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Effects of dietary thiaminase on reproductive traits in three populations of Atlantic salmon targeted for reintroduction into Lake Ontario

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Abstract

The fitness of reintroduced salmonids in Lake Ontario can be reduced by high levels of thiaminase in exotic prey consumed at the adult stage. If sensitivity to dietary thiaminase differs among the three Atlantic salmon populations targeted for reintroduction into Lake Ontario, this could significantly influence their performance. I quantified the effects of experimental diets that contained high or low (control) levels of thiaminase on thiamine concentrations, survival, growth rate, and reproductive traits (sperm and egg quality) in Atlantic salmon from the three candidate source populations. Fish that consumed the high-thiaminase diet had comparable growth rates, but lower survival and muscle thiamine concentrations than control fish. Sperm count, velocity, motility and linearity, did not differ based on diet. Adult females fed the high-thiaminase diet resulted in lower egg thiamine concentrations and embryo survival. The effects of dietary thiaminase on reproductive traits did not differ among the tested populations.

Keywords

Summary for Lay Audience

Atlantic salmon were once abundant in Lake Ontario but were driven to extinction more than a century ago. There have been multiple attempts to reintroduce Atlantic salmon into Lake Ontario, but these attempts have not yet produced a stable population. One potential obstacle is the presence of exotic fishes in Lake Ontario, including alewife and rainbow smelt. Alewife and rainbow smelt contain high levels of the enzyme thiaminase, which can lead to a thiamine deficiency in salmon that eat these fishes. Thiamine deficiency can lead to many negative health effects, including early offspring mortality and neurological disorders. Atlantic salmon from different populations naturally consume prey fishes that differ in thiaminase levels. If tolerance for dietary thiaminase differs among the three Atlantic salmon populations targeted for reintroduction into Lake Ontario, this could significantly influence their health on a high-thiaminase diet and the chances of establishing an Atlantic salmon population in Lake Ontario. To examine tolerance to thiaminase, I fed Atlantic salmon from the three candidate source populations experimental diets that contained high or low (control) levels of thiaminase and measured tissue thiamine concentrations, survival, growth rate, and reproductive traits (sperm and egg quality). Fish that consumed the high-thiaminase diet had comparable growth rates but lower survival and lower muscle thiamine concentrations than control fish. Male sperm characteristics, including sperm count, velocity, motility and linearity, did not differ based on diet. Embryo survival was lower for females fed the high-thiaminase diet, and the high-thiaminase diet was associated with significantly lower egg thiamine concentrations. The effects of dietary thiaminase on these traits did not differ among the tested populations, albeit survival was lowest for the LaHave population—predicted to be the most susceptible to dietary thiaminase—limiting their inclusion in these analyses. Regardless, the negative effects of a high-thiaminase diet across populations suggest that source population selection is unlikely to fully overcome this potential challenge for re-establishing Atlantic salmon in Lake Ontario.

Co-Authorship Statement

A version of this thesis has been submitted for publication to the Canadian Journal of Fisheries and Aquatic Sciences with Bryan Neff, Shawn Garner, Aimee Lee Houde, Chris Wilson and Trevor Pitcher as co-authors. All work described were supervised under Bryan Neff. Kim Mitchell developed the experimental design, collected and analyzed the data, and drafted the manuscript. Shawn Garner provided input to the experimental design, analyzed data and assisted in drafting the manuscript. Aimee Lee Houde contributed to the experimental design and developed the experimental feed. Chris Wilson provided the salmon and facilities where the experiment was conducted and contributed to the experimental design. Trevor Pitcher contributed to the experimental design and collection and analyses of the sperm data.

Acknowledgments

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1 Introduction

1.1 Reintroduction programs

The extirpation of species from parts of their historical range is a major issue in conservation biology, as the overall decrease in density may increase the risk of extinction due to demographic stochasticity (Melbourne and Hastings 2008). For example, eight of the ten populations of the Iberian lynx (*Lynx pardinus*) in Spain and Portugal were lost between 1985 and 2001; these extirpations were associated with a reduction in the total number of Iberian lynxes by 86-93% due to stochastic processes (Sarmento et al. 2009; Palomares et al. 2011). The removal of native species within an area may affect food web dynamics within an ecosystem and reduce biodiversity (Dar and Reshi 2013). For example, the extirpation of predatory pelagic sharks along the east coast of the USA had cascading effects, which have led to the loss of scallop (*Placopecten magellanicus*) populations (Sodhi et al. 2009). When the sharks disappeared, mesopredators such as skates and rays, increased in abundance and consumed large numbers of scallops. By 2004, the century-old fishery for scallops in North Carolina had collapsed (Myers et al. 2007). Extirpation is thus associated with a number of negative consequences, leading to great interest in reducing its occurrence and reversing its effects whenever possible.

A reintroduction program is an effort to return a species to an area where it was historically present, and is an important tool in reversing extirpation (Armstrong and Seddon 2008). Reintroductions can be done by translocating individuals from an extant population or by using captive-bred animals (Seddon et al. 2007). Several factors need to be considered to make a reintroduction successful, such as knowing the life history of a species and their reproductive tactics (IUCN/SSC 1998). As an example, knowledge of life history aided in the reintroduction of *Dianthus morisianus* in Sardinia. *Dianthus morisianus* is a plant that has been impacted by habitat loss and fragmentation, and only has one population remaining. An important life stage for *Dianthus morisianus* establishment is seedling emergence, therefore juvenile plants that survived without horticulture treatment were used for reintroduction efforts. The survival rate of the plants

was high and 65% became reproductive by the second year after planting (Cogoni et al. 2013). Reintroductions can be successful as a tool as long as all aspects of a species' biology is considered.

There have been several cases of successful reintroductions around the world, such as the reintroduction of Gray Wolves (*Canis lupus*) to Yellowstone National Park in the USA. Wolves were removed by farmers who were fearful of predation on their livestock, and were extirpated by the mid-1920s with detrimental cascading effects on the surrounding ecosystem. There was an explosion in the elk (*Cervus canadensis*) population, which resulted in the decline of several deciduous woody species, leading to the disappearance of beavers. The absence of beavers reduced the number of ponds, resulting in the decline of succulent plants, which grizzly bears use as food post-hibernation (Zahniser and Singh 2011). Reintroduction began in 1995 with the translocation of 31 wolves from Canada (Smith et al. 2003), and since then there has been a stable population of around 100 individuals, countering the negative effects (Boyce 2018). Another successful case was the reintroduction of the Eastern wild turkey (*Meleagris gallopavo silvestris*). The wild turkey was native to 39 States in the USA and southwestern Ontario, but populations declined in the late 1800's due to overhunting, and the loss of habitat caused by growing settlements. In southwestern Ontario, the turkeys disappeared by 1907, but reintroduction efforts were initiated in 1984, with birds imported from Iowa, Michigan, Missouri, New Jersey, New York, Tennessee and Vermont. By 2007, the population was estimated at approximately 70,000 turkeys, and there are now two hunting seasons each year, restoring a valuable ecosystem service that had been lost when the turkeys were extirpated (Nguyen et al. 2003; MacDonald et al. 2016).

However, many reintroduction efforts fail to achieve their desired outcomes (Deredec and Courchamp 2007; Haskins 2015). Reintroduction outcomes may differ among taxa, with 58% of freshwater fish reintroductions considered successful (Cochran-Biederman et al. 2015), whereas 44% of bird and mammal reintroductions and 19% of reptile and amphibian reintroductions were successful (Griffith et al. 1989; Dodd and Seigel 1991; Deredec and Courchamp 2007). The difference in success may partly be due to sociological factors, as birds, mammals and reptiles receive higher public support than

fish, and therefore result in more reintroduction attempts (Martin-Lopez et al. 2007; Worthington et al. 2010). The higher number of failed reintroduction attempts may lower the percentage of successes. Many other factors may influence the outcome of a reintroduction program, including the current versus the historic habitat conditions and the source population being used (i.e. how close to the historic range the source population is derived from) (Armstrong and Seddon 2008). It has become increasingly important to focus on factors that will grow the chance of a successful establishment of the reintroduced species (Seddon et al. 2007).

Choosing a source population based on its ecological and evolutionary history increases the chances of a reintroduction being successful. Houde et al. (2015a) developed a source population selection framework that evaluates the evidence for choosing a population based on either the genetic or environmental similarities between the source and target locations (pre-existing adaptation strategy), or by selecting a population or populations with high genetic diversity (adaptive potential strategy). A pre-existing adaptation strategy could use a population that is genetically similar to the extirpated population, and therefore has genes that are adapted to the target location. Alternatively, it could use a population that is from a similar environment, as it would be expected to have phenotypes that result in high fitness. For example, the reintroduction efforts of the golden paintbrush (*Castilleja levisecta*) in the Pacific Northwest were more successful when using seed sources from ecologically similar environments (Lawrence and Kaye 2011). An adaptive potential strategy will choose a population based on its ability to locally adapt to the target environment due to its high genetic variability. Multiple populations can also be used, which when mixed together create high genetic variation. For example, the Pitcher's thistle (*Cirsium pitcher*) reintroduction efforts that used multiple source populations had a high level of genetic variability lasting past the first generation. This is thought to allow the reintroduced population to have a greater capacity to adapt to the environment it was placed in (Fant et al. 2013). The choice of strategy may depend on the habitat state relative to its historical state. When the habitat is similar to the historical state, a source population that is a genetic match to the extirpated population is recommended. Although when the habitat has changed substantially, a

source population from a habitat similar to the new habitat may be the best choice (Houde et al. 2015a).

1.2   Impacts of introduced non-native species

The invasion of non-native species can change the composition of the community and inhibit the establishment of a reintroduced species. During the last century, over 180 non-native species have been introduced into the Great Lakes, including the zebra mussel (*Dreissena polymorpha*), the spiny water flea (*Bythotrephes longimanus*) and rainbow smelt (*Osmerus mordax*) (Vander Zanden and Olden 2008), and 35 have been documented to cause substantial impacts through predation, competition and trophic disruptions (Mandrak and Cudmore 2010). Non-native species may escalate changes to an ecosystem where habitat disturbances and invasions interact (Bauer 2012). For example, non-native species that are introduced have a tendency to do well in degraded habitats, such as the Great Lakes (Mandrak and Cudmore 2010), further aiding their spread.

One example of a prevalent invader in the Great Lakes is the rainbow smelt, that was introduced in 1912 as a forage fish for game fish, such as lake trout (*Salvelinus namaycush*). By the 1930s they had colonized all five Great Lakes, and hundreds of inland lakes, through accidental and intentional human-mediated processes (i.e. release as bait) (Dextrase and Mandrak 2006). In 94% of the sites, rainbow smelt have negatively impacted at least one native species, such as lake trout, walleye (*Sander vitreus*), yellow perch (*Perca flavescens*) and brook trout (*Salvelinus fontinalis*) as a result of niche overlap (Mercado-Silva et al. 2006). For example, juvenile yellow perch and rainbow smelt feed on the same prey and forage in the same habitat, and rainbow smelt have been found to decrease prey availability (Hrabik et al. 2001). Rainbow smelt have also been shown to replace native forage fish species. In Sparkling Lake in Wisconsin, cisco historically dominated the pelagic fish community, but they were replaced by rainbow smelt by 1985 (Hrabik et al. 1998). Therefore, the species has had effects across multiple trophic levels in the habitats they have invaded.

Another abundant invasive fish in the Great Lakes is the alewife (*Alosa pseudoharengus*), which were first spotted in Lake Ontario in 1873. They are believed to have used artificial channels, such as the Erie canal, to spread from the Atlantic Ocean to the Great Lakes. By the 1960s, alewives had spread to all of the Great Lakes, except for Lake Superior. They exceeded the carrying capacity of the lakes due to the lack of predators, resulting in massive die-offs (Dettmers et al. 2012). However, alewife continues to be a dominant forage fish species in Lake Ontario even when their population fluctuates (Weidel et al. 2019). Invasive zooplankton, such as the *Mysis relicta*, became a common prey species to alewife (Mills et al. 2003) further supporting the population of alewife in Lake Ontario. The high abundance of alewife had led to fishery managers searching for a way to control the population.

1.3   Salmonid communities in the Great Lakes

Since the arrival of European settlers, the Great Lakes have gone through enormous changes to their ecology due to overfishing, exotic species, eutrophication and the degradation of habitat. Historically, the salmonid community consisted of Atlantic salmon (*Salmo salar*) and lake trout, which preyed upon species like deepwater sculpins (*Myoxocephalus thompsoni*) and coregonids. After the disappearance of these native species, exotic species like alewife, rainbow smelt, and white perch (*Morone americana*) increased in population numbers (Mills et al. 2003). In an effort to control the population of alewives, chinook salmon (*Oncorhynchus tshawytscha*) were introduced in the 1970s, with the additional intention of creating a sport fishery (Fenichel et al. 2010). 32 to 54% of the stocking efforts in Lake Ontario comprised of chinook salmon from 1982 to 1999 (Mills et al. 2003). These efforts became so successful at reducing the alewife population that fishery managers became worried chinook salmon might overwhelm the prey supply, and stocking numbers decreased from 4.2 million in 1984 to 1.7 million fish in 1996 (Stewart and Ibarra 1991; O'Gorman and Stewart 1999). They were also stocked in the other Great Lakes, such as in Lake Superior, where millions of chinook salmon were introduced (Peck et al. 1999). Natural reproduction of chinook salmon has been observed in most of the Great Lakes as a result of the stocking efforts (Principe et al. 2007). In Lake Superior, chinook salmon spawning runs have been recorded in 41 tributaries. In

addition, from 1990 to 1994 75% of chinook salmon in Lake Superior and 30% of chinook salmon in Lake Michigan were discovered to be naturally produced (Peck et al. 1999). Similar patterns were seen with the introduction of coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*) (Peck et al. 1999). Although chinook salmon were originally meant to control the alewives and create a sport fishery, they became a naturalized species and an important predator within the Great Lakes system.

Lake trout were native to the Great Lakes and supported a commercial fishery, but disappeared by the 1950s (with the exception of a few regions in Lake Superior) due to overfishing and the parasitic sea lamprey (*Petromyzon marinus*) (Binder et al. 2015), which invaded Lake Ontario via the Erie Canal in the early 1800s, and the rest of the Great Lakes via the Welland Canal in the early 1900s (Christie and Goddard 2003). As sea lamprey cause mortality by latching onto a host to suck blood and leave open wounds (Christie and Goddard 2003), fishery managers looked at ways to eliminate them. The lampricide selected was 3-trifluoromethyl-4-nitrophenol (TFM) (Applegate et al. 1961), and starting in 1958 it was applied to tributaries in the Great Lakes to cease spawning runs (Applegate et al. 1961; Pearce et al. 1980; Cornelius et al. 1995), and was considered successful. For example, spawning sea lamprey runs in Lake Superior were reduced by 92% in 1978 (Smith and Tibbles 1980). Once the issue of sea lamprey was controlled, reintroduction efforts for lake trout was initiated, and numbers increased to more than 2 million fish annually in Lake Ontario between 1985 and 1992 (Stewart and Ibarra 1991; O'Gorman and Stewart 1999). There was a focus on using the Seneca population for reintroduction efforts, which had higher survival rates than other populations (Marsden et al. 1989; Elrod et al. 1995). Unfortunately, self-sustaining populations have only recovered in Lake Superior, where there were already remnant stocks, and in regions of Lake Huron (Hansen 1995; Reid et al. 2001; Bronte et al. 2002). In the other three lakes, populations continue to rely on stocking efforts (Muir et al. 2012). It is thought that the lack of establishment is affected by alewife predation on their eggs and fry (Jones et al. 1995; Krueger et al. 1995), and thiamine deficiency (Fitzsimons et al. 2007).

1.4   Atlantic salmon reintroduction in Lake Ontario

Atlantic salmon populations throughout Europe and North America have experienced extirpations or severe declines (Parrish et al 1998; Gibson 2017). Prior to 1850, Atlantic salmon were an abundant source of food for early settlers and indigenous peoples around Lake Ontario, with large tributaries, such as the Credit River, historically supporting significant Atlantic salmon runs. At the first signs of decline of the salmon, a government hatchery was opened on Wilmot Creek in 1866, and population increases were initially observed (Parsons 1973). However, Atlantic salmon were ultimately extirpated from Lake Ontario by 1898 due to habitat degradation, dam construction and over-exploitation (Crawford 2001).

Recent efforts to reintroduce Atlantic salmon into Lake Ontario have not yet resulted in a self-sustaining population, even though the causes of degradation from the 1800s and 1900s in Lake Ontario have largely been improved, as evident by the increase in water quality, the reversal of eutrophication (Beeton 2002) and the removal of dams (Ketola et al. 2000). As these habitats support populations of other salmonids, such as chinook salmon, coho salmon and steelhead (anadromous *Oncorhynchus mykiss*) (Johnson et al. 2010), other obstacles to the restoration of Atlantic salmon must exist. One potential challenge is that the prey fish community in Lake Ontario has changed considerably over the past century, and Atlantic salmon in Lake Ontario historically fed primarily on lake herring (cisco, *Coregonus artedi*) and bloater (*C. hoyi*), but these prey species have largely been replaced by alewife and rainbow smelt (Crawford 2001). These introduced fishes have much higher levels of the enzyme thiaminase, which breaks down thiamine (vitamin B1) (Tillitt et al. 2005), and the high levels may negatively affect the restoration program efforts.

1.5   Role of thiamine

Thiamine (vitamin B_1) is an environmentally-obtained nutrient that is essential for all animals (Harder et al. 2018). It occurs in the environment at extremely low concentrations, as its availability depends on factors such as pH, ultraviolet radiation and temperature. It is primarily produced by micro and macro algae, as well as by some fungi

and bacteria (Kraft and Angert 2017). After ingestion, free thiamine may be phosphorylated into thiamine mono- or pyrophosphate (di-phosphate) forms (Figure 1.1). Thiamine pyrophosphate is the active form of this vitamin and acts as a cofactor for enzymes necessary for carbohydrate metabolism (Amcoff et al. 1999), and also plays a central role in nerve function (Brown et al. 1998).

Thiamine has a critical role in metabolic function and neural development, leading to many well-documented effects of thiamine deficiency (Depeint et al. 2006). Interest in the subject started in the early 1900s as thiamine deficiency causes beriberi in humans and polioencephalomalacia (PEM) in livestock. Beriberi is induced by several causes, such as the intake of mostly thiamine-poor food such as milled rice, alcoholism, pregnancy and malnutrition (Fattal-Valevski 2011). The disease presents itself as either wet beriberi, which involves the cardiovascular system, or dry beriberi, which involves the nervous system. Early symptoms of the Beriberi disease progression in humans include fatigue, irritation and poor memory. Later stages of the disease involve symptoms such as polyneuritis (disorder of the peripheral nerves), bradycardia (slow heart rate), peripheral edema (swelling of peripheral vascular system), cardiac enlargement, and ophthalmoplegia (paralysis of eye muscles) (Fattal-Valevski 2011). Thiamine also may affect sperm quality in humans, where the concentration of spermatozoa is positively correlated to the thiamine diphosphate concentration in semen (Gangolf et al. 2010). Overall, thiamine has been linked to a number of important biological processes, especially during reproduction and early development (Wilson 1997).
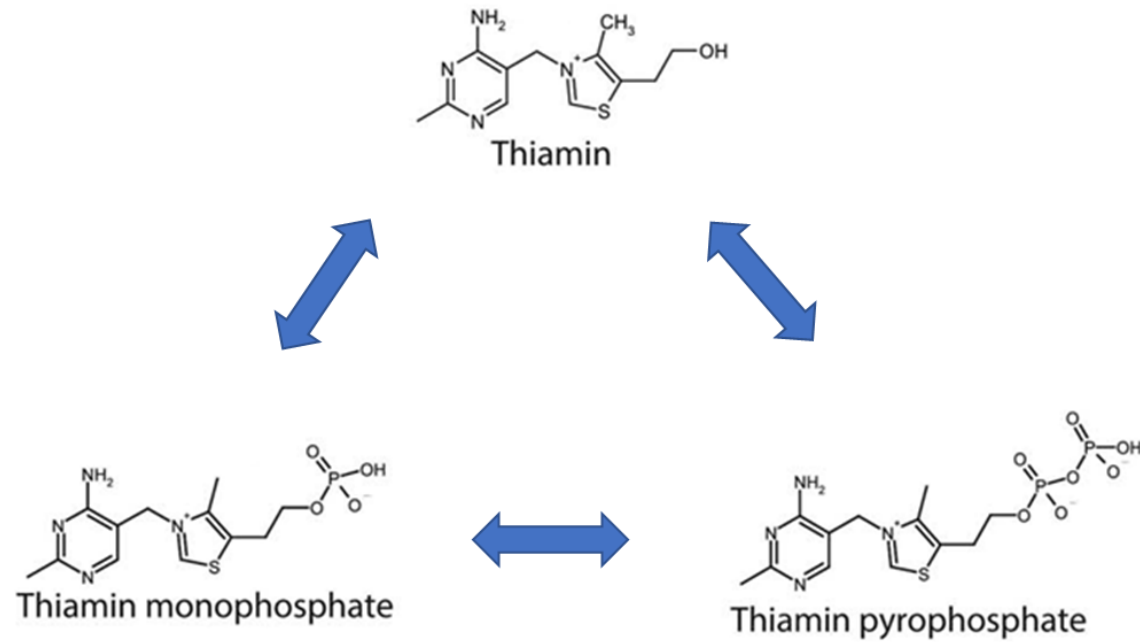
Figure 1.1 Shown are vitamers that can be derived from thiamine through phosphorylation. Free thiamine (thiamin) is phosphorylated into thiamine monophosphate and thiamine pyrophosphate (diphosphate) (TDP), which is the active form of this vitamin, and acts as a cofactor for enzymes necessary for carbohydrate metabolism. Figure is adapted from Kraft and Angert (2017).
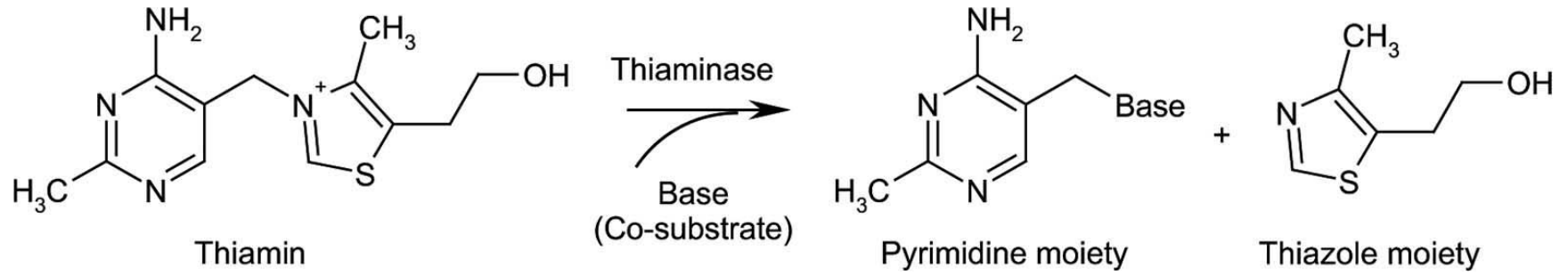
Figure 1.2 Shown is the degradation of thiamine (thiamin) into its components via the enzyme thiaminase. Figure is adapted from Kraft and Angert (2017).

Thiaminase I and thiaminase II modify thiamine; the former degrades the vitamin by replacing the thiazole moiety with a variety of nucleophiles, while the latter breaks down thiamine into its constituent parts with the use of water as the co-substrate. Both cases eliminate thiamine's biological function (Hanes et al. 2007; Kraft and Angert 2017) (Figure 1.2). The function of thiaminase is not well-understood. Thiaminase I is found in shellfish, clams, ferns, and fish (via certain species of *Bacillus* and *Clostridium* in the guts). Thiaminase II is only found in bacteria, and breaks down thiamine but not thiamine pyrophosphate, which is the active form (Bitsch 2003). The ability to produce thiaminase may allow certain bacteria, fungi and plants to outcompete thiamine auxotrophs (organisms that cannot synthesize thiamine) by reducing the availability of thiamine in the environment, and having the ability to salvage components required for thiamine synthesis (Kraft and Angert 2017). Fishes that have thiaminase-producing bacteria in their gut may obtain thiamine through this symbiotic relationship (Harder et al 2018), as a positive correlation between thiamine content and thiaminase activity has been reported in alewife (Tillitt et al 2005). Although the relationship between thiaminase and thiamine has been well studied, the importance of thiaminase is still poorly understood.

Consumption of resources containing thiaminase, and specifically thiaminase I, is known to induce thiamine deficiency in animals (Harder et al. 2018; Sannino et al. 2018). Thiamine deficiency in sheep was caused by the consumption of water ferns (*Marsilea* spp.) (McCleary and Chick 1977; Fattal-Valevski 2011; Kraft and Angert 2017). In American alligators (*Alligator mississippiensis*) ingesting gizzard shad (*Dorosoma cepedianum*), clutches with high concentrations of thiamine had increased hatch rates compared to clutches with low thiamine concentrations (Sepúlveda et al. 2004; Ross et al. 2009). In birds that eat alewives, thiamine deficiency during development results in polyneuritis, a disorder of the peripheral nerves, and embryonic mortality (Wilson 1997; Munot et al. 2013).

Thiamine deficiency has been well-documented in salmonids, including lake trout, Atlantic salmon, chinook salmon, coho salmon, steelhead, and brown trout (*Salmo trutta*)

(Harder et al. 2018). In the Great Lakes basin, the disorder is referred to as Early Mortality Syndrome (EMS) (Werner et al. 2006), and was first linked to reproductive failure in wild fish in the mid-1990s (Honeyfield et al. 2016). Egg total thiamine concentrations in salmonids within the Great Lakes region were found to be inversely correlated with alewife abundance in diet (Riley et al. 2011), emphasizing the effects of a high-thiaminase diet. Lake trout reared on a diet of alewife produced eggs with low thiamine concentrations, which resulted in increased fry mortality (Honeyfield et al. 2005). Salmon in regions of the Baltic Sea have displayed up to 90% of mortality in fry prior to the onset of exogenous feeding (Karlsson et al. 1999). This disorder is known as M74 syndrome, and is thought to be caused by adult salmon feeding on the European sprat (*Sprattus sprattus*), which are rich in thiaminase and make up more than 50% of their diet (Harder et al 2018). However, a diet high in sprat may not be the only factor in inducing thiamine deficiency. Young sprat contain approximately half the amount of total thiamine as older sprat (range for sprat aged 1–5 years: 16–122 nmol total thiamine/g fat; aged 6–13 years: 48–248 nmol/g fat) (Keinänen et al. 2012). Further, the combination of high fat and low thiamine concentrations can lead to oxidative stress in Atlantic salmon, when easily oxidized fatty acids are not balanced by antioxidants like thiamine (Lukienko et al. 2000). An unbalanced diet that consists of fatty prey fishes with low thiamine levels may thus be another risk factor for the development of thiamine deficiency in salmonids.

In salmonids, thiamine deficiency has been linked to reduced activity, abnormal movement patterns and loss of equilibrium during multiple life stages (Fisher et al. 1995; Fitzsimons et al. 1995; Fisher et al. 1996; Ketola et al. 2000; Brown et al. 2005; Fitzsimons et al. 2007; Ketola et al. 2008). Young salmonids appear most sensitive to the effects of thiamine deficiency, probably because of their small thiamine stores and the importance of thiamine during development (Morito et al. 1986; Ketola et al. 2008). The reproductive phase is thus critically important because females that are deficient in thiamine cannot provision their eggs with sufficient thiamine. Interestingly, a study of Atlantic salmon found that the concentration of thiamine was 5 times higher in milt (sperm and seminal plasma) than in eggs, although the purpose of thiamine in milt was unclear, as there is little transfer of thiamine from the male to the offspring (Koski 2002).

Instead, thiamine might affect sperm performance and fertilization success, although neither effect has been examined before.

Sperm metrics (i.e. sperm velocity) can be used to assess male reproductive function. For species that fertilize eggs externally, such as with Atlantic salmon, sperm are exposed to many environmental stressors (i.e. temperature, pH) that reduce the likelihood that sperm will reach an egg (Morisawa et al. 1983; Pennington 1985; Billard et al. 1986). Males that produce faster sperm have been shown to fertilize more eggs, suggesting that sperm velocity is an important component of male reproductive function (Simmons and Fitzpatrick 2012). Sperm motility is affected by ATP production, and ATP is created by the oxidative phosphorylation in the mitochondria of the sperm or by glycolysis in the sperm flagellum (Fitzpatrick and Lüpold 2014). Thiamine acts as a cofactor for enzymes in several metabolic pathways, such as the production of ATP (Amcoff et al. 1999). Therefore, the quality of the sperm may be affected by the levels of thiamine in males.

Hatcheries have developed a few methods to deal with low thiamine concentrations in salmonid eggs. The most common method is the immersion of fertilized eggs in a thiamine solution during "water hardening", which is the stage where the egg membranes become rigid. Immersion in a 1% thiamine hydrochloride (HCl) solution increased the total egg thiamine concentrations by 14 times in Atlantic salmon eggs (Wooster et al. 2000). This method was found to eliminate thiamine deficiency symptoms and mortalities (Wooster et al. 2000; Harder et al 2018). Fitzsimons et al. (2005) demonstrated that injecting Coho salmon with either a thiamine solution (50 mg thiamine/kg bodyweight) had 27 times higher egg thiamine concentrations than the control treatment with a saline solution. In addition, this method doubled the survival of salmon migrating up the Platte River compared to the control salmon due their larger thiamine stores. Although immersions and injections with thiamine solutions are a solution to treating thiamine deficiency in the short-term, they are not a long-term solution and are not practical for fishes that are breeding in natural habitats rather than in hatcheries.

1.6 Population sources and stocking program

Local adaptation may lead to differences in tolerance for high-thiaminase prey among conspecific populations. Some evidence suggests that potadromous (freshwater lake migrating) populations of Atlantic salmon display fewer symptoms related to thiamine deficiency despite consuming rainbow smelt (Dimond and Smitka 2005). In contrast, anadromous (ocean migrating) populations of Atlantic salmon have a diverse diet that includes capelin (*Mallotus villosus*), sand eels (Ammodytidae), krill (Euphausiacea), and amphipods (Amphipoda) (Rikardsen and Dempson 2011), and saltwater prey fishes have been shown to have lower thiaminase levels than freshwater prey fishes (Neilands 1947; Ceh et al. 1964). Consequently, anadromous populations of Atlantic salmon might be predicted to have lower tolerance for dietary thiaminase than potadromous populations and be more susceptible to thiamine deficiency. However, the extent that thiaminase tolerance varies among populations during the critical reproductive phase has never been measured using a common experimental procedure.

Three North American source populations of Atlantic salmon (LaHave River, NS, Lac St. Jean, QC, and Sebago Lake, ME) are currently being used for reintroduction efforts into Lake Ontario (Dimond and Smitka 2005). The Sebago and St. Jean populations are native to freshwater lakes (potadromous) and primarily consume rainbow smelt, a high thiaminase-containing prey fish, but are not known to suffer from a thiamine deficiency. Conversely, the LaHave population, the focus of the initial restoration efforts in Lake Ontario, is anadromous and has a more diverse diet that is predicted to be lower in thiaminase (Rikardsen and Dempson 2011). A previous study examined the effects of a high-thiaminase diet in sub-adult (2 year-old) Atlantic salmon from these three populations after they had consumed either a high-thiaminase or low-thiaminase (control) diet for 8 months (Houde et al. 2015b). The high-thiaminase diet led to a significant decline in tissue thiamine concentrations and swimming performance, but no difference in growth rate or survival. As predicted, the high-thiaminase diet was associated with a greater decline in tissue thiamine concentrations in the LaHave population than in the Sebago and St. Jean populations (Houde et al. 2015b).

1.7   Hypotheses

Here I build on this earlier research by examining the critical reproductive phase during which the most serious effects of thiamine deficiency are expected. I hypothesized that the three populations of Atlantic salmon would differ in reproductive traits following exposure to high- and low-thiaminase diets, and predicted that potadromous populations (Sebago and St. Jean) would tolerate a high thiaminase diet better than an anadromous population (LaHave) due to pre-existing adaptations in their ancestral habitats. I used these data to explore the importance of using tolerance to a high-thiaminase diet as a criterion to inform brood stock selection for the Lake Ontario Atlantic salmon restoration program.

2 Methods

2.1 Experimental diets

Two diets were prepared: (i) a high-thiaminase diet that mimics the thiaminase activity found in alewife and rainbow smelt and (ii) a low-thiaminase (control) diet that mimics the thiaminase activity of the historical diet of bloater and herring. The specific composition and preparation of these diets followed Honeyfield et al. (2005) and Houde et al. (2015b) (Table 2.1). Both diets contained the same formulation of ingredients, with the exception that the high-thiaminase diet included the bacteria *Paenibacillus thiaminolyticus*—isolated from Lake Michigan alewives by Honeyfield et al. (2002)—while the control diet did not include *P. thiaminolyticus.*

Both diets were fish meal based and contained all the necessary nutrients required for the salmon. Bacterial broth inoculated with *P. thiaminolyticus* was prepared using liquid media (yeast extract 1.0 g/L and 8.0 g/L Difco nutrient broth, Becton Dickinson, Mississauga, Ontario) and was used for the high-thiaminase diet. The broth was then incubated for 96 h at 37°C, which resulted in a final bacterial count of $1.1 \times 10^8 \pm 9.2 \times 10^7$ cfu/ml. An autoclaved broth was prepared without *P. thiaminolyticus* to be used for the control diet. Dry ingredients were thoroughly mixed with a Hobart mixer (Hobart Ltd, Don Mills, Ontario, Canada) prior to the addition of 5.3 L of broth and 2.7 L of deionized water. The feed was then transported to the University of Western Ontario in London, Ontario and incubated for 24 hours at room temperature to allow the bacteria to break down the thiamine in the feed. Water was then added until the feed had a dough-like consistency. The feed was pelletized using a meat grinder and placed in a food dehydrator for 12 hours. Thiaminase activity was estimated to be 6,800 pmol/min per gram of feed based on the data provided in Honeyfield et al. (2005). Dehydrated feed was transported to Codrington to be fed to the experimental salmon.

Table 2.1. The composition of the experimental diets for the Atlantic salmon (*Salmo salar*). Bacterial cultures were added to the thiaminase feed. Table is adapted from Houde et al. (2015b).

Variable	Control (%)	Thiaminase (%)
Diet composition		
Fish meal, herring	32.0	32.0
Starch	30.0	30.0
Corn gluten meal	18.0	18.0
Blood flour	8.6	8.6
Fish oil	8.0	8.0
Dextrin	1.0	1.0
Choline chloride	0.5	0.5
Vitamin premix	0.5	0.5
Mineral premix	0.2	0.2
Ascorbyl-2-polphosphate	0.2	0.2
Betaine-HCl	1.0	1.0
Bacterial thiaminase	None	Trace

2.2 Experimental fish

Three Atlantic salmon populations were examined in this study: the Sebago Lake population from Maine, USA (43.8ºN, 70.5ºW), the Lac St. Jean population from Québec, Canada (48.6ºN, 72.0ºW) and the LaHave River population from Nova Scotia, Canada (44.3ºN, 64.4ºW). The LaHave population has been maintained in the Ontario Ministry of Natural Resources and Forestry (MNRF) hatchery system since 1995, the Sebago population since 2006, and the St. Jean population since 2007 (MNRF 2011; Houde et al. 2015b).

The fish used in this study were produced in November 2011 at the MNRF Harwood Fish Culture Station. For additional detail about the care and rearing of these fish through the sub-adult stage see Houde et al. (2015b). Briefly, the fish were transferred to the MNRF Codrington Fisheries Research Facility in spring 2012, and were held separately by population under a natural photoperiod and seasonal temperature variation (water was supplied by Marsh Creek). The salmon were initially fed a commercial diet (Corey Aquafeeds, Fredericton, New Brunswick, Canada). In October 2013, 96 salmon from each population were anaesthetized with buffered MS-222 (tricaine methanesulfonate, 0.1 g/L, Syndel, Nanaimo, British Columbia, Canada) and marked with uniquely numbered vinyl anchor tags (Floy Tag & Mfg., Seattle, Washington, USA). The fish were then divided equally among six tanks (n=48 fish per tank; 16 fish from each population per tank). Over the next four weeks the salmon were transitioned to the experimental diets, with three tanks receiving the high-thiaminase diet and three tanks receiving the control diet. Fish were fed at a rate of 1% of body mass per day and maintained on these diets for the entirety of the experimental period. Salmon mortalities were removed daily from the tanks and kept frozen.

I assessed the characteristics of the fish as they began to reach sexual maturity, with measurements beginning in October 2015. At that point, 142 of the original 288 fish survived and 35% of the surviving fish had lost the anchor tag that was used to identify their population origin. Consequently, all fish were anesthetized and marked with a

uniquely numbered passive integrated transponder (PIT) tag inserted into the abdominal cavity. Anchor tags were then removed if present and used to identify population origin (n=93). For fish that had lost their anchor tag (n=49), a fin clip was collected for genetic determination of population origin by the MNRF DNA lab at Trent University (Peterborough, Ontario) using 12 microsatellite loci (Houde et al. 2016). These unknown individuals were assigned to one of the three populations using Structure 2.1 (Pritchard et al. 2000) based on reference samples of at least 100 broodstock individuals from each population. Q value (% probability of group membership) was well over 80%.

Body mass and length were measured at three times: October 2015, April 2016 and October 2016. Specific growth rate was calculated based on mass for the intervals between each pair of measurement as the natural log of mass on the second measurement date minus the natural log of mass on the first measurement date, and then expressed as a percent change in body mass per day (i.e. by dividing by the number of days in the measurement interval).

2.3   Reproductive characteristics

Gametes were collected from mature fish between October 28 and November 18, 2016 after the fish had been fed the experimental diets for three years. When fish reached sexual maturity—males freely expressed milt, females had free-flowing eggs within the abdominal cavity—they were anaesthetized and gametes were expelled by gently pressing on the abdomen, taking care to avoid contamination by water, urine or faeces. Egg diameter was measured using 15 eggs per each mature female using ImageJ version 1.51 (NIH, Bethesda, MD, USA, available at rsb.info.nih.gov/ij/) and a mean was determined. A subsample of unfertilized eggs (approximately 50 eggs) was immediately frozen on dry ice for thiamine analysis. There were few mature females from the LaHave population, so this population was excluded from experimental components that required eggs.

Within 6 hours of collection, sperm from each male was activated by combining 0.2 µl of milt with 10 µl of water, and then milt was placed on a 2X-CEL glass slide (Hamilton Thorne, Massachusetts, USA) with a cover slip. Sperm activity was recorded using a

digital black and white video camera (XC-ST50, Sony, Japan) connected to an external phase-contrast microscope (CX41 Olympus, Melville, New York, USA) with a 10× magnification negative-phase objective. During observations a heat exchanger maintained the sperm at the same temperature as the hatchery water (7ºC). HTM-CEROS sperm analysis software (version 12, CEROS, Hamilton Thorne Biosciences, Beverly, Massachusetts, USA) was used to measure sperm characteristics based on 60 frames of video at 5 and 10 seconds post-activation (Johnson et al. 2013). I first measured sperm count as the average number of sperm cells observed in the video (minimum sperm size = 3 pixels, minimum contrast = 11). Sperm motility was measured as the proportion of the sperm cells that were progressively motile. Sperm velocity was measured as the average velocity of the sperm cells over its smoothed cell path (average pathway velocity). Sperm linearity was measured as the departure of the path of the sperm cell from a straight line, with high values indicating sperm cells that followed a straighter path towards a potential egg. Larger values for motility, velocity and linearity indicated higher sperm quality. For each sperm characteristic, the value for a male was calculated as an average based on all sperm cells at each activation timepoint.

Sperm and egg viability were quantified using experimental crosses (Figure 2.1). The eggs of each mature female were divided equally into four replicates of approximately 200 eggs. Each replicate was fertilized using the milt of a single male from the same population as the female, with two males from the high-thiaminase diet and two males from the control diet. The same four males were used to fertilize the eggs of all females from a population. Fertilizations were performed by adding approximately 1 mL of milt to each egg replicate and then adding stream water to activate the milt. After 1 minute the eggs were rinsed with stream water and immersed for 30 minutes in a disinfecting bath of 0.5% Ovadine (Syndel, Nanaimo, BC, Canada). The eggs from each cross were then transferred to separate sections in flow-through Heath incubation trays. Dead or unfertilized eggs and alevins were removed and recorded every two days until the swim-up stage (i.e. immediately before complete yolk absorption). At this time, all alevins were euthanized with an overdose of MS-222. Survival was calculated separately for the egg

stage, which spanned fertilization to hatching, and for the alevin stage, which spanned hatching to swim-up.

2.4 Thiamine analysis

All surviving adult salmon were euthanized with an overdose of MS-222 in April 2017 and a sample of muscle tissue was collected. Thiamine concentrations were then measured in two tissues: adult muscle (spring 2017) and unfertilized eggs (fall 2016). Over half of the egg samples were damaged during transport, so offspring thiamine measurements are available for only a subset of the mature females in our study. All thiamine measurements were performed in the laboratory of Dr. Jacques Rinchard at the College at Brockport, State University of New York, and followed the methods of Brown et al. (1998) and Futia et al. (2017). First, 1.2-3.9 g of tissue was mixed with tricholoracetic acid, boiled in a water bath for 10 minutes and centrifuged. Extracts were washed with ethyl acetate and hexane, and then oxidized with sodium hydroxide and potassium ferricyanide. After extraction, high-performance liquid chromatography (HPLC) was used to determine thiamine concentrations. The system included a delivery pump, automatic sample injector, Hamilton PRP-HI column (150×4.1 mm; 5-µm mesh size) with attached guard column (25×2.3 mm; 12 to 20-mm mesh size) and a fluorometric detector (375-nm excitation wavelength and 433-nm emission wavelength). Standard curves using analytical standards of free thiamine (TH), thiamine monophosphate (TMP) and thiamine pyrophosphate (TPP) were prepared fresh daily and used to convert HPLC fluorescence values into concentrations. The concentrations of the three vitamers were then summed to determine the total thiamine concentration. A subset of the samples (n=48) were analyzed in duplicate and showed high repeatability in the measurement of thiamine concentrations (coefficient of variation = 5.0%).

2.5 Statistical analysis

All analyses were performed in JMP (version 4.04). First, I used chi-square tests to compare between experimental diets and populations the proportion of the fish that survived until October 2015. I next compared body length, body mass, specific growth rate, total thiamine concentration in muscle tissue, sperm count, sperm motility, sperm

velocity, sperm linearity and egg thiamine concentrations using linear models that included diet, population, and the diet $\times$ population interaction as factors. Sex was included in the initial model for total thiamine concentration in muscle tissue, but the factor was non-significant and was removed from the final model. To examine survivorship in the experimental crosses, survivorship was first calculated as percent survival by family during the egg and alevin stages of development. 32 families had 100% mortality during the egg stage and were thus excluded from analyses of survivorship during the alevin stage. Survivorship was then analyzed separately for the egg and alevin stages using linear models that included paternal diet, maternal diet and population as factors. All higher-order interaction terms were included in the initial survivorship models and then removed if non-significant.
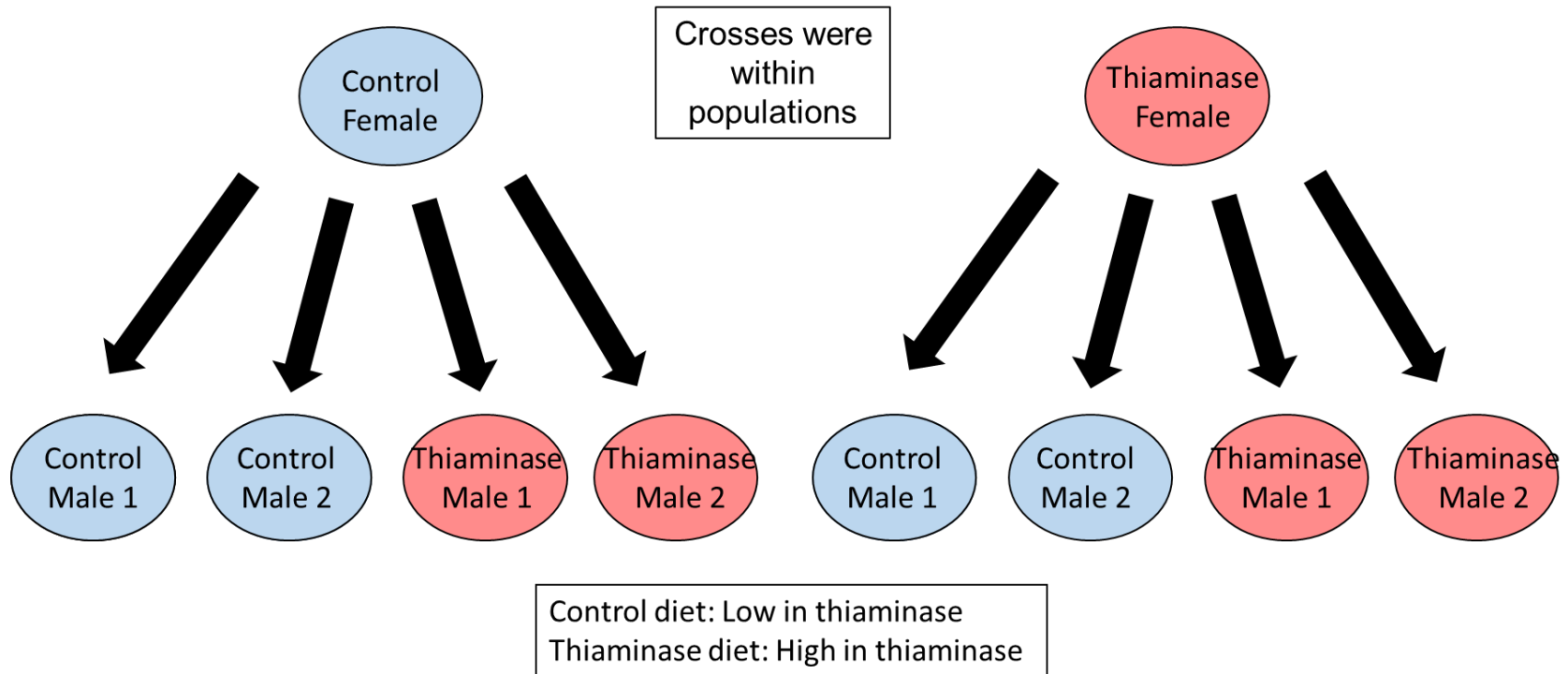
Figure 2.1. An illustration of the experimental crosses completed. Mature females from the high- and low-thiaminase diets were paired within populations, and the eggs of each female were divided equally into four batches. Two batches were fertilized with the milt of two males from the high-thiaminase treatment and the remaining two batches were fertilized with the milt of the two males from the low-thiaminase diet. To control for sperm quality, the same group of males per population was used for the crosses.

3   Results

3.1   Adult metrics

At the first sampling point in October 2015, a significantly greater proportion of the fish fed the control diet were alive (56%, n = 80 of 144) relative to the fish fed the high-thiaminase diet (42%, n = 61 of 144; $\chi 2$ = 5.01, p = 0.025; Table 3.1). Independent of diet, the proportion of the fish that were alive was significantly lower for the LaHave population (17%, 16 of 96) than for the Sebago (65%, 62 of 96) or St. Jean populations (66%, 63 of 96; $\chi 2$ = 60.1, p < 0.001). There was little mortality after the first sampling point (see Table 3.1).

Body length did not differ significantly based on diet in either October 2015 ($F_{1,136}$ = 0.80, p = 0.37), April 2016 ($F_{1,126}$ = 0.91, p = 0.34), or October 2016 ($F_{1,125}$ = 0.003, p = 0.95; Table 3.1). There were significant differences in body length among populations in October 2015 ($F_{2,136}$ = 6.36, p = 0.002), April 2016 ($F_{2,126}$ = 5.01, p = 0.008), and October 2016 ($F_{2,125}$ = 15.8, p < 0.001; Table 3.1). Fish from the St. Jean population were consistently shorter than fish from the LaHave or Sebago populations. Body length did not show a significant interaction between population and diet in October 2015 ($F_{2,136}$ = 2.14, p = 0.12), April 2016 ($F_{2,126}$ = 2.54, p = 0.08), or October 2016 ($F_{2,125}$ = 0.61, P = 0.43; Table 3.1).

Body mass did not differ significantly affected by diet in either October 2015 ($F_{1,136}$ = 2.25, p = 0.13), April 2016 ($F_{1,126}$ = 3.05, p = 0.08), or October 2016 ($F_{1,125}$ = 0.07, p = 0.80; Table 3.1). There were significant differences in body mass among populations in October 2015 ($F_{2,136}$ = 12.86, p < 0.001), April 2016 ($F_{2,126}$ = 7.01, p = 0.001), and October 2016 ($F_{2,125}$ = 21.7, p < 0.001; Table 3.1). Fish from the LaHave and Sebago populations were consistently heavier than fish from the St. Jean population. Body mass did not show a significant interaction between population and diet in October 2015 ($F_{2,136}$ = 1.44, p = 0.24), April 2016 ($F_{2,126}$ = 1.51, p = 0.23), or October 2016 ($F_{2,125}$ = 1.68, p = 0.19; Table 3.1).

Table 3.1. Body length and mass of Atlantic salmon (*Salmo salar*) from three populations that were fed a control or high-thiaminase diet. Data are presented for three sampling times and comprise the number of surviving fish, total length (mean ± SD) and body mass (mean ± SD).

Time	Measure	Control diet			High-thiaminase diet		
		LaHave	Sebago	St. Jean	LaHave	Sebago	St. Jean
Oct 2015	Number	12	34	34	4	28	29
	Length (mm)	331 ± 39	320 ± 25	308 ± 22	317 ± 29	340 ± 23	318 ± 26
	Mass (g)	326 ± 103	349 ± 84	293 ± 58	312 ± 62	415 ± 84	326 ± 87
Apr 2016	Number	11	34	34	4	24	25
	Length (mm)	360 ± 42	338 ± 26	328 ± 24	346 ± 35	363 ± 27	339 ± 31
	Mass (g)	443 ± 131	434 ± 111	383 ± 82	435 ± 114	533 ± 123	427 ± 126
Oct 2016	Number	11	34	34	4	24	24
	Length (mm)	449 ± 44	410 ± 37	387 ± 29	420 ± 26	432 ± 34	392 ± 34
	Mass (g)	1027 ± 250	888 ± 246	684 ± 151	915 ± 151	1011 ± 261	712 ± 184

Diet did not affect specific growth rate for the period between October 2015 and April 2016 ($F_{1,126}$ = 1.26, p = 0.26; Figure 3.1a). However, between April 2016 and October 2016 specific growth rate was significantly higher for the fish fed the control diet than fish fed the high-thiaminase diet ($F_{1,125}$ = 6.30, p = 0.013; Figure 3.1b). There were significant differences in specific growth rate among populations for the period between October 2015 and April 2016 ($F_{2,126}$ = 3.09, p = 0.049; Figure 3.1a), with growth rate significantly higher for the LaHave population than the Sebago population and St. Jean intermediate to the other two populations. There were also significant differences in specific growth rate among populations for the period between April 2016 and October 2016 ($F_{2,125}$ = 24.8, p < 0.001; Figure 3.1b), with growth rate significantly higher for the LaHave population than both other populations, and significantly higher for Sebago than St. Jean. Specific growth rate did not show a significant interaction between population and diet for either the period between October 2015 and April 2016 ($F_{2,126}$ = 0.75, p = 0.48; Figure 3.1a), or the period between April 2016 and October 2016 ($F_{2,125}$ = 0.11, p = 0.89; Figure 3.1b).

Total thiamine concentrations were significantly lower in the muscle of Atlantic salmon fed the high-thiaminase diet than in salmon fed the control diet ($F_{1,95}$ = 21.2, p < 0.001; Table 3.2). Total thiamine concentrations in muscle also differed significantly among all three populations ($F_{2,95}$ = 39.6, p < 0.001; Table 3.2), being lowest in the St. Jean population, intermediate in the Sebago population and highest in the LaHave population. Total thiamine concentrations in the muscle did not show a significant interaction between population and diet ($F_{2,95}$ = 0.02, p = 0.98; Table 3.2). Similar patterns were observed when the three thiamine vitamers were instead compared individually (analysis not shown).
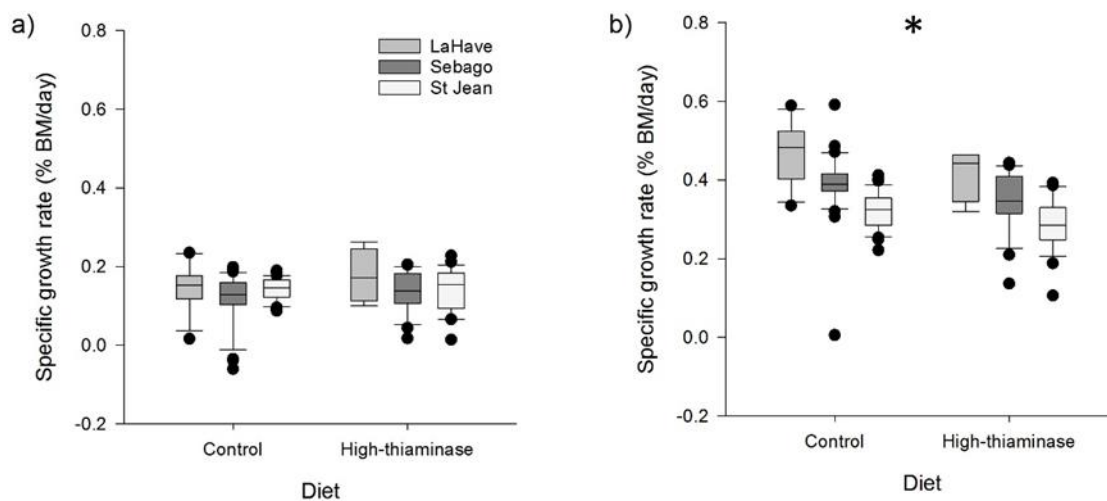
Figure 3.1. Specific growth rate in Atlantic salmon (*Salmo salar*) from three populations that were fed a control or high-thiaminase diet. The panels show specific growth rate between October 2015 and April 2016 (a) and between April 2016 and October 2016 (b). The box plots show the median, 25th and 75th percentiles, with whiskers indicating the 10th and 90th percentiles and individual points used to show data outside this interval. Specific growth rate is expressed as the percentage change in body mass per day. Asterisk (*) indicates significant diet effects. N = 58 salmon for the control diet and N = 43 salmon for the high-thiaminase diet.

Table 3.2. Concentrations of thiamine vitamers in the muscle of Atlantic salmon (*Salmo salar*) from three populations that were fed a control or high-thiaminase diet. Data comprise the number of individuals measured, and the concentrations of free thiamine, thiamine monophosphate, thiamine pyrophosphate, and the total across the three thiamine vitamers. Thiamine concentrations differed significantly among the three populations and were significantly lower for salmon fed the high-thiaminase diet than salmon fed the control diet (see text for details).

Measure	Control diet			High-thiaminase diet		
	LaHave	Sebago	St. Jean	LaHave	Sebago	St. Jean
Number	10	23	25	4	20	19
Free thiamine	0.8 ± 0.3	0.2 ± 0.3	0.2 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1
Thiamine monophosphate	3.1 ± 0.5	2.0 ± 0.8	1.4 ± 0.4	1.8 ± 0.3	1.5 ± 0.5	1.0 ± 0.3
Thiamine pyrophosphate	39.9 ± 8.6	35.0 ± 8.4	25.2 ± 4.7	34.1 ± 6.9	27.8 ± 4.9	18.6 ± 3.7
Total Thiamine	43.8 ± 9.2	37.1 ± 9.3	26.8 ± 5.2	36.1 ± 6.7	29.4 ± 5.3	19.7 ± 4.0

Note: Concentrations are presented as mean $\pm$ SD and are measured in nmol thiamine / g tissue.

3.2 Reproductive metrics

Sperm count was not significantly affected by diet ($F_{1,24} = 0.87$, p = 0.36), population ($F_{2,24} = 0.11$, p = 0.89), or the interaction between diet and population ($F_{2,24} = 1.11$, p = 0.35; Figure 3.2). Sperm motility did not differ significantly based on diet at either 5 seconds ($F_{1,24} = 0.11$, P = 0.74; Figure 3.3a) or 10 seconds post-activation ($F_{1,25} = 2.20$, p = 0.15; Figure 3.3b). Sperm motility did not differ significantly among populations at either 5 seconds ($F_{2,24} = 0.37$, p = 0.69; Figure 3.3a) or 10 seconds post-activation ($F_{2,25} = 0.00$, p = 1.00; Figure 3.3b). Finally, sperm motility did not show a significant interaction between diet and population at either 5 seconds ($F_{2,24} = 0.56$, p = 0.58; Figure 3.3a) or 10 seconds post-activation ($F_{2,25} = 0.93$, p = 0.41; Figure 3.3b).

Sperm velocity did not differ significantly based on diet at 5 seconds post-activation ($F_{1,24} = 3.10$, p = 0.09; Figure 3.3c), but at 10 seconds post-activation sperm velocity was significantly higher for fish fed the high-thiaminase diet than fish fed the control diet ($F_{1,25} = 4.58$, p = 0.042; Figure 3.3d). Sperm velocity differed significantly among populations at 5 seconds post-activation ($F_{2,24} = 4.84$, p = 0.017; Figure 3.3c), but did not differ significantly among populations at 10 seconds post-activation ($F_{2,25} = 0.94$, p = 0.40; Figure 3.3d). Finally, sperm velocity did not show a significant interaction between diet and population at either 5 seconds ($F_{2,24} = 0.05$, p = 0.95; Figure 3.3c) or 10 seconds post-activation ($F_{2,25} = 2.31$, p = 0.12; Figure 3.3d).

Sperm linearity did not differ significantly based on diet at either 5 seconds ($F_{1,24} = 0.89$, p = 0.35; Figure 3.3e) or 10 seconds post-activation ($F_{1,25} = 0.01$, p = 0.93; Figure 3.3f). Sperm linearity did not differ significantly among populations at 5 seconds post-activation ($F_{2,24} = 0.57$, p = 0.57; Figure 3.3e), but did differ significantly among populations at 10 seconds post-activation ($F_{2,25} = 3.63$, p = 0.041; Figure 3.3f). Finally, sperm linearity showed a significant interaction between diet and population at 5 seconds post-activation ($F_{2,24} = 3.48$, p = 0.047; Figure 3.3e), as within the St. Jean population linearity was significantly higher for individuals on the control diet than the high-

thiaminase diet. At 10 seconds post-activation no significant interaction between diet and populations was observed ($F_{2,25} = 0.37$, $p = 0.69$; Figure 3.3f).

Egg diameter was significantly lower in the eggs of Atlantic salmon fed the high-thiaminase diet than in salmon fed the control diet ($F_{1,30} = 6.04$, $p = 0.020$; Table 3.3). Egg diameter was significantly higher in the Sebago population than in the St. Jean population ($F_{1,30} = 16.1$, $p < 0.001$; Table 3.3). Egg diameter did not show a significant interaction between population and diet ($F_{1,30} = 0.19$, $p = 0.67$; Table 3.3).

Total thiamine concentrations were significantly lower in the eggs of Atlantic salmon fed the high-thiaminase diet than in salmon fed the control diet ($F_{1,13} = 19.1$, $p < 0.001$; Table 3.3). Total thiamine concentrations in eggs did not differ significantly between populations ($F_{1,13} = 0.16$, $p = 0.16$; Table 3.3). Total thiamine concentrations in the eggs did not show a significant interaction between population and diet ($F_{1,13} = 1.16$, $p = 0.30$; Table 3.3). Similar patterns were observed when the three thiamine vitamers were instead compared individually (analysis not shown).

Examining the experimental crosses, there was no effect of paternal diet on family survivorship at the egg ($F_{1,120} = 0.04$, $p = 0.84$; Figure 3.4a) or alevin stage ($F_{1,84} = 0.07$, $p = 0.79$; Figure 3.4a). Maternal diet, however, had a significant effect on family survivorship at the egg stage ($F_{1,120} = 29.2$, $p < 0.001$; Figure 3.4b); survivorship was lower for the eggs of females fed a high-thiaminase diet than for the eggs of females fed a control diet. Maternal diet was not associated with family survivorship at the alevin stage ($F_{1,84} = 3.05$, $p = 0.084$; Figure 3.4b). Population had a significant effect on family survivorship at both the egg stage ($F_{1,120} = 63.7$, $p < 0.001$; Figure 3.4c) and alevin stage ($F_{1,84} = 34.8$, $p < 0.001$; Figure 3.4c), with higher survivorship in the Sebago population than the St. Jean population. All higher order interactions were non-significant ($p > 0.05$) and were removed from the final models of survivorship.
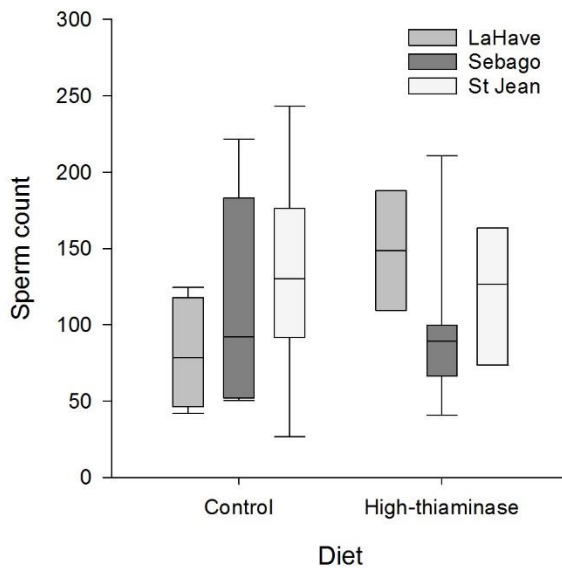
Figure 3.2. Sperm count in Atlantic salmon (*Salmo salar*) from three populations that were fed a control or high-thiaminase diet. Sperm count was measured as the average number of sperm cells observed during the video observations of sperm activity. The box plots show the median, 25th and 75th percentiles, with whiskers indicating the 10th and 90th percentiles. N = 24 males for control group and N = 25 males for high-thiaminase group.
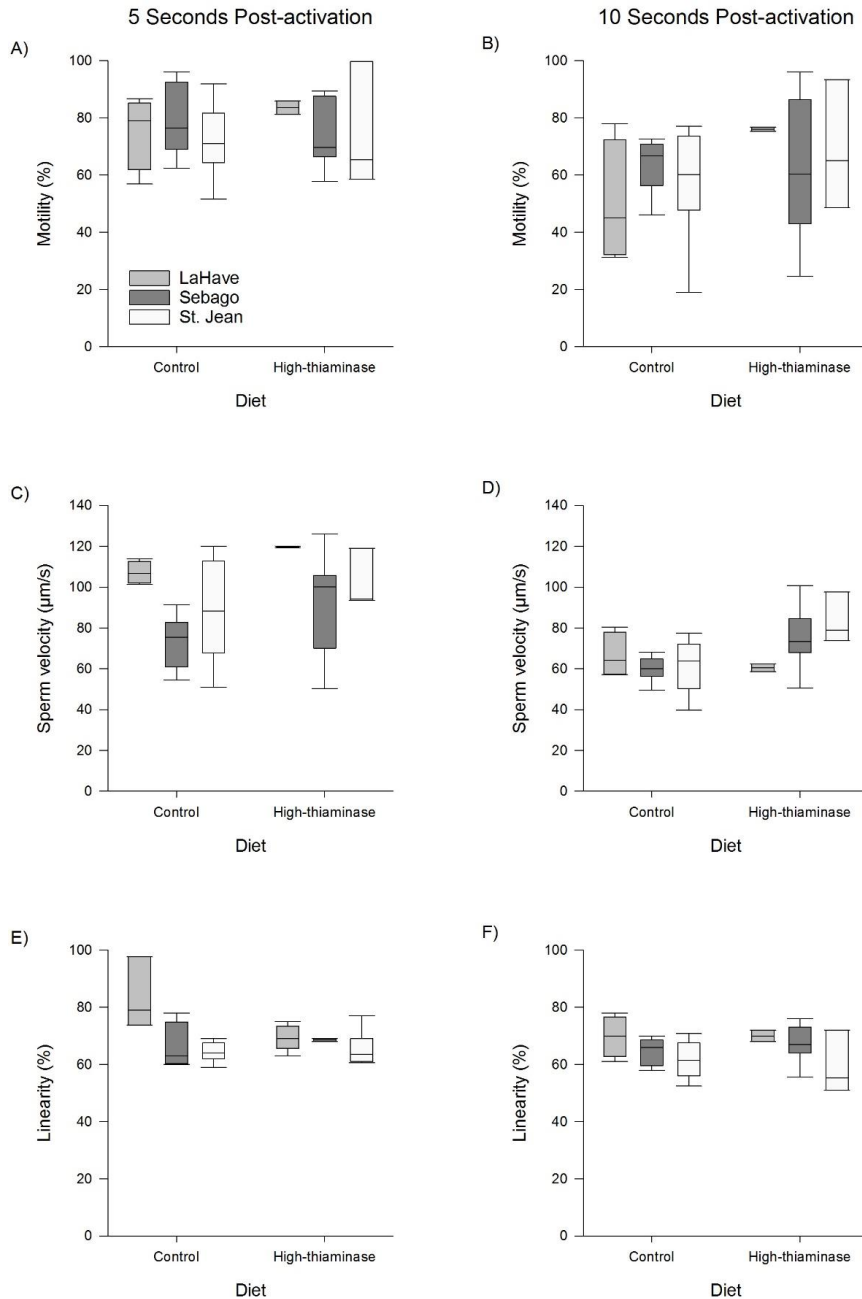
Figure 3.3. Sperm activity in Atlantic salmon (*Salmo salar*) from three populations that were fed a control or high-thiaminase diet. The panels show sperm motility at 5 seconds post-activation (a) and 10 seconds post-activation (b), sperm velocity at 5 seconds post-activation (c) and 10 seconds post-activation (d), and sperm linearity at 5 seconds post-activation (e) and 10 seconds post-activation (f). The box plots show the median, 25th and 75th percentiles, with whiskers indicating the 10th and 90th percentiles. N = 24 males for control group and N = 25 males for high-thiaminase group.

Table 3.3. Diameter and thiamine concentrations in the eggs of Atlantic salmon (*Salmo salar*) from females from two populations that were fed a control or high-thiaminase diet. Data comprise the number of females whose eggs were measured for each trait, egg diameter, and the concentrations of free thiamine, thiamine monophosphate, thiamine pyrophosphate, and the total across the three thiamine vitamers.

Measure	Control diet		High-thiaminase diet	
	Sebago	St. Jean	Sebago	St. Jean
Number (thiamine/diameter)	5/7	5/12	6/9	1/6
Diameter	6.73 ± 0.26	6.31 ± 0.35	6.46 ± 0.22	6.11 ±0.13
Free thiamine	15.9 ± 3.5	15.1 ± 2.9	10.0 ±1.9	5.5
Thiamine monophosphate	0.3 ± 0.03	0.3 ± 0.03	0.3 ± 0.06	0.3
Thiamine pyrophosphate	0.9 ± 0.2	0.9 ± 0.1	1.0 ± 0.3	0.9
Total Thiamine	17.1 ± 3.6	16.4 ± 2.9	11.3 ± 2.0	6.7

Note: Values are presented as mean ± SD and are measured mm for egg diameter and in nmol thiamine / g tissue for thiamine concentrations.

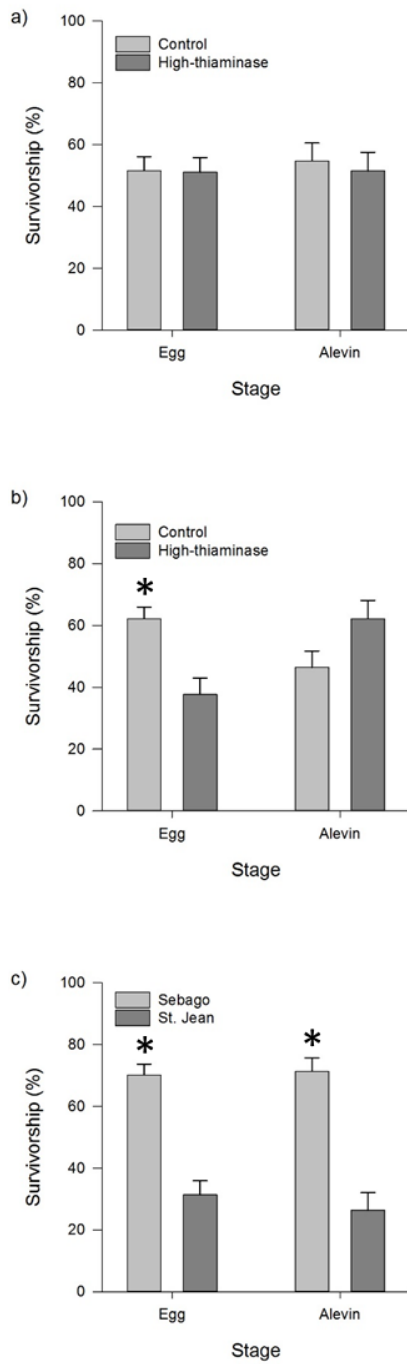**Figure 3.4.** Survivorship of experimental crosses at the egg and alevin stage in Atlantic salmon (*Salmo salar*) from two populations that were fed a control or high-thiaminase diet. Survivorship is shown as a function of paternal diet (a), maternal diet (b), and population (c). Data are plotted as mean ± SE. Asterisk (*) indicates significant effects. N = 120 families for egg stage and N = 84 families for alevin stage.

4   Discussion

4.1   Impacts of thiamine deficiency in adults

High levels of dietary thiaminase are often associated with thiamine deficiency and a range of negative consequences (Harder et al. 2018). In my study, the high-thiaminase diet was associated with a decline in total thiamine levels in muscle of about 7.5 nmol/g, and across populations the concentrations averaged 20-36 nmol/g on the high-thiaminase diet. In an earlier study of the same populations at the sub-adult stage (Houde et al. 2015b), the high-thiaminase diet was associated with a reduction in muscle thiamine levels of about 6 nmol/g, with the populations averaging 3-6 nmol/g on the high-thiaminase diet. A previous study of adult coho salmon, steelhead and lake trout in the Great Lakes found that total thiamine levels in muscle averaged about 0.5 to 8 nmol/g, with external symptoms of thiamine deficiency (e.g. wiggling and lethargy) typically observed when muscle concentrations were less than 1 nmol/g (Brown et al. 2005). Thiamine concentrations in the current study were thus relatively high compared to previous studies, potentially due to lower energetic costs from living in a hatchery environment. Despite this, the significant difference in thiamine concentrations between diets suggests that the high-thiaminase diet may still be associated with symptoms of thiamine deficiency.

One potential consequence of thiamine deficiency is reduced growth rate, as was observed in rainbow trout exposed to a low-thiamine experimental diet (Morito et al. 1986). In contrast, a low-thiamine diet was not associated with a difference in growth rate in sub-adult Atlantic salmon (Houde et al. 2015b). In adult Atlantic salmon, I found that a low-thiamine diet was associated with a significant reduction in specific growth rate during one of the two measurement intervals. However, neither body length nor mass differed between the two diets at any point. Body length and mass might obscure differences between diets if many of the smaller fish fed the high-thiaminase diet died, leading to similar body length and mass (calculated based only on the surviving fish), but not growth rate (calculated at the level of individual fish). However, the fish that died

were slightly larger at the start of our experiment than the fish that survived, which suggests that selective mortality cannot explain the difference between growth metrics. Instead, it appears that the effects of a high-thiaminase diet on growth were minor in our study. The strong effect observed in rainbow trout likely reflects the near zero concentration of thiamine in the experimental diet in that study, which was associated with acute clinical symptoms that included loss of appetite and eventually death (Morito et al. 1986). Increased mortality associated with a high-thiaminase diet was also observed in our study of adult Atlantic salmon, albeit less frequently and after longer exposure to a low-thiamine diet than in the rainbow trout study. Together these data suggest that thiamine deficient diets can reduce survival in fishes, but that these diets do not have a strong influence on growth rate outside of cases in which serious clinical symptoms are also present.

4.2   Impacts of thiamine deficiency on reproductive traits

Maternal thiamine deficiency has been widely associated with offspring mortality in salmonids (Fisher et al. 1995; Fitzsimons et al. 1995; Fisher et al. 1996; Ketola et al. 2000; Brown et al. 2005; Fitzsimons et al. 2007). Consistent with these previous studies, I found that a high-thiaminase diet led to significantly higher offspring mortality during the egg to alevin stage. Egg thiamine concentrations were lower for the high-thiaminase diet than the low-thiaminase diet, consistent with thiamine deficiency being the cause of the increased mortality. Previous research has suggested a critical threshold for egg thiamine concentrations of around 1 nmol/g in salmonids (Fisher et al. 1996; Honeyfield et al. 2005), and the present study indicates that negative effects may be seen at even higher concentrations. The high-thiaminase diet was also associated with smaller egg diameter, similar to a study that found that wild-caught Atlantic salmon that exhibited symptoms of thiamine deficiency had smaller eggs than salmon that did not exhibit symptoms (Amcoff et al. 2000). Smaller egg diameter might be an adaptive response to low maternal thiamine levels, as it would enable the eggs to have higher thiamine concentrations than if the same maternal thiamine resources were distributed among larger eggs. However, low maternal thiamine levels still led to reduced egg thiamine concentrations and lower survival, suggesting that any benefit to smaller egg diameter is unable to fully mitigate

the effects of maternal thiamine deficiency. Overall, our data add to the strong evidence in salmonids that a high-thiaminase diet is associated with increased mortality prior to the onset of exogenous feeding.

Thiamine has previously been detected in the milt of Atlantic salmon, but its function is not well-characterized (Koski 2002). I tested the hypothesis that thiamine promotes sperm performance and successful fertilization. If this hypothesis is true, I predicted that a high thiaminase diet would result in reduced sperm performance and lower fertilization success. My data did not support these predictions. I found no significant effect of dietary thiaminase on sperm count or sperm motility, and sperm velocity was actually higher for fish fed the high-thiaminase diet at one of the two measurement points. There was one significant result in the predicted direction: in the St. Jean population, sperm swam in a more linear path at 5-seconds post-activation for the control diet than the high-thiaminase diet. However, this difference was absent at 10-seconds post-activation and thus provides only weak support for the hypothesis. Male diet was likewise unrelated to fertilization success, with no difference in mortality during the egg to alevin stage (or during the alevin to fry stage). Therefore, my data do not support the hypothesis that thiamine promotes sperm performance or successful fertilization, meaning that the importance of thiamine in milt remains uncertain.

4.3   Population differences

I hypothesized that susceptibility to thiamine deficiency might differ among populations that are locally adapted to consume high-thiaminase prey fishes at different rates. However, I saw similar effects of the high-thiaminase diet on muscle thiamine concentrations and growth rate among the three populations, and similar effects of the high-thiaminase diet on egg thiamine concentrations and offspring mortality in the Sebago and St. Jean populations. The absence of differences among populations may relate in part to the low numbers of fish from the LaHave population, which at the sub-adult stage had the greatest reduction in tissue thiamine levels on the high-thiaminase diet (Houde et al. 2015b) and was predicted to be the most susceptible to a high-thiaminase diet. The LaHave population had poor survival regardless of diet, leading to sufficient sample sizes for only the St. Jean and Sebago populations, which share a similar diet in

their native habitats and were not predicted to differ in susceptibility to thiamine deficiency. Future studies seeking to identify local adaptation to dietary thiaminase would benefit from assessing more individuals and additional populations that differ in predicted susceptibility to dietary thiaminase. Regardless of the low power to document differences in susceptibility to thiamine deficiency among populations, my data indicate that dietary thiaminase has negative effects on multiple traits even in Atlantic salmon populations with long-term exposure to high-thiaminase prey fishes.

4.4   Caveats of the study

Due to timing and availability of resources, there were several research directions and questions that could not be pursued. First, my tests would have had more statistical power with larger sample sizes, especially with the LaHave population. The salmon had been fed the experimental diets for three years prior to the start of my study, and by then there were significantly fewer LaHave individuals compared to the Sebago and StJean population. Having only four (4) salmon in the LaHave high-thiaminase diet group severely restricted the amount of data I could collect and the analyses I could perform. Specifically, the LaHave population had to be omitted from the egg thiamine and egg survival comparisons. With a higher sample size, the LaHave population could have been included in the analysis looking at offspring survivorship. It would have been helpful if more fish from the three populations were initially placed on the experimental diets to ensure that I would have enough LaHave salmon to measure reproductive traits with high statistical power for all three populations.

Second, it would have been advantageous to test thiamine concentrations in more tissues than muscle and eggs. My liver samples were lost due to shipping issues, and liver samples normally have higher concentrations of thiamine than muscle due to liver thiamine being more conserved (Futia et al 2017). Measuring thiamine concentrations in the blood of the adults would have allowed me to monitor thiamine concentrations over time, starting from when they were placed on the experimental diet to when they spawned. Additionally, measuring thiamine in a subset of alevins that were taken at hatch prior to the onset of mortality would have been a useful analysis. That way the thiamine levels at each development stage could have been mapped, showing insight into the

thiamine concentrations associated with low survival across multiple developmental stages.

Third, due to the limited number of tanks we could use to rear the salmon in the hatchery, the populations had to be mixed to create 3 replicates per diet. A downside to this is that some populations may be more aggressive and better competitors for food, resulting in potential higher growth rates. For example, eight populations of wild guppies in Trinidad were found to differ in the levels of agonistic behaviours, and particularly fought over food (Seghers and Magurran 1991). Populations may also be genetically inclined to have faster growth rates. For example, the Sebago and StJean populations have been documented to gain mass faster than the LaHave population within a 10-month period (Houde et al. 2015a). Larger individuals can outcompete smaller individuals within a tank for food, meaning not all salmon would be equally fed their intended feed allotment, which would affect the results. Ideally, each population would have its own tanks so that one population could not outcompete the others for food.

Furthermore, a population's tolerance to thiaminase may be affected by the number of generations it has been reared in captivity. All populations in the MNRF hatchery system are fed a commercial diet without thiaminase, and maintaining the ability to tolerate thiaminase is unnecessary. As time goes on, generations of captive populations that are tolerant to thiaminase in the wild may lose that ability, since there is no benefit to maintaining it in a hatchery. For example, hatchery steelhead have been found to be larger, smolt at a younger age, have higher egg-to-smolt survival and lower smolt-to-adult survival than their wild counterparts (Kostow 2004). Differences in phenotypes and survival may occur between hatchery and wild stocks. This means that although wild Sebago and StJean salmon may be tolerant to thiaminase, their counterparts in the hatchery may not have that same tolerance. Preferably, salmon directly from the wild would have been used to ensure the effects of captivity did not alter the results.

Finally, with more resources and if the logistics could be coordinated, additional populations other than the LaHave, Sebago and StJean population would have been valuable to include. There may be other populations that show greater differences in

susceptibility to dietary thiaminase. For an example, the St. Mary's River off of Lake Huron has returning adults and a healthy population of Atlantic salmon derived from the donor West Grand Lake population. These returning adults have approximately 25-35% of its females displaying thiamine deficiency (Dimond and Smitka 2005), which is a low percentage. The St. Mary's River population feed on alewife in Lake Huron (Werner et al. 2006), and so may be able to tolerate a high-thiaminase diet. Additional anadromous populations would also be useful to test the hypothesis that they have a lower tolerance for dietary thiaminase than potadromous populations. More research on other potential source populations could increase the chance of a successful reintroduction.

4.5   Implications for reintroduction efforts

The selection of a suitable source population is an important contributor to the success of a reintroduction program (Houde et al. 2015c). The three populations used in this study are all being considered for the Atlantic salmon reintroduction program, and it is important to test multiple aspects of each populations' suitability for reintroduction. In previous studies, the Sebago population was deemed to be the most likely to establish a self-sustaining population in Lake Ontario, due to their greater growth performance and survival when in competition with other salmonids that are present in Lake Ontario tributaries (Van Zwol et al. 2012; Houde et al. 2015b). My study did not identify differences among populations for most of the metrics that I measured, with the exception of offspring survivorship, where the Sebago population had higher survival than the St Jean population. Although not significant, the Sebago population also had higher egg thiamine concentrations than the St Jean population on the high-thiaminase diet. Despite the fact the results do not show that the Sebago population is more tolerant to the high-thiaminase diet than the other populations, my results support the trend that the Sebago population has a performance that matches or exceeds the other populations and may be best-suited for reintroduction efforts in Lake Ontario.

Another suggestion to aid reintroduction efforts is the creation of a comprehensive monitoring program of returning Atlantic salmon in Lake Ontario. At the moment, data of returning adults comes from anecdotal evidence (Dimond and Smitka 2005). The use of fish counters and direct fish sampling methods is a common method employed to

monitor the movement of salmonids during the spawning period (Silva et al 2018), however there are more modern methods that can be used to monitor species that are rare to capture, such as Atlantic salmon. Environmental DNA (eDNA) has been shown to successfully estimate the abundance of aquatic species (Lacoursière-Roussel et al. 2016), which could potentially be used for the monitoring of Atlantic salmon in Lake Ontario.

Most importantly, returning salmonids in Lake Ontario should have their thiamine levels monitored over time. Salmonid species in Lake Ontario, such as chinook salmon, coho salmon and lake trout, have recently been tested for thiamine levels and continue to display thiamine deficiencies. Thiamine analyses can be used in conjunction with stomach content analyses to get a fuller picture of the diet of salmonids (Futia and Rinchard 2019). However, traditional methods to measure thiamine can be costly and take a long time to obtain results. New methods, such as the rapid thiamine method, could be used instead of HPLC as a more cost-effective analyses for thiamine. The rapid thiamine method has been found to be well correlated with the HPLC method results when measuring thiamine in salmon eggs (Zajicek et al. 2005). This tool can be used for monitoring thiamine levels in Atlantic salmon eggs, and give the best estimates of the thiamine status in Lake Ontario.

4.6 Future directions

The abundance of forage fish species (both non-native and native) have a significant influence on the thiamine status of Atlantic salmon in Lake Ontario. The inverse correlation of the abundance of thiaminase-rich prey species and thiamine levels in salmonids (Ketola et al. 2009) make it imperative that more research is done on forage fish community structure in Lake Ontario. The restoration of native forage fish species in Lake Ontario may be just as important as the restoration of Atlantic salmon, as the successful establishment of native forage species could aid the establishment of predators like Atlantic salmon. To date, there are no targeted cisco assessment programs in Lake Ontario, with more research needed on cisco behaviour, distribution and impediments (George et al. 2018). The re-establishment of native species, such as cisco, should be pursued in combination with Atlantic salmon reintroduction efforts.

Other vitamins may a play a role in thiamine deficiency, and there is the potential for other essential nutrient deficiencies to exist. Thiamine, vitamin E and vitamin A all play a role as antioxidants. Alewife have previously been shown to contain low levels of vitamin E, which might also have negative consequences for predators that feed on the alewife. A sufficient amount of vitamin E may help to conserve thiamine reserves because both vitamins are known to act as cellular antioxidants (Honeyfield et al 2012). Low availability of vitamins acting as an anti-oxidative, may increase the need for thiamine (Harder et al 2018), further stressing predatory fish like Atlantic salmon. It is evident that more research needs to be done on the interactions of thiamine with other vitamins.

One last future direction that could be pursued is the effect of thiamine deficiency on the immunology of Atlantic salmon. There may be a relationship between thiamine deficiency and susceptibility to infectious diseases. In lake trout, thiamine deficiency has been shown to reduce immune responses, and may cause more physiological responses than previously thought (Ottinger et al. 2012). In the offspring of Lake Ontario steelhead, the bacteria *Flavobacterium* spp. was shown to cause high alevin mortality (Futia et al. 2017). Several pathogens can inflict both hatchery-reared and wild fish, and so it would be important to determine if thiamine deficiency makes Atlantic salmon more susceptible to outbreaks.

4.7   Conclusion

Thiamine deficiency is a disorder that affects many Great Lakes salmonids and has been linked to the consumption of introduced prey fishes that are high in the enzyme thiaminase (Harder et al. 2018), with negative consequences for Atlantic salmon in particular (Ketola et al. 2000). Our research quantified the effects of thiaminase consumption in multiple populations of Atlantic salmon, and was conducted in large part to address the objective of the Lake Ontario Atlantic Salmon Restoration Program to identify the source population that is best-suited for reintroduction (i.e. following Houde et al. 2015c). With few fish available from the LaHave population—historically stocked at the highest frequency—I was unable to fully evaluate the contribution of high-thiaminase diets to reintroduction outcomes. However, high-thiaminase diets led to high

mortality even in the populations (Sebago and St. Jean) that naturally consume these prey fishes, so source population selection is unlikely to entirely overcome issues associated with high-thiaminase diets in the Great Lakes. With thiaminase in naturalized invasive species an ongoing concern for Great Lakes fisheries management (Ketola et al. 2000; Zimmerman and Krueger 2009), restoration of native forage species would benefit rehabilitation efforts for multiple species.

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Appendices

The experimental protocols used in the thesis research were developed in accordance with the guidelines of the Canadian Council on Animal Care, the Animal Care Committee at the University of Western Ontario, and the Committees of the Ontario Ministry of Natural Resources and Forestry.

University of Western Ontario

Animal Use Protocol #2010-2014 (2010-present)

"Behavioural and molecular ecology of fishes"

Ontario Ministry of Natural Resources and Forestry

Animal Use Protocol #115

"Genetic adaptations to current thiaminase diets in candidate strains of Atlantic salmon for reintroduction into Lake Ontario"

Curriculum Vitae

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Mitchell, K.T., Pitcher, T.E., Wilson, C.C., and Neff, B.D. (2016, June). *Reproductive effects of thiamine deficiency in three populations of Atlantic salmon.* Oral presentation at the annual meeting NoWPaS Salmonid Network workshop in Sacre-Coeur, Quebec, Canada.

Pending Publication:

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