminase) collected from each of the four treatments following the completion of the experiment.

Results:

Alewife pond experiment:

Mean alewife thiaminase activities were not influenced by the treatments in this experiment. Despite temperature differences due to the experimental treatments, the mixed-model analysis comparing control and treatment alewife showed that there was no relationship between alewife thiaminase activity and the prolonged exposure to cold temperatures (Mixed-model; $F_{20,20} = 0.37$, $p = 0.55$; see Figure 4.1).

Pond temperatures were substantially altered by the presence or absence of aeration (i.e. surface ice). Temperatures in the control ponds (#230 and #231; not aerated) remained below 3ºC for approximately 12 days just prior to and following ice formation while the treatment ponds (#229 and #232; aerated) remained below 3ºC for approximately 8 weeks – and below 2ºC for approximately 7 of those 8 weeks (Lepak and Kraft 2008).

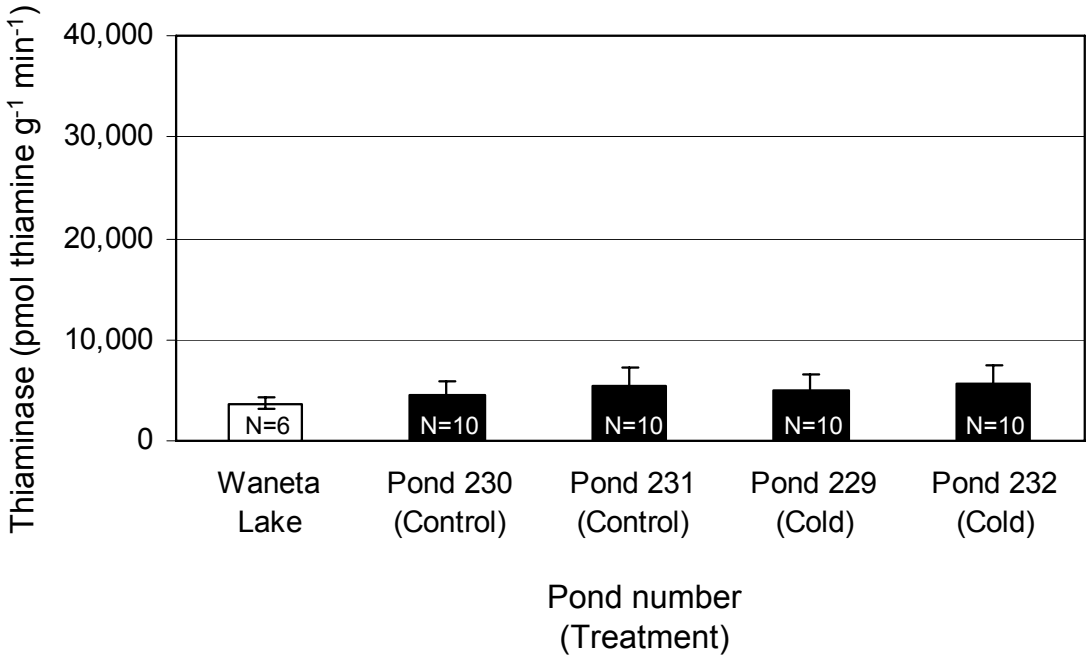

Figure 4.1. Mean (± S.E.M.) alewife thiaminase values in pmol thiamine·g^{-1}·min^{-1}, from the source population (open bar; Waneta Lake) and control and treatment experimental ponds (black bars). The number of fish tested (N) to establish each mean is shown at the base of the individual bars.

Previous results showed that alewife survival and condition (as measured by water content) did not differ between temperature treatments. However, alewife from the treatment ponds had significantly lower circulating lymphocyte counts (approximately 40% lower) when compared to alewife from the control ponds, indicating a treatment effect on alewife immune systems (Lepak and Kraft 2008).

Mean alewife water content following the experiment (71 ± 2%, N = 45) was within 2% of the alewife water content (69 ± 3%, N = 30) of the source population (Waneta Lake) and within the range of other alewife previously measured in freshwater systems (Hartman and Brandt 1995). Mean alewife thiaminase activity (approximately 5,200 pmol·g^{-1}·min^{-1}) following the experiment was within the range

of thiaminase activities observed for wild-caught populations in freshwater systems (ranging from 1,700 to 7,000 pmol·g^{-1}·min^{-1}; Tillitt et al. 2005).

Gizzard shad pond experiment:

Mean gizzard shad thiaminase activities were found to be significantly higher in fish from ponds that were treated with nutrients (approximately 30,000 ± 1,800 pmol·g^{-1}·min^{-1}) relative to control ponds (22,000 ± 2,500 pmol·g^{-1}·min^{-1}) and those that had sediments (24,000 ± 1,900 pmol·g^{-1}·min^{-1}) or nutrients and sediments (22,000 ± 2,100 pmol·g^{-1}·min^{-1}) added based on a multiple contrast comparison (ANCOVA; $F_3 = 5.14$, $p < 0.01$; see Figure 4.2).

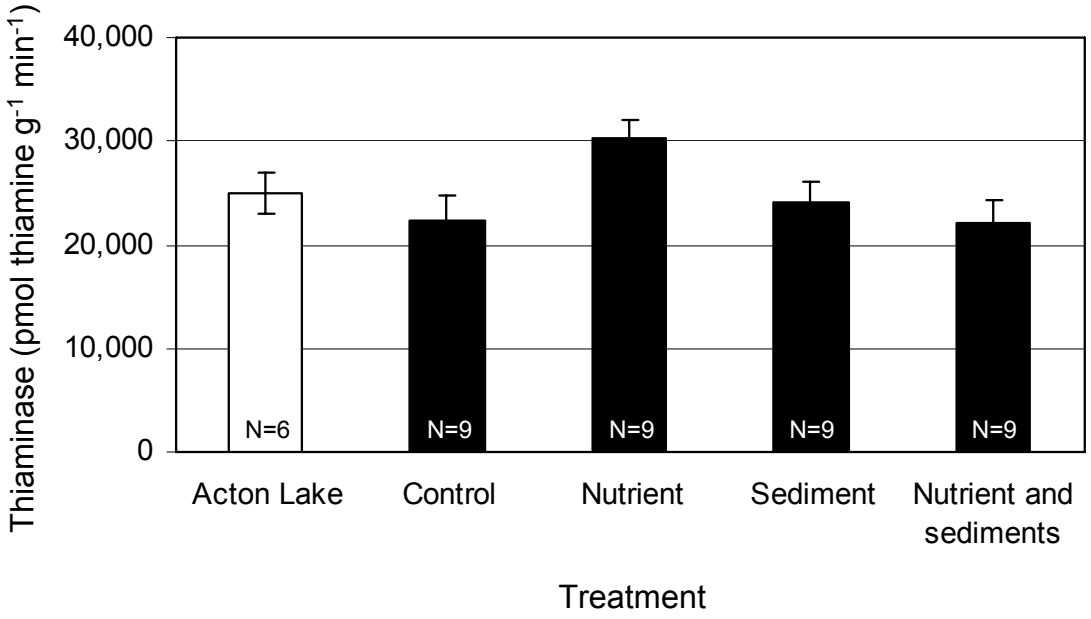

Figure 4.2. Mean (± S.E.M.) gizzard shad thiaminase values in pmol thiamine·g^{-1}·min^{-1}, from the source population (open bar; Acton Lake) and the various treatments for the gizzard shad pond experiment (black bars). The number of fish tested (N) to establish each mean is shown at the base of the individual bars.

Chlorophyll levels (μg L^{-1}), primary productivity (mg C m^{-2} d^{-1}), total phosphorus (μ P L^{-1}) and suspended solids (mg L^{-1}) were significantly greater in ponds with nutrient additions (+N), sediment additions (+S) and additions of nutrients and sediments (+N+S) than in control ponds (M. Vanni personal comm.). Gizzard shad excretion rates of P (μgP g^{-1} h^{-1}) and N (μgN g^{-1} h^{-1}) were significantly higher for gizzard shad collected from ponds treated with nutrient additions alone relative to all other ponds (M. Vanni personal comm.).

Gizzard shad condition (as indicated by Fulton's K) was significantly influenced by treatment (ANOVA; $F_3 = 87.60$, $p < 0.01$) with gizzard shad from ponds with nutrients and/or sediments added being in better condition than those from control ponds. Since gizzard shad thiaminase and condition factor were influenced by treatment, we conducted a comparison to evaluate whether gizzard shad thiaminase was related to condition. An ANCOVA analysis ($F_1 = 3.17$, $p = 0.09$) showed that the relationship was not significant at the level of $\alpha = 0.05$. These results were confirmed using a Monte Carlo randomization simulation showing that the relationship was significant at the level of $\alpha = 0.10$ (an F value of 3.04 was required to confirm this) but not at $\alpha = 0.05$.

The mean thiaminase activity of gizzard shad collected from Acton Lake prior to stocking was 25,000 ± 4,700 pmol·g^{-1}·min^{-1}. Though thiaminase activities in gizzard shad from the nutrient addition treatments were significantly greater (30,000 ± 1,800 pmol·g^{-1}·min^{-1}) relative to those from the other treatment and control ponds, they were within the range of thiaminase values observed from the source population (20,000 to 35,000 pmol·g^{-1}·min^{-1}).

Discussion:

The results observed in this study did not support the hypothesis that prolonged cold temperatures and low food availability would result in increased clupeid thiaminase activity. Following the experiments, alewife and gizzard shad held in pond systems had thiaminase activities that were within the range of the source populations they were taken from originally. The only significant treatment effect on clupeid thiaminase was the increase in mean thiaminase activity in gizzard shad from the ponds where nutrients alone were added.

Increases in mean thiaminase activities have been observed in alewife held in captivity that were provided with a high quality diet in the form of commercial pellet food for periods of three weeks or more (Lepak et al. 2008; Fitzsimons personal comm.; Honeyfield et al. personal comm.). The mean thiaminase activities of these alewife were on the order of two to three-fold higher than any thiaminase activities observed for wild-caught populations in freshwater systems (ranging from 1,700 to 7,000 $pmol \cdot g^{-1} \cdot min^{-1}$; Tillitt et al. 2005). These alewife were not exposed to any external sources of thiaminase found in natural aquatic systems (e.g., cyanobacteria, zooplankton or other dietary sources) and their food was heat treated, denaturing any thiaminase found in the feed (Lepak et al. 2008). Furthermore, a study conducted by Arsan and Malyarevskaya (1969) to investigate the effect of blue-green algae on thiaminase activity in silver carp (*Hypophthalmichthys molitrix*) showed that fish fed with five different diets (consisting of blue-green algae, green algae, mixed food, protococcoid algae and daphnia) had intestine and liver tissue with mean thiaminase activities at least two-fold higher than fish held under the same conditions without any available food source.

Similarly, gizzard shad from the experiment described in this Chapter that were provided exclusively with a high quality food source (high N and P content; M.

Vanni personal comm.) in the ponds where only nutrients were added had mean thiaminase levels significantly higher than gizzard shad from the other treatments. Excretion rates of both N and P were significantly higher in gizzard shad from ponds where only nutrients were added relative to fish from the other treatments, supporting the supposition that a high quality diet was consumed by gizzard shad in ponds where only nutrients were added (M. Vanni personal comm.). Gizzard shad held in ponds where sediments were added were likely relying heavily on these inputs for energy. Once gizzard shad reach 30 mm their digestive tracts elongate to facilitate the digestion of poor quality lake sediments (Heinrichs 1982; Mundahl and Wissing 1987) and non-larval gizzard shad (like those used in this study) can rely almost entirely on sediment detritus (Higgins et al. 2006). This assumption is supported by the lower excretion rates of N and P in gizzard shad from all of the treatments relative to those in which exclusively nutrients were added.

Thiaminase activities in gizzard shad within this study were similar to those of the source population and the within the range of the limited data available from gizzard shad previously tested for thiaminase (Tillitt et al. 2005; Honeyfield et al. 2008; J. Ross et al. unpublished data; S. Marquenski, unpublished data). It is important to note that the mean thiaminase activity of gizzard shad held in control ponds – those that did not receive nutrient or sediment additions and were subsequently food-limited (M. Vanni personal comm.) – were not elevated in thiaminase relative to the source population in Acton Lake. These data suggest that the composition of clupeid diets (e.g., the proportion of N and P or possibly other components like thiamine content) may differentially impact the resulting activity of thiaminase. These findings are compelling, and identifying the mechanisms behind these observations could prove useful to better understand thiaminase expression and variability in natural systems.

The only consistent finding across the set of experiments described in this Chapter and analogous data from tank experiments collected by Lepak et al. (2008), D. Honeyfield (unpublished data) and J. Fitzsimons (unpublished data) is that clupeids that had high quality diets available to them were higher in thiaminase relative to those that did not. Alewife and gizzard shad held under pond conditions had mean water contents, Fulton's K and thiaminase activities that were within the range of wild-caught fish (Hartman and Brandt 1995; Tillitt et al. 2005; Vanni unpublished data); while alewife held in the laboratory by Lepak et al. (2008) had mean water content values lower than any other published values and thiaminase activities higher (more than double) than any group of alewife collected from natural freshwater systems (Hartman and Brandt 1995; Tillitt et al. 2005). There was one exception to this; gizzard shad held in ponds where only nutrients were added had significantly higher mean thiaminase activity than fish from the other treatments. However, these gizzard shad had thiaminase activities within the range of natural variation found in fish from other natural systems as well.

Alewife and gizzard shad held under conditions that were hypothesized to increase thiaminase activities (prolonged cold temperatures and low food availability) responded to experimental treatments. Alewife held in prolonged cold temperatures had significantly lower counts of circulating lymphocytes relative to alewife held in warmer water temperatures. Gizzard shad that were held in ponds where nutrient and sediment additions were absent had significantly lower Fulton's K values relative to gizzard shad held in ponds where nutrients and/or sediments were added. These results were expected, however, there was no evidence linking these responses to thiaminase activity in clupeids.

The isolation of thiaminase-positive bacteria from alewife viscera represents an intriguing potential source of alewife thiaminase (Honeyfield et al. 2002). Bacterial

communities and their gene expression within fish viscera are altered by different feeding regimes and fish condition (Syvokienë & Mickënienë 1999). Additionally, thiaminolytic bacteria have been shown to out-compete other types of bacteria in the gastrointestinal tracts of ruminants under certain feeding regimes (increased carbohydrate consumption), ultimately resulting in thiamine deficiency (Brent 1976). The results of this set of experiments suggest that clupeids that are feeding on high quality energy sources with specific compositions have the potential to harbor high levels of thiaminase activity. We suggest that this phenomenon could be related to internal conditions in clupeids affecting bacterially produced thiaminase. Thus, clupeid feeding conditions may result in the proliferation of thiaminolytic bacterial populations and/or altered expression of thiaminase by these bacteria. Although this is speculation, the presence of thiaminolytic bacteria within alewife as a primary source of thiaminase should be explored.

The characterization of internal clupeid microbial communities and their expression of thiaminase in response to experimental treatments may provide important insights into thiaminase research. Thiaminase is primarily found in the visceral tissues of alewife and gizzard shad and only small amounts of thiaminase have been detected in muscle tissue (D. Honeyfield unpublished data; S. Marquenski unpublished data). Evaluations of clupeid thiaminase might benefit greatly from the inclusion of genetic components to identify and quantify the microbial species present in these visceral tissues, and to characterize microbial expression of thiaminase. We suggest that future experiments be designed that vary clupeid feeding rates and diet composition (especially with respect to nitrogen and thiamine content) while characterizing changes in clupeid internal microbial communities and microbial expression of thiaminase.

Understanding the dynamics of thiaminase expression in clupeids will aid ongoing efforts to re-establish sustainable, naturally reproducing salmonine communities in the Great Lakes and maintain naturally reproducing salmonine communities around the world. Given the dependency of salmonines on clupeids, the threat that they pose to salmonine natural reproduction and the ongoing spread of clupeids across the United States and Canada, it will be important for scientists, managers and stakeholders to recognize and acknowledge the importance of these prey fish. For example, further introductions of alewife carry serious, yet unpredictable implications related to salmonine communities because of variation in alewife population size and thiaminase content. Continuing efforts to evaluate clupeid thiaminase variability and using whole-system manipulations and other innovative techniques will provide applicable results that could lead to the remediation of the negative impacts that clupeids have on salmonines and possibly other predators.

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CHAPTER FIVE
CHANGES IN METHYLMERCURY BIOACCUMULATION IN AN APEX PREDATOR IN RESPONSE TO REMOVAL OF AN INTRODUCED PISCIVORE

Abstract:

We evaluated methylmercury (MeHg) concentrations in a native top predator, lake trout *Salvelinus namaycush* before and after the large-scale removal of an introduced predator, smallmouth bass *Micropterus dolomieu*. The removal was initiated in a 270-hectare Adirondack lake in 2000 and has continued to the present. Following the removal, lake trout switched from consuming primarily invertebrates to consuming fish. Previous studies have shown that diet switches and reductions in predator abundance have resulted in increased growth and decreased mercury concentrations due to "growth dilution". Instead, we observed a significant increase in lake trout MeHg concentrations despite observed increases in lake trout growth rates. Bioenergetics simulations predicted similar increases in MeHg when simulating lake trout growth and diet conditions during the pre- and post-removal periods. Higher MeHg in prey fish (post-removal diet) relative to invertebrates (pre-removal diet) was the most important factor influencing lake trout MeHg concentrations, though some evidence for growth dilution was observed. The proportion of MeHg relative to total mercury varied between lake trout, prey fish and invertebrates, therefore it was necessary to account for this in order to draw appropriate conclusions about empirical and theoretical findings in this study. The data presented in this manuscript provide empirical and theoretical evidence that changes in food web structure and dynamics influence MeHg concentrations in top predators.

Introduction:

Mercury contamination in fish is a serious issue affecting fisheries in the northeastern United States and is of concern worldwide to human and ecosystem health (Bodaly et al. 1993; Johnston et al. 2003; Kamman et al. 2005). The northeastern U.S. receives high levels of atmospheric mercury deposition (Chen et al. 2005; Kamman et al. 2005) and mercury levels in aquatic ecosystems are unlikely to change in the near future (Hunter et al. 2003). Due to bioaccumulation, mercury concentrations are particularly high in large piscivores, i.e. long-lived, slow-growing fish that feed at the top of aquatic food webs. These large piscivores with high mercury concentrations are frequently targeted for harvest and consumption by anglers.

Methylmercury (MeHg), the primary form of mercury found in sport fish, is toxic to humans and is readily absorbed by the blood stream before being distributed to the brain, nervous system and tissues. An important distinction between MeHg and other forms of mercury is that MeHg is the form that bioaccumulates most readily because it is absorbed by living tissue. Thus, MeHg poses a direct threat to humans when it is consumed. Most studies of mercury in sport fish generally assume that the form of mercury is > 95% MeHg and cite Bloom (1992) as a reference. This assumption is widely accepted and due to higher costs associated with MeHg analyses, total mercury (T-Hg) is often measured as a proxy for MeHg. The terms used in this paper include: "MeHg" (when referring to the methylated form of mercury), "T-Hg" (when referring to total mercury) and "mercury" (when more than one form of mercury is being discussed).

Mercury bioaccumulation in fish is dependant upon many factors, including fish diet (Harris and Bodaly 1998; Johnston et al. 2003; Swanson et al. 2003), fish trophic position (Power et al. 2002), food web structure (Johnston et al. 2003;

Swanson et al. 2003) and energy sources (Power et al. 2002). Stable isotope studies have established that increased trophic position and enriched $\delta^{13}C$ signatures in predators are correlated with increased concentrations of T-Hg (Power et al. 2002; Swanson et al. 2003). Previous studies have shown that the mercury content in a predator's diet is largely responsible for the mercury accumulated within the tissue and that subsequent changes in diet, metabolism, and growth rate can effectively alter mercury concentrations in fish (Harris and Bodaly 1998; MacRury et al. 2002; Trudel and Rasmussen 2006).

The removal of certain fish (predominantly piscivores) from lakes has been proposed as a means to reduce T-Hg concentrations in fish (Gothberg 1983; Verta 1990; Rask 1996). Reductions in fish abundance have been shown to be effective at lowering fish T-Hg concentrations in small lake systems (< 25 ha), but the mechanisms behind subsequent decreases in T-Hg accumulation were not well understood (Gothberg 1983; Verta 1990; Rask 1996). Verta (1990) suggested that "growth dilution" was the primary factor leading to decreased T-Hg concentrations in the fish remaining after removal. Growth dilution is a process whereby an increase in growth rate leads to lower concentrations of MeHg in fish at a given size or age. For example, growth dilution would occur if the MeHg concentration in a fish's diet remained constant while the energy content of the diet was increased and/or predator growth efficiency was increased. These circumstances could result from an increased abundance of prey items with higher energy density or increased food availability, which could occur in response to a decrease in predator density. The resulting increase in fish growth – without a corresponding increase in prey consumption – would subsequently decrease the overall dietary intake of MeHg per unit of fish tissue growth. Although the outcome of this proposed mechanism would be lower MeHg

concentrations in the tissues of faster-growing individuals, this mechanism was not verified in the aforementioned studies (Gothberg 1983; Verta 1990; Rask 1996).

Fish community composition, abundance and food web interactions in aquatic ecosystems are frequently altered by managers of sport fisheries, other anthropogenic impacts, and stochastic natural events (such as fish die-offs). All of these factors have the potential to alter fish density, fish size and age structure through changes in competition for resources such as available food and habitat. By quantifying the effects of an intense fishery manipulation – removal of a dominant non-native piscivore – on food web linkages and the bioaccumulation of MeHg in lake trout, we evaluated the influence of changes in food web dynamics on MeHg concentrations in a piscivore population. This large-scale manipulation provided an opportunity to identify factors influencing MeHg concentrations in lake trout, a dominant native predator in north temperate lakes.

Methods:

Study Site:

For five decades, non-native smallmouth bass replaced lake trout as the dominant top predator and reduced native prey fish abundance in the littoral zone of Little Moose Lake, a 270 hectare oligotrophic lake in the Adirondack Mountains (New York, USA). An intensive electrofishing effort was initiated in Little Moose Lake in spring 2000 and has continued to the present, resulting in a 90% reduction of adult smallmouth bass abundance with over 60,000 individuals removed to date (Weidel et al. 2007). Prior to smallmouth bass removal, lake trout consumed primarily benthic invertebrates and daphnids. The abundance of littoral prey fish increased in response to smallmouth bass removal, (Weidel et al. 2007); stable isotope and diet data showed

that lake trout began to take advantage of this energy source shortly after removal was initiated (Lepak et al. 2006).

Collections, mercury analysis and statistical comparisons:

In order to characterize the effect of smallmouth bass removal on changes in mercury bioaccumulation in lake trout, we evaluated the T-Hg content of samples collected from Little Moose Lake from 2000 – 2006. We did not find evidence for growth dilution in these samples, therefore sought more detail regarding the form of mercury in our fish and invertebrate samples. Thus lake trout collected in 2007 were analyzed for T-Hg and MeHg and a correction factor was applied to the empirical measurements of T-Hg in lake trout from previous years (2000 – 2006) for comparison across years. Because archived tissue samples were frozen and partially dessicated, results from all fish tissue samples used in this study are reported on a dry weight basis.

Lake trout diet items including prey fish and invertebrates were tested for T-Hg and a subset of these (collected from lake trout diets in 2003 – 2007) were analyzed for MeHg content. Newly collected and archived samples of lake trout, prey fish and invertebrates were dried prior to T-Hg and MeHg analyses. For lake trout, only skinless muscle tissue was analyzed. Lake trout diet items were tested for T-Hg by homogenizing at least 25 individual invertebrate prey items (*e.g.*, chironomids, ephemerids (*Hexagenia*) and daphnids) or two to three fish prey, (*e.g.* rainbow smelt *Osmerus mordax*, smallmouth bass and pumpkinseed sunfish *Lepomis gibbosus*) taken directly from lake trout stomachs. A sub-sample of the homogenate from invertebrate prey (chironomids, ephemerids and daphnids) was also analyzed for MeHg . Additional smallmouth bass (n = 15) were analyzed for MeHg as part of another project, and these data were used to allocate the MeHg proportion of T-Hg in

smallmouth bass and pumpkinseed sunfish from lake trout diets. Additional MeHg data obtained from yellow perch *Perca flavescens* in a nearby lake were used to estimate the MeHg proportion of T-Hg in rainbow smelt because they have similar feeding patterns and information regarding rainbow smelt MeHg concentrations in Little Moose Lake was unavailable.

Samples analyzed for T-Hg were prepared by HNO_3 digestion and further oxidation with BrCl prior to analysis. Samples for MeHg analysis were first alkaline digested and an aliquot of the digestate was oxidized with BrCl for analysis of T-Hg. T-Hg was analyzed by modified EPA 1631, oxidation with $SnCl_2$ reduction, purge, gold trap collection and CVAFS detection. Methylmercury was analyzed using modified EPA 1630, aqueous phase ethylation, purge, Tenax trap collection GC separation and CVAFS detection. Certified reference materials, Dorm-2, IAEA350, IAEA142 and SRM1566b biota tissues were analyzed for quality assessments. Duplicate and matrix spike samples were prepared and analyzed for monitoring the precision and accuracy of the analyses. Duplicate samples varied slightly but never more than 4% from the original values with a mean of 1.8 ± 1.8%, and no systematic bias was evident.

Lake trout were collected using a combination of gillnetting, angling and electrofishing in 2000 – 2007. Sagittal otoliths were extracted from each lake trout and sagittal sections were prepared and mounted for age interpretation (Secor 1991). A subset (approximately 50) of these otoliths was aged by two readers independently and any discrepancies in age were discussed until the age was agreed upon. The remaining otoliths were interpreted by a single reader (one of the two mentioned above) with six years of experience ageing salmonids using otoliths.

Baseline stable isotope signatures developed following Lepak et al. (2006) were used to determine lake trout $\delta^{13}C$ and trophic position. Lake trout $\delta^{13}C$ was

determined using a two-source mixing model following Vander Zanden et al. (1999). Lake trout trophic position was determined using diet information obtained in 2002 (the most recent substantial collection of lake trout for diet analysis, N = 93) following Lepak et al. (2006). Lake trout diets in 2005 – 2007 were quantified as described in Lepak et al. (2006).

A principal component analysis was conducted to develop a single, multivariate index that could represent changes in lake trout length, weight and age in models accounting for the influence these factors on key response variables (Niles 1973; Cooch et al. 1999). Due to high correlations between these three size-age metrics, they cannot be used as independent predictors. This index (referred to as PC1, the size-age principal component) was used to account for differences in lake trout length, weight and age by year in accounting for changes in MeHg through time. A second principal component incorporating lake trout length, weight and age was also developed, but had little to no explanatory value for the variation in lake trout MeHg (Pearson correlation coefficient; -0.01, $p = 0.92$) and therefore was not used in further analyses. Changes in lake trout $\delta^{13}C$ and trophic position were also evaluated in models accounting for the size-age principal component, year, and relevant interaction terms. A linear regression analysis was conducted to evaluate changes in lake trout growth over time. All statistical analyses (principal components analysis, ANCOVA's, Pearson correlation coefficients and linear regression analyses) were conducted using SAS (SAS Institute Inc.).

Bioenergetics simulations:

Bioenergetics simulations were developed to compare empirical values of lake trout MeHg with estimated values to identify potential mechanisms responsible for changes in lake trout MeHg concentration. Specifically, the Wisconsin Fish

Bioenergetics Model 3.0 (see Hanson et al. 1997) was used to estimate individual lake trout MeHg concentration using lake trout diet and growth as observed under two different scenarios – 1) prior to, and 2) following the smallmouth bass removal. Under both scenarios MeHg concentrations and the energy content of prey items were varied (± 10%) to evaluate the relative importance of the influence of these factors on lake trout MeHg concentrations. We also compared pre and post-removal estimates of lake trout prey energy density, lake trout growth efficiency and lake trout prey MeHg concentration to evaluate the relative importance of these factors in determining lake trout MeHg concentration under the changing conditions related to the smallmouth bass manipulation described in this and previous studies (Lepak et al. 2006; Weidel et al. 2007).

The pre-removal simulation was designed to mimic the growth of individual lake trout prior to the initiation of the smallmouth bass removal using the mean age (12.5 years) of 15 lake trout collected in 2000 that grew to a mean weight of 761 grams. The diet composition of fish collected in 2000 was similar to that of fish collected in 1996 and 1997 (N = 99 and 89 respectively). Similar amounts of chironomids, ephemerids (primarily *Hexagenia*) and daphnids (see Lepak et al. 2006) were found in diets from fish collected in both time periods, therefore these data were used in simulations of the pre-removal diets. The post-removal simulation used the maximum growth rate observed following the smallmouth bass removal in an attempt to evaluate the likely maximum potential impact of growth dilution on individual lake trout MeHg concentrations. The maximum mean growth rate of Little Moose Lake lake trout was observed in fish collected in 2006 with a mean age of 7.5 years (mean weight: 745 grams). Because diet data collected from lake trout in 2005 – 2007 were limited with respect to sample size and seasonality and the observed prey fish species were different from the prior substantial collection of diet data in 2002 (N = 93), diets

from lake trout collected in 2002 were used to develop the post-removal diets in the simulation. The most common three diet items (accounting for > 70% of lake trout diet dry weight) were set to comprise 100% of the diets for the purposes of the simulations under both pre- and post-removal conditions (Table 5.1). The remaining

Table 5.1. Lake trout pre- and post-removal diet compositions. The three most abundant diet items were set to represent 100% of lake trout diets. Estimates of energy densities (J/g), total mercury (µg/g) and percentage of total mercury that was in the methylated form are shown.

Pre-removal diet	Proportion	Energy (J/g)	Mercury (µg/g)	% Methyl
Chironomids	63%	2747	0.023	31%
Hexagenia	34%	4706	0.040	36%
Daphnia	3%	2514	0.036	25%
Post-removal diet				
Rainbow smelt	43%	4865	0.030	100%
Smallmouth bass	39%	4186	0.030	81%
Pumpkinseed sunfish	18%	4186	0.038	81%

diet items were diverse and no information was available regarding their MeHg concentrations. Estimates of energy densities for diet items were compiled from the literature (Cummins and Wuycheck 1971; Rand et al. 1994; Hanson et al. 1997).

Bioenergetics simulations were used to estimate the mass of the diet items necessary for an individual lake trout to consume to achieve the growth observed under pre- and post-removal conditions. These values were estimated daily and were then used to estimate the MeHg concentration in a lake trout using the following equation:

$$MeHg_{LT} = [(\alpha * C * MeHg_{prey}) - K] / W \qquad (1)$$

where $MeHg_{LT}$ is daily lake trout MeHg concentration (μg/g), α is the assimilation efficiency of MeHg, C is daily consumption in grams, $MeHg_{prey}$ is the lake trout prey items MeHg concentration (μg/g), K is the MeHg elimination rate and W is the estimated lake trout weight in grams. These simulations were carried out for 12.5 and 7.5 years for the pre and post-removal conditions respectively. The cumulative numerator divided by daily lake trout weight in equation 1 provides daily estimates of lake trout MeHg concentrations through time. The assimilation efficiency of MeHg was assumed to be 0.8, reflecting the assimilation efficiency of sulfur-containing proteins, to which MeHg is covalently bound (Brett and Groves 1979; Harris et al. 2003; Trudel and Rasmussen 2006). The elimination rate (K) of MeHg was calculated using the following equation developed by Trudel and Rasmussen (2001):

$$\text{Ln}(K) = 0.066(T) - 0.2*\text{Ln}(W) - 5.83 \qquad (2)$$

where K is the elimination rate of MeHg, T is the water temperature and W is the weight of the fish. Water temperatures to which fish were exposed were estimated using data obtained from a temperature logger placed at a depth of 10 meters in Little Moose Lake in 2004-2005.

We assumed that lake trout were not sexually mature for the purposes of the simulation (maturation ranges from approximately 7 to 11 years of age in this population, J. Lepak, J. Robinson and N. Smith unpublished data), therefore no mercury losses occurred due to spawning. Other investigators have observed that elimination of T-Hg from eggs and sperm is negligible in lake trout relative to other losses (Trudel and Rasmussen 2001). The bioenergetics simulations also assumed that

the majority of MeHg in lake trout tissue came from dietary sources and not through exchange from the water column (Harris and Bodaly 1998; Johnston et al. 2003).

Results:

Empirical findings:

Lake trout collected from 2000 – 2007 were between 362 and 551 mm in length with a mean length of 456 ± 3 mm (1 SEM); diets of these predators following shortly after the initiation of the smallmouth bass removal (2002-2007) were consistently dominated by fish prey (> 70% by dry weight). Based on the diet data available from 15 lake trout collected each year from 2005 – 2007, lake trout primarily consumed smallmouth bass, followed (in order of respective abundance) by small lake trout, rainbow smelt, slimy sculpin *Cottus cognatus* and pumpkinseed sunfish. Based on data collected in 2007, the mean percentage of MeHg relative to T-Hg in the 2007 lake trout was 91.7 ± 2.8% (1 SEM). This value was used to convert lake trout T-Hg concentrations measured in previous years to MeHg concentrations.

No trend was observed over time in the first principal component (PC1) encompassing lake trout size and age (e.g. length, weight and age) (ANCOVA; $F_1 = 0.41$, $p = 0.52$). A model developed to evaluate the importance of year and the lake trout size-age principal component upon lake trout MeHg (MeHg = year + PC1 + year * PC1) indicated that PC1 was the most important factor associated with lake trout MeHg (ANCOVA; $F_1 = 25.13$, $p < 0.01$) followed by year (ANCOVA; $F_5 = 3.19$, $p = 0.01$) and the interaction term (ANCOVA; $F_5 = 1.99$, $p = 0.09$). A linear regression analysis showed that from 2000 – 2007 lake trout MeHg concentrations increased significantly ($F_1 = 6.83$, $p = 0.01$; see Figure 5.1). The interaction between PC1 and year was included in this analysis because it was significant at $\alpha = 0.1$, but including

or excluding the interaction term did not change the significance of the increase in lake trout MeHg concentrations over time.

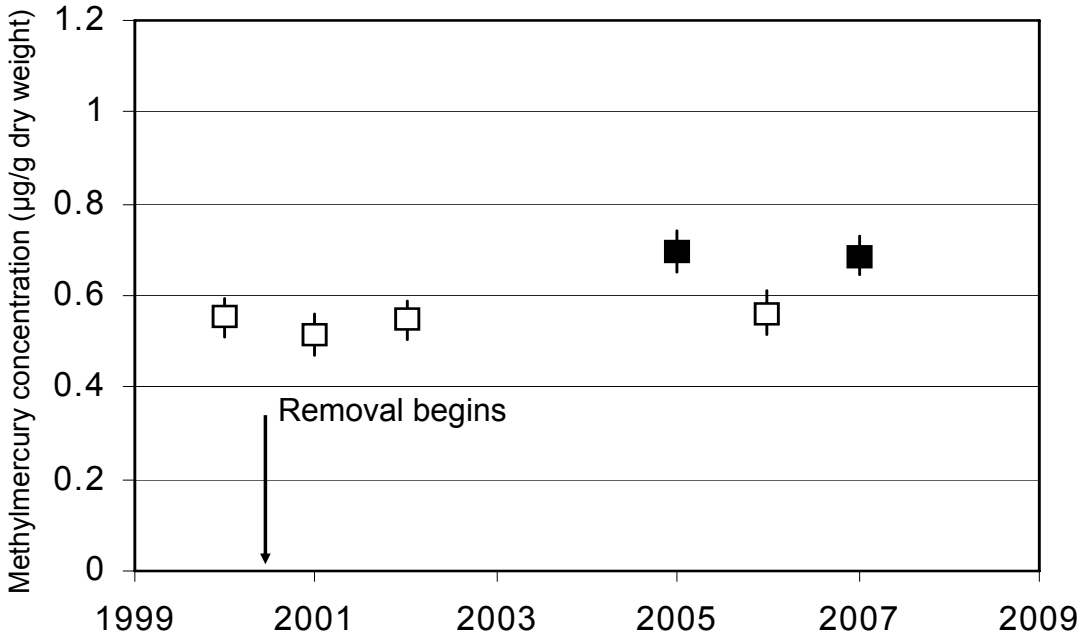

Figure 5.1. Lake trout MeHg by year. Least squares mean annual lake trout MeHg (µg/g) is presented in terms of dry weight. Filled boxes are significantly higher than the open boxes at α = 0.05. N = 15 for all years. Error bars are one SEM.

A model developed to evaluate the influence of year and PC1 upon lake trout $\delta^{13}C$ ($\delta^{13}C$ = year + PC1 + year * PC1) indicated that the size-age principal component was the most important factor for predicting lake trout $\delta^{13}C$ (ANCOVA; $F_1 = 7.80$, p = 0.01) followed by year (ANCOVA; $F_5 = 3.07$, p = 0.01) and the interaction term (ANCOVA; $F_5 = 1.60$, p = 0.17). A linear regression analysis showed that from 2000 – 2007, lake trout $\delta^{13}C$ increased significantly ($F_1 = 7.83$, p = 0.01; see Figure 5.2). Including or excluding the interaction term did not change the significance of the increase in lake trout $\delta^{13}C$ over time.

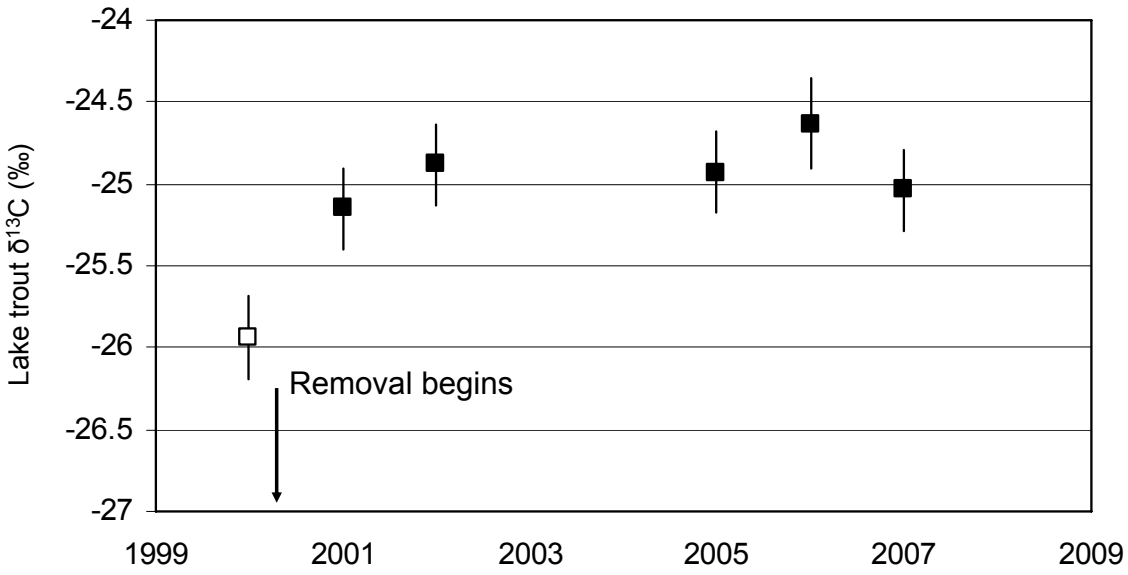

Figure5.2. Lake trout $\delta^{13}C$ by year. Least squares mean annual lake trout $\delta^{13}C$ (‰) is presented. Filled boxes are significantly higher than the open box at $\alpha = 0.05$. N = 15 for all years. Error bars are one SEM.

A model developed to evaluate the importance of year and PC1 for predicting lake trout trophic position (trophic position = year + PC1 + year * PC1) showed that year was the most important factor (ANCOVA; $F_5 = 4.87$, $p < 0.01$) followed by PC1 (ANCOVA; $F_1 = 4.38$, $p = 0.04$) and the interaction term (ANCOVA; $F_5 = 1.87$, $p = 0.11$). A linear regression analysis showed that from 2000 – 2007, lake trout trophic position has not increased significantly ($F_1 = 0.03$, $p = 0.87$; see Figure 5.3). Earlier findings showed an initial increase in lake trout trophic position (Lepak et al. 2006), but this trend has not continued through time. The interaction between PC1 and year

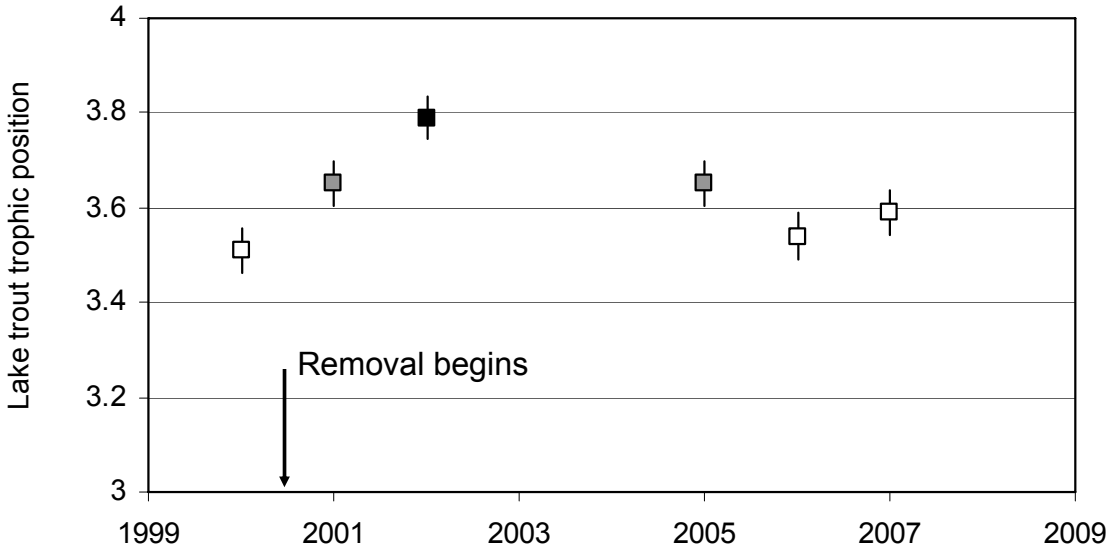

Figure 5.3. Lake trout trophic position by year. Least squares mean annual lake trout trophic position is presented. Boxes shaded differently are significantly different at α = 0.05. N = 15 for all years. Error bars are one SEM.

was included in this analysis because it was nearly significant at α = 0.1, but including or excluding the interaction term did not change the significance of the increase in lake trout MeHg over time.

Lake trout $\delta^{13}C$ and trophic position were correlated (Pearson correlation coefficient; 0.42, $p < 0.01$). Because we were interested in the importance of diet source and trophic position relative to changes in lake trout MeHg concentration, the effects of $\delta^{13}C$ and trophic position were evaluated individually. Individual models were developed for $\delta^{13}C$ and trophic position (MeHg = $\delta^{13}C$ + year + PC1 + year * PC1 and MeHg = trophic position + year + PC1 + year * PC1, respectively). Neither lake trout $\delta^{13}C$ nor trophic position effectively explained the observed variation in lake trout MeHg (ANCOVAs; $F_{1,1}$ = 1.25 and 0.61, p = 0.27 and 0.44 respectively). In both cases, PC1 was the most important factor for predicting lake trout MeHg (ANCOVAs; $F_{1,1}$ = 25.07 and 21.45, p's < 0.01 respectively) followed by year

(ANCOVAs; $F_{5,5}$ = 3.27 and 3.32, p's = 0.01 respectively) and the interaction term (ANCOVAs; $F_{5,5}$ = 2.06 and 1.91, p = 0.08 and 0.10 respectively). Trophic position explained more of the variability in lake trout MeHg concentration than $\delta^{13}C$, but this relationship was not significant. The interactions between PC1 and year in both analyses were significant at $\alpha = 0.1$ and were included, but including or excluding the interaction terms did not change the outcome of these results.

Lake trout age at length decreased significantly from 1996 – 2007 (Figure 5.4). Combining data from 1996 – 1997, 2000 – 2002 and 2005 – 2007, a linear regression analysis revealed a significant decline in lake trout age interpreted from otoliths after accounting for individual length (F_7 = 54.76, p < 0.01; Note that only lake trout from 400 – 500 mm in length were used from the 1996 and 1997 collections, adding 68 and 126 lake trout to the data set respectively).

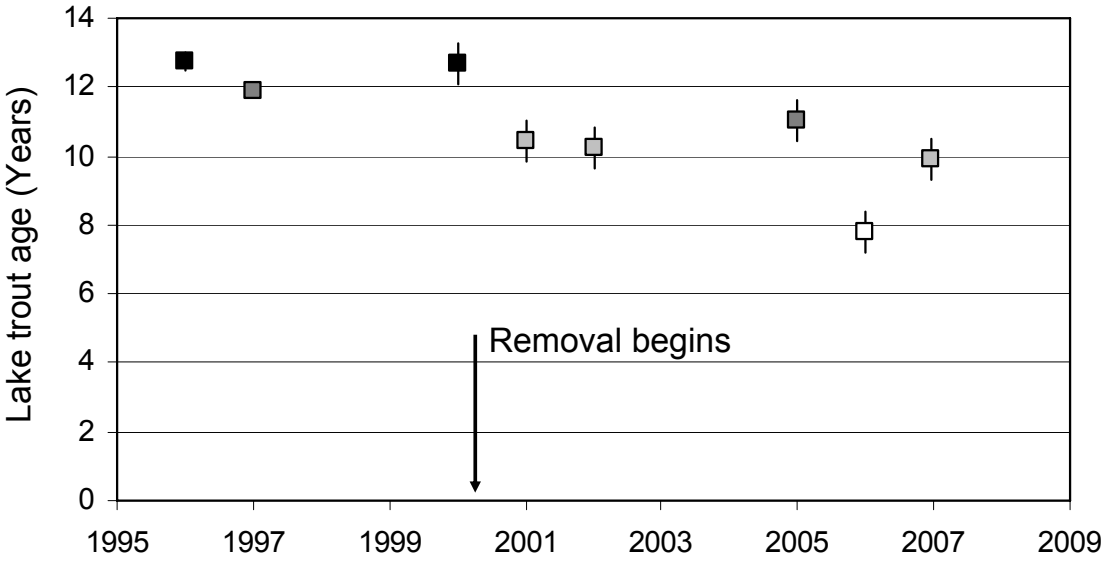

Figure 5.4. Lake trout age by year. Least squares mean annual lake trout age is presented. Boxes shaded differently are significantly different at α = 0.05. N = 68 and 126 for 1996 and 1997 respectively and 15 for all other years. All lake trout from 1996 and 1997 were between 400 and 500 mm. Error bars are one SEM.

Bioenergetics simulation findings:

Simulation results indicated that the most important factor evaluated in this study that influenced MeHg concentrations in lake trout following the smallmouth bass removal was the shift in lake trout diet to food sources that had higher concentrations of MeHg (Table 5.2). Increasing energy density in lake trout prey items (and subsequently growth efficiency of lake trout) resulted in decreases in lake trout MeHg concentrations that were of similar magnitude to those associated with MeHg increases caused by increasing MeHg concentrations in lake trout prey items (Table 5.3). However, the weighted-mean estimates of MeHg concentration in lake trout prey items was almost three-fold higher in prey fish (post-removal diet) relative to invertebrates (pre-removal diet). This mechanism driving lake trout MeHg concentration up was relatively large by comparison with the changes in lake trout diet

Table 5.2. Lake trout prey energy content, growth efficiency and prey item MeHg concentration estimates pre- and post-removal. Energy content and MeHg concentrations shown are weighted means; the sum of (proportion of total * J/g and MeHg concentration respectively). The increase in estimated lake trout prey energy content, growth efficiency and prey item MeHg concentration estimates are shown. The expected influences of these increases on lake trout MeHg concentration are listed respectively.

	Pre-removal	Post-removal	Increase	Mechanism drives
Energy content of prey items (J/g)	3747	4030	7.5%	[MeHg] down
Growth efficiency (%)	5%	10%	100%	[MeHg] down
MeHg in prey items (ng/g)	9.7	27.9	288%	[MeHg] up

Table 5.3. Influence of proportional changes in lake trout prey energy content and MeHg concentration relative to baseline bioenergetics simulations for pre- and post-removal conditions. Growth efficiency is represented by growth divided by total consumption. Total exposure of lake trout to MeHg from prey items is shown (Total MeHg) and the resulting lake trout MeHg concentration based on bioenergetics simulations are shown in dry weight. The percent change in lake trout MeHg concentration resulting from varying lake trout prey energy content and MeHg concentration is shown as a difference from the respective baseline estimation for pre and post-removal simulations.

Pre-removal conditions	Age (Years)	Initial weight (g)	Final weight (g)	Growth (g)	Total Consumption (g)	Growth efficiency (%)	Total MeHg (µg)	MeHg concentration in dry weight (µg of MeHg/g)	Change (%) from baseline
Energy content (-10%)	0-12.5	10	761	751	15757	4.77	121.75	0.65	12.0 (+)
MeHg (+10%)	0-12.5	10	761	751	14073	5.34	119.61	0.64	10.0 (+)
Baseline	0-12.5	10	761	751	14073	5.34	108.73	0.58	0.0
MeHg (-10%)	0-12.5	10	761	751	14073	5.34	97.86	0.52	10.0 (-)
Energy content (+10%)	0-12.5	10	761	751	12719	5.90	98.27	0.52	9.7 (-)
Post-removal conditions									
Energy content (-10%)	0-7.5	10	745	735	8038	9.14	179.41	0.98	13.6 (+)
MeHg (+10%)	0-7.5	10	745	735	7192	10.22	176.57	0.96	10.0 (+)
Baseline	0-7.5	10	745	735	7192	10.22	160.52	0.87	0.0
MeHg (-10%)	0-7.5	10	745	735	7192	10.22	144.46	0.79	10.0 (-)
Energy content (+10%)	0-7.5	10	745	735	6508	11.29	145.26	0.79	9.6 (-)

energy density (~ 300 J/g or 7.5% increase) and subsequent change in growth efficiency (increasing from 5 to 10%) that resulted in lower estimated lake trout consumption that could have reduced overall lake trout MeHg concentrations (Table 5.2).

The bioenergetics simulations showed that although lake trout are growing faster following the smallmouth bass removal, they are still expected to have higher MeHg concentrations relative to the period before the removal. This prediction matches the empirical data obtained from lake trout collected from Little Moose Lake. The pre- and post-removal bioenergetics simulations estimated lake trout MeHg concentrations of 0.58 µg/g dry weight and 0.87 µg/g dry weight, respectively, while the empirical least squares means of lake trout MeHg concentrations pre- and post-removal were 0.54 µg/g dry weight and 0.65 µg/g dry weight, respectively.

A simulation using T-Hg values for lake trout prey, instead of measured MeHg concentrations showed that lake trout would have reached a MeHg concentration of

1.76 µg/g dry weight under pre-removal conditions (compared to 0.58 µg/g dry weight using measured MeHg concentrations as reported above). A simulation using T-Hg values for lake trout prey, instead of measured and estimated MeHg values, showed that lake trout MeHg concentrations would have reached 0.99 µg/g dry weight under post-removal conditions (compared to 0.87 µg/g dry weight using estimated MeHg concentrations as reported above).

Discussion:

Given previous observations of T-Hg growth dilution in empirical studies and corroborative theoretical predictions (Göthberg 1983; Verta 1990; Rask et al. 1996; Borgmann and Whittle 1992; Simoneau et al. 2005), our observations of increased growth and increasing MeHg concentrations in lake trout were unexpected. We expected that increased lake trout growth – reflected by a decrease in age at length – observed in lake trout following the smallmouth bass removal would result in lower MeHg concentrations. Instead, our results support observations that growth dilution has a smaller influence on mercury concentrations in predatory fish than corresponding changes in fish diets that substantially influence mercury intake (Harris and Bodaly 1998; Stafford and Haines 2001; MacRury et al. 2002). In our study the changes in fish diets influenced lake trout MeHg concentrations because lake trout began consuming prey fish that had higher concentrations of MeHg relative to invertebrate prey that previously dominated their diets. These changes occurred despite lake trout post-removal diets being higher in energy content resulting in higher lake trout growth efficiency and estimated lower overall consumption (as supported by the bioenergetics simulations) relative to pre-removal conditions. Although simulation results indicated that increasing lake trout prey energy content and/or lake trout growth efficiency result in decreases in lake trout MeHg concentration, the

empirical and theoretical results presented here show that this mechanism is not as important as the increase in lake trout diet MeHg concentration for determining lake trout MeHg concentration in our study system.

Varying the energy content of lake trout diets in the bioenergetics simulations showed that the potential for growth dilution may occur under certain circumstances. For example, it is probable that fish relying on prey with the same MeHg concentration that have increased energy content or decreased capture and handling costs would grow faster and have lower MeHg concentrations than fish consuming prey with lower energy content or increased capture and handling costs. This mechanism may explain why lake trout collected from Little Moose Lake in 2006 had relatively low MeHg concentrations when compared to lake trout collected in 2005 and 2007. The group of lake trout collected in 2006 had the fastest growth of any group evaluated in this study in conjunction with concentrations of MeHg comparable to values found in lake trout prior to and immediately following the initiation of the smallmouth bass removal. However, over the course of this study this effect was generally counteracted by the increase in lake trout diet MeHg concentrations.

T-Hg in fish is generally assumed to be primarily (~95%) in the form of MeHg (Bloom 1992). However, this is not the case with invertebrates and prey fish (Huckabee et al. 1979; Hildebrand et al. 1980; J. Loukmas pers comm.; Lepak unpublished data) and even in some fish predators such as lake trout and smallmouth bass (Lepak unpublished data). In our study we found that diet items varied widely in their proportion of MeHg relative to T-Hg, especially when comparing invertebrates and fish prey. Our ability to interpret empirical observations of lake trout MeHg concentration would have been confounded without taking into account differences in prey MeHg concentration. For example, if T-Hg measurements were used for invertebrate diet items the bioenergetics simulations would have predicted that lake

trout collected during the pre-removal period – consuming invertebrates with T-Hg concentrations similar to prey fish but with less MeHg – were higher in T-Hg concentrations than those collected during the post-removal period. The conclusion drawn from such simulations would therefore have been that growth dilution was very important and accounts for large differences in lake trout MeHg concentration.

Similar to previous observations based on three years of data (2000 – 2002; Lepak et al. 2006), lake trout from Little Moose Lake continue to consume prey fish and obtain energy from littoral energy sources in subsequent years (2005 to 2007). However, the trophic position of lake trout captured in 2006 and 2007 was similar to the trophic position observed at the onset of smallmouth bass removal, despite the fact that trophic position initially increased in 2001 and 2002 (Lepak et al. 2006). We speculate that lake trout may now be consuming littoral prey items that are lower in trophic position than those consumed in 2001 and 2002, such as smaller prey fish or prey fish consuming diet items with lower trophic positions relative to those consumed prior to the initiation of the smallmouth bass removal. It is possible that littoral prey fish are now lower in trophic position due to increased competition for food, given that the relative abundance of these fish has increased substantially in response to the bass removal (Weidel et al. 2007). These changes in trophic position suggest that food web changes are continuing to occur in response to the large-scale removal of a non-native dominant predator, but the mechanism behind these observations has not been identified.

Previous studies have shown that T-Hg concentrations in lake trout are positively correlated with activity (Trudel and Rasmussen 2001; Trudel and Rasmussen 2006). Rennie et al. (2005) used a contaminant model and two other independent measures, to demonstrate that a slow-growing yellow perch population was approximately 30% more active and twice as contaminated with MeHg relative to

a fast-growing yellow perch population found in a similar system. Both populations were feeding on benthic invertebrates and significant differences were not found in prey availability. Although we have no direct measures of changes in lake trout activity during the course of this study, we have observed increases in littoral prey fish abundance and consumption indicating that these prey are now more readily available. Similarly, we expect that the activity costs of lake trout feeding on prey fish would be lower than for lake trout feeding on invertebrates because a single prey fish provides the same amount of energy as gained by consuming hundreds or more individual invertebrate prey items. This is supported by the observation of Pazzia et al. (2002) that found piscivorous lake trout grew more efficiently than lake trout consuming invertebrates and that greater activity costs for non-piscivorous lake trout foraging on small prey items accounted for this difference. It is therefore hard to invoke increased activity associated with foraging on abundant prey fish as accounting for increased MeHg bioaccumulation, by comparison with evidence that lake trout in Little Moose Lake that were feeding on invertebrates were significantly lower in MeHg concentrations than those feeding on prey fish.

An important result of our study demonstrates the need to verify the proportion of T-Hg that consists of MeHg, which is the form of mercury that is of primary concern with regard to human health. We specifically note the commonly used, but flawed assumption that MeHg comprises > 95% of the T-Hg content of fish. Sport fish, prey fish and especially invertebrates have variable proportions of T-Hg that is in the form of MeHg (Huckabee et al. 1979; Hildebrand et al. 1980; J. Loukmas pers comm.; Lepak unpublished data). As such, MeHg analysis will be necessary for accurately measuring and predicting MeHg bioaccumulation in predatory fish until strong relationships can be established between total and MeHg across a wide variety of prey organisms.

The results from this study suggest that changes in food web structure and dynamics can have significant influences on MeHg concentrations in top predators such as lake trout. Specifically, alterations in fish density can influence MeHg concentrations in individuals remaining within a lake system. This mechanism could potentially be used to increase growth rates while maintaining or reducing levels of T-Hg intake, resulting in "growth dilution" as has been observed previously (Göthberg 1983; Verta 1990; Rask 1996). But more importantly, several food web components must be characterized before changes in MeHg concentrations in fish can be understood in response to changes in prey consumption and growth. For example, the Little Moose Lake manipulation showed that knowing the T-Hg content of fish predators and their prey is not sufficient for determining the effects of food web changes on MeHg concentrations in lake trout. Instead, it is necessary to understand the energy and MeHg content of the prey resources that become more or less available as a result of changes in lake food web dynamics. Only by understanding these characteristics will it be possible to evaluate how alterations in lake food webs influence the MeHg concentrations in sport fish.

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