ANSWERING KEY UNCERTAINTIES TO SUCCESSFUL REINTRODUCTION OF ARCTIC GRAYLING TO MICHIGAN STREAMS

By

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ABSTRACT

Arctic Grayling Thymallus arcticus were extirpated from Michigan in 1936. Several factors led to their demise including over-fishing, habitat destruction from large-scale logging, and introductions of Brook Trout Salvelinus fontinalis and Brown Trout Salmo trutta. Subsequently, Brook and Brown trout have become the dominant salmonid species in Michigan streams. Past attempts to restore Grayling to their historic home waters in Michigan were unsuccessful. Recent advances in the understanding of Grayling have led to successful reintroductions in Montana, particularly through the use of remote site incubators. These successes have renewed interest in restoring naturally reproducing populations to Michigan, but many unknowns need to be addressed for such efforts to succeed. Determination of the timing of imprinting and the potential for recognition and preference of familiar water are key to determining the time at which Grayling are to be stocked into receiving waters. Predation and competition with resident trout may be two of the greatest challenges to overcome in re-establishing a self-sustaining population of Arctic Grayling in Michigan's cold and cool water streams. This study addresses these key factors through examination of plasma thyroxine levels in sub-yearling Grayling, water choice trials, predation trials with age-1 Brook and Brown trout, and competition trials with Brook and Brown trout of the same year class. Grayling were incubated in East Lansing, Michigan from eyed eggs, provided by Ruth Burnett Sport Fish Hatchery in Fairbanks, Alaska. Grayling were found to have a peak in plasma thyroxine levels corresponding to the eyed-egg life stage, indicative of a time of potential olfactory imprinting. Predation did not differ between predator species and was found to decline over time as Grayling develop and increase in biomass. Brown Trout were found to have a significant competitive effect on Grayling resulting in decreased growth and high mortality while Brook trout did not affect Grayling growth or mortality. These data will help determine the time at which Grayling are to be stocked into receiving waters and to guide suitable streams for reintroduction, thus increasing the likelihood of a successful reintroduction.

Copyright by NICOLE M. WATSON 2024 This dissertation is dedicated to my spouse, Tom Watson, whose unfailing belief in me made this dream and research possible. Thank you for going on this crazy journey of life with me. And to my dogs, whose warmth and steadfast love have supported me in ways only a dog person understands.

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vi

TABLE OF CONTENTS

INTRODUCTION	
REFERENCES	
CHAPTER 1: IMPRINTING AND WATER CHOICE	
REFERENCES	
CHAPTER 2: PREDATION	
REFERENCES	
APPENDIX A: CHAPTER 2	
CHAPTER 3: COMPETITION	
REFERENCES	
APPENDIX B: CHAPTER 3	
CONCLUSION	

INTRODUCTION

There is renewed interest in the restoration of self-sustaining populations of Arctic Grayling *Thymallus arcticus* to select Michigan streams. Grayling hold both historic and cultural significance to the state of Michigan and Native American tribal communities. Many unknowns must be addressed to increase the likelihood of success with this reintroduction. The overarching purpose of this study was to gain insight into potential impediments to successful reintroduction of Arctic Grayling to Michigan streams. A primary goal of this research was to better understand several key areas of uncertainty associated with Grayling reintroduction efforts in Michigan, namely early life imprinting in Grayling and potential levels of predation and competition between resident trout and Grayling.

Several attempts have been made to reintroduce the species to Michigan waters, most recently in the late 1980s. Each of these attempts failed. Historic attempts of reintroduction began as early as 1877 (Jerome 1879). From 1900-1933 over three million fry from Montana were introduced to Michigan rivers and lakes with no evidence of reproduction occurring naturally post-stocking. This was followed by attempts in 1934-1941 when approximately 70,000 yearlings were stocked into lakes and stream, failing to reproduce. Following a lull in reintroduction attempts, approximately 300,000 fingerlings, eyed eggs, and fry were stocked in Lake Manganese and French Annie Creek in Keweenaw County from 1958-1960, again failing to reproduce (Nuhfer 1992). Failure of the 1980s attempt was attributed to predation by resident fish, competition with resident fish for resources, illegal harvesting, and outmigration (Nuhfer 1992) The extent of these potential effects was never quantified (Troy Zorn, Fisheries Research Biologist, Michigan Department of Natural Resources, personal communication). A common theme amongst each attempt is outmigration and failure in natural reproduction. Vincent (1962) noted that competition

over spawning was unlikely a factor due to differences in timing and substrate preferences between Grayling and competitors and it is unknown whether any of the Grayling had survived to maturity (Troy Zorn, personal communication). However, it was noted that early-life competition and predation are likely the primary contributors to lack of recruitment in Grayling reintroductions that should be considered.

How does this contemporary attempt differ from those previous? This attempt incorporates a multi-tier plan that incorporates research, conservation strategies, partner relationships, education, and outreach (MAGI 2017). By focusing on the Montana Arctic Grayling Recovery Program model established in 1987 (MAGR 2007), Michigan has aimed for a successful reintroduction of Grayling to the state. The Michigan Arctic Grayling Initiative (MAGI) involves approximately 50 collaborators with a common goal: *"to restore self-sustaining populations of Arctic Grayling within its historic range in Michigan."* (MAGI 2017). Four key focus areas are implemented as part of the plan: research, management, fish production, and outreach. Each of these aspects brings a uniqueness to this contemporary reintroduction attempt. Advancements in technology and knowledge specific to Arctic Grayling will strengthen the likelihood of a successful reintroduction and allow for a deeper understanding of their requirements. Further, this attempt builds upon the knowledge learned through examination of previous attempts and using successful efforts as models.

Stakeholder involvement has been a novel aspect of this effort. Through a combination of presentations at professional symposia and conferences (12 presentations), outreach presentation to public stakeholder groups (43 presentations), and through alternative outreach avenues such as guest sessions on podcasts (4 podcasts), I have utilized a robust suite of communication of research efforts undertaken.

Rapid outmigration was a common problem leading to lack of recruitment in early reintroduction attempts (Vincent 1962; Kaya 1992; Nuhfer 1992). Hatchery-reared Grayling stocked into Montana and Michigan waters both showed dramatic migrations, disappearing shortly after stocking (Kaya 1992; Nuhfer 1992). Outmigration may have been influenced by several factors, one of which is unfamiliar water. This may have been linked to insufficient recognition of "home waters" and fish being reared in hatchery settings prior to release. Although rearing of Grayling is more efficient in a hatchery setting, this may have inadvertently led to outmigration from streams. Kaeding and Boltz (2004) found that remote-site incubators (RSIs) were helpful in reestablishing resident Grayling stocks in Montana streams. Rearing Grayling to fry life stage on stream water through the use of RSIs resulted in Grayling establishing residency at or near where they emigrated from the RSI. Thus, use of RSIs as part of a Michigan-based reintroduction effort may facilitate early-life imprinting, decrease the likelihood of outmigration, and increase site fidelity. Recent studies with Pacific salmon species suggest these fish imprint on spawning waters at hatch and swim-up life stages, which ultimately enables the adult salmon to return to spawn in the exact riffle they hatched from (Dittman et al. 2015). These findings and previous work on Pacific salmonids (Dittman et al. 2015) suggest that physiological (hormone level) changes occur during this period which enable fish to "imprint" on target waters. Elevated thyroid hormone levels in the blood plasma of salmon appear provide some evidence of when imprinting is occurring (Tilson et al. 1994; Lema and Nevitt 2004; Havey et al. 2017). Knowing when such elevations occur can aid in identifying key life stages at which imprinting is likely to occur, but these key times are unknown for Grayling, therefore, one of the goals of this study is to determine key times in which Grayling may be imprinting and determine "home water" recognition.

Interactions with resident trout, namely Brook Trout Salvelinus fontinalis and Brown Trout Salmo trutta that have flourished since the extirpation of Grayling, may be the greatest challenge to successful reintroduction. Predation by resident trout, particularly abundant juvenile trout on Grayling eggs and recently hatched fry, may be a significant hurdle to reestablishing Grayling populations to Michigan streams. Juvenile fish often numerically predominate a population in tributaries that are suitable to young Grayling (Zorn and Nuhfer 2007; Nuhfer et al. 2017; Zorn et al. 2020). Given their size, Grayling fry are more likely to be vulnerable to dense populations of smaller trout that often occur in Michigan streams. It is due to these higher densities that it is critical to understand predatory interactions, including potential predation rates, between the species. Previous research from Montana has shown negative association between density of Brown Trout 10" and larger (McCullough 2017) and Grayling density. Zorn and Nuhfer (2007) demonstrated declines in age-0 and age-1 Brook Trout populations in the Au Sable River system in association with higher densities of age-3 and older Brown Trout which can be attributed to their predation on young Brook Trout. However, little evidence exists in regards to consumption rates of fry and other young fish in diet analysis due to the rapid digestion rate of such diet items (Trippel and Beamish 1987; Hansel et al. 1988; Scharf et al. 1998; Waraniak 2017). Such data may be prone to under-estimation of consumption rates for similar reasons. Studies in Montana have shown predation on young Grayling by other fishes but not during fry stage (Nelson 1954; McMichael 1990; Kaya 1992). Specifically, there is no previous research regarding vulnerability of Grayling fry to smaller-sized resident trout, therefore this research fills a critical knowledge gap.

Competition can be described as interactions between organisms through the common use of a limited resource (Pianka 1976). Previous research from streams in Montana have shown negative association between adult Grayling and Brown Trout (McCullough 2017), but little evidence of negative association between Grayling and Brook Trout between 100-250mm (Byorth and Magee 1998). Fausch and White (1986) found negative associations between age-0 Coho Salmon Oncorhynchus kisutch and both Brook and Brown trout. Coho Salmon fry emerge approximately two weeks earlier than both Brook and Brown trout and at a relatively larger size (Fausch and White 1986). Relative total lengths at emergence for Grayling, Brook Trout, and Brown Trout are 7.00-15.00 mm, 28.45-30.55 mm, and 27.24-29.21 mm, respectively (Fausch and White 1986; Northcote 1995). The approximate time of emergence for Brook and Brown trout in Michigan streams is between early April through early May (Zorn and Nuhfer 2007). Time of emergence for Grayling in Michigan is expected to be early spring, with historic documentation providing a spawning range from March to May (Metcalf 1961; Vincent 1962). Thus, competition between age-0 resident trout, potentially having a distinct size advantage early in the growing season, could adversely affect Grayling survival through aggressive interactions, resulting in young Grayling being pushed into less profitable microhabitats, but the effects of this are unknown. Therefore, this research will address this knowledge gap through interspecific competition trials.

This study assessed three potential key impediments to successful Grayling reintroduction in Michigan streams: early-life imprinting in Grayling, predation by young resident trout, and competition between age-0 Grayling and age-0 Brook and Brown trout. The three core components of this study included:

 Determination of the early-life stages at which Grayling are most likely to be imprinting to target waters through the use of plasma thyroid hormone analysis and water choice trials. I tested the hypothesis that imprinting occurs during early-life stages corresponding to elevated levels of plasma thyroid hormone levels. I also tested the hypothesis that young Grayling will show positive association to their "home waters" as compared to unfamiliar water sources.

- Examination of the predatory interactions between young (age-1) Brook and Brown trout, and newly emerged Grayling fry and to test the hypothesis that predation will differ between the two trout species.
- Examination of the competitive interactions between age-0 Grayling and Brook and Brown trout of the same year class through testing the hypothesis that the presence of resident trout species will result in changes in growth.

Michigan's Historic Arctic Grayling

Arctic Grayling were once the dominant salmonid in streams of the northern half of Michigan's Lower Peninsula (Vincent 1962). In the Lower Peninsula, their range extended primarily north of the White River, Lake Michigan drainage, and Rifle River, Lake Huron drainage, where they were found primarily in headwater streams. They were also found in Hersey Creek, a tributary of the Muskegon River, south of the White River (Vincent 1962). In the Upper Peninsula, their presence was documented in the Otter River, and possibly in Little Carp Lake, and East Branch of the Ontonagon River; all of which are located near the Keweenaw Peninsula. However, Vincent (1962) notes that the presence of Grayling in Little Carp Lake and the East Branch of the Ontonagon was only documented by a single source and may be questionable. Fluvial, stream resident, Grayling were the predominant life history found in the Laurentian Great Lakes region but rare occurrences of adfluvial populations were documented with catches reported in Lake Michigan near Charlevoix, MI, in the Traverse Bay region, and in Lake Huron in Thunder Bay (Hallock 1877; Harris 1884; Whitaker 1886; Bissell 1890; Henshall 1919; Creaser and Creaser 1934; Nuhfer 1992).

Attention to the presence of Grayling in northern Michigan streams began around 1854 (Whitaker 1886). Several factors then led to their rapid extirpation from Michigan including rampant over-exploitation, high intensity logging, and introduction of non-native salmonids (Vincent 1962; Nuhfer 1992; Tingley 2010). Over-exploitation was the primer to their ultimate demise. Accounts include railroad boxcar loads of Grayling being shipped to metropolis areas, wagonloads collected and hauled away, and individual fishing parties catching approximately 5,000 Grayling over the course of four weeks (Creaser and Creaser 1934; Vincent 1962). Over-exploitation was rampant, without regulation, and incessant. Grayling, as a species, may be prone to over-exploitation as declines associated with harvest intensity have been documented in both Montana and Alaska (Wojcik 1955; Vincent 1962). Over-exploitation was likely the initial cause of decline while habitat loss, predation, and competition quickly became additional stressors.

Extensive logging and habitat alterations of the region began around 1880, after overexploitation was occurring (Vincent 1962). Impoundments and other obstructions are detrimental to Grayling survival due to their tendency to migrate within watersheds with habitats associated to various life history requirements, i.e., migration to smaller tributaries for spawning. Logging practices of the late 1800's to early 1900's included clear-cutting of forests, sloughing into rivers to be held behind impoundments until spring high water events, then floated downstream to mills (Vincent 1962). Tree harvesting, as historically practiced as clear-cutting, may have resulted in increases in water temperature through reduced canopy cover. Further habitat destruction was the result of floating logs through the practice of "log drives", common throughout the region during this time. The timing of these log drives coincided with Grayling spawning. Deepening of the channel in regions previously containing riffles, removal of large-woody debris (LWD) within the channel and banks, and the erosion caused by the movements of the logs themselves resulted in widespread alterations to the stream ecosystems (Vincent 1962). Both the Manistee and Au Sable Rivers were documented to have a substrate largely consisting of clean sand with small clusters of gravel and rocks with dark loam along the banks, prior to the impacts of logging. Major habitat loss occurred as a result of logging: banks and stream beds were scoured, destabilization of the stream bed and associated sediments, increased turbidity, and loss of vegetation both within the stream and associated riparian zone drastically altered the ecosystems. In addition to the impacts from over-exploitation and habitat destruction due to logging, the introduction of non-native salmonids to the region further stressed the already vulnerable population (Vincent 1962).

Introduction of exotic salmonids to the native Grayling range in Michigan likely stressed the already vulnerable population. Brook Trout are the dominant salmonid in the Upper Peninsula of Michigan but were historically rare in the Lower Peninsula until after 1890. Dispersal of Brook Trout into the Lower Peninsular waters seems to have been a result of both natural range expansion and primarily through anthropogenic introductions (Vincent 1962; Westerman 1974; Zorn et al. 2018). The earliest recorded stocking of Brook Trout to a Lower Peninsular stream was to the South branch of the Tobacco River in 1870 (Mershon 1923). Rainbow Trout *Oncorhynchus mykiss* were first introduced to Michigan waters with the Au Sable River being the first recipient watershed in northern Michigan in 1876. In subsequent years, their introduction included several additional watersheds (Smedley 1938). Brown Trout, native to Europe, were first introduced to Michigan in 1884 with stocking occurring in the Baldwin River with rapid expansion of stocking sites in subsequent years (Zorn et al. 2018). As noted by Vincent (1962), it is important to note that in the majority of rivers and streams, Grayling populations were declining before the introduction of these species. While competition may not have been the most influential cause of decline of Grayling in Michigan, it is clear that the combination of over-exploitation, habitat destruction primarily due to logging, and the introduction of exotic salmonids all contributed to the extirpation of Arctic Grayling from Michigan.

Arctic Grayling Biology

Arctic Grayling represents one of five commonly accepted species of the genus Thymallus and one of the three subfamily complex of Salmonidae: Thymallinae, Salmoninae, and Coregoninae. The remaining four commonly accepted species classifications within the genus Thymallus include: T. brevirostris (Mongolian Grayling), T. grubii (Amur Grayling), T nigrescens (Kosogol Grayling), and T. thymallus (European Grayling) (itis.gov 2020). Taxonomic classifications at the species level has been debated among the scientific community with the number of extant species ranging from 2 to 14 (Gardiner 2000; Nelson 2006; Kottelat and Freyhof 2007; Gum et al. 2009; Ma et al. 2016; Froese and Pauly 2019). Based on mitochondrial analysis, divergence of Coregoninae and Thymallinae was approximately 46.11 million years ago and is suggestive of a Thymallinae-Coregoninae sister relationship (Ma et al. 2016). Arctic Grayling is a holoarctic species found in North America, north-eastern Siberia, and northern Asia (Vincent 1962; Northcote 1995). It is the only species of the grayling complex found in North America in which their native range predominantly includes northern regions of Canada and Alaska. Two isolated populations, thought to be glacial relicts, historically occupied ranges in Montana and Michigan during the last glaciation (McPhail and Lindsey 1970). Of these two relict populations, only the Montana population currently occupies a small portion (5%) of its historic range while the Michigan population was extirpated in 1936 (Vincent 1962; Kaya 1992).

Arctic Grayling are easily distinguishable by their large, colorful dorsal fin, containing a total of 17-25 rays. Upon maturity, males display a larger and more elongated dorsal fin than females, with the fin often reaching or exceeding the adipose fin when depressed and with pelvic fins nearly reaching the anal pore. The dorsal fin of the female is also highly colored but shorter in overall length and anteriorly taller than the posterior portion (McClane 1978). Their tail is deeply forked and have large, cycloid scales with a lateral line scale count of 77-98. Length varies depending on range but can be up to 610 mm and weigh up to 2.3 kg. They have a moderate sized, square-shaped mouth with small teeth and black markings on the lower jaw. Body coloration varies from silver, gold, blue, purple with various shades of iridescent colors with dorsal region often darker in color. Individuals often have several irregular black "v" spots on sides, unique to each fish. Pelvic fins have several orange to red colored stripes. Dorsal fin coloration is often dark with several rows of orange, blue, and green spots with a red or orange edge. Males are more vivid in coloration during spawning. Female fins are shorter in length. Juveniles have 10-19 dark, narrow parr marks with irregular narrow dark marks above and below the primary row (McPhail and Lindsey 1970; Scott and Crossman 1973; Morrow 1980; Mecklenburg et al. 2002; Page and Burr 2011).

Arctic Grayling inhabit clear, cold-water systems. The optimal temperature range is 9.5-16°C with a lethal temperature tolerance of 29.3°C (Hubert et al. 1985; Lohr et al. 1996; Danhoff et al. 2017; Goble et al. 2018). Unlike other salmonids, Grayling may not be restricted by dissolved oxygen (DO) levels. Bendock (1980) noted Grayling inhabiting waters with DO levels of 0.6 mg/L while overwintering. Hubert et al. (1985) estimated optimal summer DO levels of 6 mg/L, noting suitable levels are above 4 mg/L with a critical low of 2 mg/L. Grayling are known to have three different life-history patterns: stream-resident, in which they exhibit limited migration, primarily remaining in small tributary streams less than 10 m wide; fluvial, inhabiting small tributaries primarily for spawning and rearing with migration to rivers greater than 10 m wide; and adfluvial, in which they inhabit lakes for the majority of the year with migration to small tributaries for spawning and rearing (McPhail and Lindsey 1970; Scott and Crossman 1973; Jessop and Lilley 1975; Krueger 1981; Northcote 1995; Stewart et al. 2007; Tingley 2010). A fully lacustrine, lake dwelling, life history has also been documented, but is not as common, with spawning occurring within a lake system in the absence of flowing inlet or outlet tributaries (Bendock 1984; Armstrong 1986; Northcote 1995; Stewart et al. 2007; Tingley 2010). Abiotic habitat conditions for Grayling in Michigan were similar to those required by other, more northern populations. The substrate of "Grayling streams" in Michigan typically consisted of coarse sand with some amounts of gravel. Water depth was typically less than 1.52 m with velocities in the range of 30-60 cm/s.

Grayling are opportunistic feeders, but primarily insectivores. Juvenile and adult Grayling have been known to consume smaller fish, voles, and shrews (McPhail and Lindsey 1970). Fry begin exogenous feeding within 4 days of hatching. In laboratory or hatchery settings, feeding begins within 24 hours of hatching. During this stage, their diet largely consists of zooplankton with particle size increasing through maturation, transitioning to macroinvertebrates as their gape size allows (Jones et al. 2003).

Spawning occurs in the spring with water temperatures ranging from 4-16°C with daily means of 6-10°C (Stewart et al. 2007). Spawning typically occurs over unembedded gravel, without construction of a redd, but has also been observed over sand, clay, or cobble substrates. Eggs are small, 2.5 mm in diameter and slightly adhesive, allowing them to adhere to the substrate.

Water velocity in spawning habitat is typically 30-80 cm/s. Incubation period in stream water temperatures with a daily mean of 8.8°C is 13-18 days, however, the degree-days from fertilization to hatching varies widely in the literature from 144 to 268 (McPhail and Lindsey 1970; Stuart and Chislett 1979, as cited in Northcote 1995). Hatching is a short duration event, typically lasting no more than 24 hours (Brown 1938). Alevin have an extremely small yolk-sac compared to other salmonids, and as such, initiate feeding shortly after hatching. Fry emerge approximately 3-4 days after hatching and are 7-15 mm in length, inhabiting shallow pools and side channels with substrate consisting of sand and fine gravel and calm waters with low flow (Stewart et al. 2007). Early life history, particularly the first 14 days post-hatch, requires stable conditions as fry are susceptible to increases in velocities which may result in being flushed out of nursery refugia to less suitable habitat conditions (Nelson 1954, Stewart et al. 2007). Sexual maturity is reached at age 2-6. Mean fecundity is 12,359 eggs/kg body weight (Falk et al. 1982, as cited in Stewart et al. 2007). Historic populations of Grayling in Michigan were described to be prolific with high fecundity. Records from Jerome (1879) show 3,555 eggs were collected from a 9 oz female Grayling in Michigan. This equates to a fecundity of 13,933 eggs/kg body weight for this historic Grayling from Michigan, which falls within the current fecundity range of 6,475 to 16,887 eggs/kg body weight (Falk et al. 1982, as cited in Stewart et al. 2007). While much smaller than other salmonid fry, growth was documented to be typically faster throughout the first year than other salmonids for Montana populations (Brown 1938). This was further noted in historic Michigan populations in which an average total length of 86 mm by the end of the first growing season (Creaser and Creaser 1934). Length at age in Michigan populations was historically recorded as: age-1, 152mm; age-2, 254-305mm; age-3, 330-381mm; age-4, 406-432mm (Norris 1883).

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CHAPTER 1: IMPRINTING AND WATER CHOICE

Introduction

Salmonids are widely known to utilize a homing strategy when navigating from various waters. This homing behavior, which is facilitated by imprinting to various olfactory cues, increases the reproductive efficiency by returning to habitats previously known to be suitable (Hasler and Scholz 1983). Returning to familiar habitats through various life stages of the fish allows for a level of predictability of conditions. Since the natal site was suitable for spawning and rearing previously, it is likely to provide suitable habitat again (Hasler and Scholz 1983). The reduction in inefficient or unsuccessful reproduction due to unsuitable conditions increases the likelihood of multigenerational survival of the population.

Imprinting is primarily driven by smelling odorants in the water (Hasler et al. 1978). Odorant cues can include natal stream water odorants; conspecific odorants such as pheromones, amino acids, steroids, prostaglandins, bile acids and salts; and artificial substances such as morpholine and phenethyl alcohol (Hara 1992; Bett and Hinch 2016). The Olfactory Imprinting Hypothesis comprises three basic concepts: streams have unique odors due to unique chemical compositions, juvenile salmonids imprint the odor of natal streams prior to migrations and reference points during out-migration, and adult salmonids use these imprinted cues to return to the reference points and natal streams (Hasler and Wisby 1951; Harden Jones 1968). Similarly, the Pheromone Hypothesis recognizes that adult migrants may utilize conspecific cues as a component of natal homing, but often secondarily to imprinted natal stream cues (Nordeng 1971; Nordeng 2009; Bett and Hinch 2016).

Regardless of the substance, the critical component is that the fish has the ability to smell and remember the odor. In order to smell the substance, the odorant binds to the receptors on the epithelia of the peripheral olfactory organs (Hara 1992). This initiates a signal to be transmitted to the olfactory bulb via the olfactory nerve, thus generating a response within the bulb. The interpretation of the cue and commitment to memory occurs in the brain (Hara 1992; Morin and Døving 1992; Laberge and Hara 2004; Bett and Hinch 2016). Fish have the ability to detect odorants at very low concentrations, from 10⁻⁷ to 10⁻⁹ for amino acids, 10⁻⁸ to 10⁻¹⁰ for bile acids, 10⁻¹² to 10⁻¹³ for certain steroid pheromones, and 10⁻⁹ to 10⁻¹³ for prostaglandins (Hara 1992). Such odors are critical to olfactory imprinting, but in their absence, environmental cues such as flow, temperature, and dissolved oxygen levels can provide guidance to potentially suitable habitat in the event of straying (Keefer and Cuadill 2014; Bett and Hinch 2016). Imprinting to navigational cues including olfactory-based cues such as natural imprinted natal cues, pheromones, natural chemical odors, conspecific cues, and non-olfactory environmental cues may all play critical roles within the hierarchy of navigation (Stabell 1981; Hasler and Scholz 1983; Bett and Hinch 2015; Bett and Hinch 2016).

Bett and Hinch (2016) propose the theory of the Hierarchical Navigation Hypothesis to explain the complexity of salmonid migration. This hypothesis combines the Olfactory Imprinting Hypothesis and the Pheromone Hypothesis while including a component when neither odorants of the two primary hypotheses are available, non-olfactory environmental cues. This provides a threetier navigational system that may be utilized in a successional manner by salmonids. As described, salmonids rely on imprinted natal cues (Olfactory Imprinting Hypothesis) as their primary navigational guide; conspecific cues (Pheromone Hypothesis) as a secondary guide which have the potential to act as a surrogate if imprinted natal cues are absent or as reinforcement when they are present; and in the event that neither imprinted or conspecific cues are present, non-olfactory environmental cues can provide navigational guidance (Bett and Hinch 2016).

The timing of imprinting is critical to understand, particularly in the context of reintroductions and restorations that may rely upon hatchery rearing (Tilson et al. 1994) such that stocking occurs prior to imprinting which may decrease the chances of outmigration or straying. Although the timing of imprinting has been studied in various salmonids, the timing at which Arctic Grayling actively imprint on their natal waters is yet unknown (Northcote 1995; Kaeding and Boltz 2004). Increased thyroid hormone levels have been shown to induce imprinting in several salmonid species (Scholz 1980; Hasler and Scholtz 1983). Further, Hasler and Scholz (1983) found elevations in blood plasma thyroid hormone levels corresponded to times at which imprinting occurred. They also found the timing of imprinting could be artificially induced by elevating thyroid hormone levels by injection of thyroid stimulating hormones. Thyroid hormones bind to receptors in the brain cell nuclei resulting in the formation of permanently imprinted memories (Scholz et al. 1985). Dickhoff et al. (1978) found that increases in plasma thyroxine was associated with imprinting of young Coho Salmon. Thus, elevations in blood plasma thyroid hormone (thyroxine) levels can be indicative of times of olfactory imprinting (Lema and Nevitt 2004). However, Kitano et al. (2010) found stream resident Three-spine Stickleback Gasterosteus aculeatus have a lower plasma thyroxine level than migratory life history conspecifics which suggests physiological differences may occur between life history variants, including those involved with imprinting.

Tilson et al. (1994) utilized whole body thyroxine content and blood plasma thyroxine concentrations to determine the critical period of olfactory imprinting of Kokanee Salmon *Oncorhynchus nerka*. Thyroxine levels were found to peak at the time of hatching, swim-up, and at the parr-smolt transformation (PST) when yearlings. Tilson et al. (1994) exposed the fish to either phenethyl alcohol or morpholine at one of the following stages: fertilized egg, eyed egg,

hatch, alevin, swim-up, fry, and smolt. The fish were held until reaching sexual maturity then tested using a Y-maze in which they were exposed to water with and without the artificially imprinted cue. They found fish significantly homed to the odorant if they were exposed at hatch, alevin, and swim-up stages signifying that these stages corresponded with times of olfactory imprinting (Tilson et al. 1994). Other studies have also used artificial chemical odorants, specifically morpholine, in association with elevations in blood plasma thyroxine, to test imprinting in pre-emergent and young salmonids with recognition by adult stages (Hassler and Kucas 1988; Tilson et al. 1994; Dittman et al. 2015). By exposing fish to artificial odorants at times in which they are known to have elevated levels of thyroxine, the fish became imprinted to the odorant.

Water choice experiments have further explored the importance of imprinting in young salmonids to water preferences and homing at the mature life stage (Brannon et al. 1984; Rottiers and Lemm 1985; Keefe and Winn 1991; Arnesen and Stabell 1992, Nevitt et al. 1994; Tilson et al. 1994; Bett and Hinch 2015; Jutfelt et al. 2017). Hassler and Kucas (1988) artificially imprinted Coho Salmon to morpholine during the parr-smolt transition period during hatchery rearing and found high returns of morpholine-imprinted fish in subsequent years as water at the hatchery fish ladder was treated with similar concentrations of morpholine during the spawning season. Laboratory examination of the preference to familiar water was further explored by Keefe and Winn (1991) in which native Brook Trout were collected from two distinct streams, one with an anadromous population and one with freshwater residents, to determine if there was a preference to home water versus that of the unfamiliar stream through the use of a simple Y-maze.

The response to olfactory cues may have seasonal fluctuations such that during nonmigratory periods, olfactory sensitivity to cues may decrease and, similarly, during times of migration this sensitivity increases. Døving et al. (1980) found seasonal fluctuations in response to olfactory cues in which both Arctic Char *Salvelinus alpinus* and European Grayling *Thymallus thymallus* exhibited minimal olfactory sensitivity starting in late August with an increase in sensitivity resuming in January. Johnson and Li (2010) have similarly found that aquatic organisms often exhibit temporal variation in their responsiveness to chemical cues. This suggests the potential for seasonal fluctuations in olfactory sensitivity and response to cues that may closely mimic ontogenetic shifts of the fish.

Nuhfer (1992) noted that rapid outmigration of Grayling upon release was a problem common to previous reintroduction attempts in Michigan. A theory as to the cause is that the young hatchery-reared Grayling had imprinted to the hatchery water and thus lacked appropriate navigational cues or olfactory familiarity important for establishing stream residency. When stocked into unfamiliar stream water, the lack of familiar olfactory cues for establishing residency may have resulted in outmigration. Post-stocking outmigration of young Grayling was also noted as a potential impediment to successful attempts in Montana (Kaya 1992; Kaeding and Boltz 2004).

Evidence of the importance of imprinting can be seen through the successful establishment of stream resident Grayling exhibiting site fidelity with the use of remote site incubators (RSIs) in the restoration of Arctic Grayling in Montana (Kaeding and Boltz 2004). Implementation of remote site incubators (RSIs) allow for embryonic salmonids to imprint to the stream at which they are to be introduced and their use has shown success (Donaghy and Verspoor 2000; Kaeding and Boltz 2004; Magee et al. 2005; Al-Chokhachy et al. 2009, Kirkland 2012; Cayer and McCullough 2014; Arnold et al. 2017). Both laboratory and field studies have demonstrated natal imprinting as early as prehatch eyed-stage of salmonid embryos, hatching, and emergent life stages (Bodznick 1978; Courtenay 1989; Tilson et al. 1994; Bentzen et al 2001; Quinn et al. 2006). This leads to the belief that such early life exposure to cues may be critical to Grayling, indicating that the timing of imprinting may occur during these early life stages. Information is sparsely available regarding early life history of Arctic Grayling, in general (Kratt and Smith 1977) and little is known regarding imprinting of Grayling which has left these behaviors unresolved. Further understanding of Grayling imprinting and subsequent water choice selectivity aims to better understand the issue of rapid outmigration upon stocking and thus further increase the likelihood of a successful reintroduction.

Understanding if and when Grayling imprint will allow for increased understanding of when it is most critical for young fish to be stocked into their receiving waters. By stocking prior to imprinting, the likelihood of emigration will be reduced. This will aid in determining rearing techniques that are both effective and efficient to the reintroduction of Grayling. For example, if it is found that Grayling imprint at hatching, it is imperative that they are stocked to the receiving waters prior to hatching and thus explains the success of reintroductions that have implemented RSIs. This study aimed to determine the time or times at which embryonic and young Grayling may be actively imprinting through examination of plasma thyroxine (T4) levels. Further, water choice experiments were used to examine preference for familiar water sources through the use of an artificially imprinted odorant, morpholine, and other cues such as conspecific odor.

Methods

Fish Rearing

Grayling for this study were obtained annually from the Ruth Burnett Sport Fish Hatchery as eyed eggs and reared in the Michigan State University Containment facility: 5,000 in 2018; 10,000 in 2019 and 2021 (no fish in 2020 due to COVID-19 pandemic). Michigan Department of

23

Natural Resource (MI DNR) personnel assisted Alaska Fish and Game (ADF&G) personnel in the collection of eggs from adult Grayling from the Chena River which were held in temporary ponds. I transported eggs from Fairbanks, AK to East Lansing, MI once they reached eyed-egg stage. Rearing methods closely followed protocols set forth by Travis Hyer and Charles Pratt (Ruth Burnett and William Jack Hernandez Sport Fish Hatcheries, Alaska Department of Fish and Game, respectively, personal communications). Eggs were reared in heath-style incubation trays with trout mesh screens. Fish were transferred (ponded) to a rearing tank located below the incubation trays 48 hours after 50% of the eggs have hatched. The rearing tank was a low-flow, shallow depth tank equipped with a fine-mesh screen on a bottom-drawn outflow standpipe. The ponding time of 48 hours was selected as it represented a hybridization of the rearing techniques used at both Alaska hatchery facilities. Flow rate and depth of water was increased corresponding with fry growth and development and based upon the recommendations of Travis Hyer and Charles Pratt, ADF&G (personal communications).

A 24hr Artemia solution drip was immediately started upon ponding with newly hatched alevin keying in on the feeding drip within 24 hours (personal observation). Six additional feeding lines further distributed the Artemia solution to decrease localized competition for food. Each of the additional lines were operated by dosing pumps in which set volumes (50 mL) of solution were administered every 10 minutes from a single dosing line. Each line dosed solution once every hour; with six lines to provide additional feed every 10 minutes. Transitioning from Artemia to Bio-Oregon trout mash began 10 days after ponding with full transition from Artemia to 100% trout mash at 14 days post-ponding. Changes in dry feed size and amount fed corresponded to increases in growth of fry and was based upon the recommendations of Bio-Oregon[®] feeding guides for trout and salmon (https://www.bio-oregon.com/assets/FeedingGuidelines.pdf). Dry feed was

administered via Eheim[®] automatic fish feeders. Each feeder was programed to dispense a measured amount of food 1-4 times per day, dependent on the daily feeding requirements of the fish. Placement of the feeders as well as timing of food dispension rotated daily. Fresh food was placed in each feeder daily. Feeding amounts were regularly monitored to decrease the likelihood of overconsumption by Grayling as they are prone to feeding beyond satiation to the point of rupture (Kindschi and Barrows 1990; Travis Hyer, personal communication).

Artificial Odorant

In 2019 and 2021, morpholine (ReagentPlus®, \geq 99%) C₄H₉NO, an artificial amino acid, was added to the recirculating system to obtain a concentration of approximately 5.70x10⁻⁸. This dilution was decided upon after consultation with A. Dittman (Environmental and Fisheries Sciences, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, personal communication) as it fell within the range of detectability in previous salmonid research: $5.00x10^{-5}$ (Rehnberg et al. 1985; Hassler and Kucas 1988), $5.70x10^{-6}$ (Dittman, personal communication), $5.70x10^{-7}$ (Dittman, personal communication) $5.70x10^{-10}$ (Wisby 1952; Dizon et al. 1973; Hasler and Scholz 1983). As no previous research was found regarding the use of morpholine within the family Thymallidae, caution was used and the first administration occurred on 04 Jun 19, two days after swim-up. The entire system was "refreshed" every 25 days with additional supplementation with each water change due to the potential to degrade (MSDS documentation). In 2021, the inoculation of the recirculating system occurred five days prior to Grayling eggs being placed into the incubation trays.

Sampling for Imprinting - Plasma Thyroxine Levels

Specimens for thyroid hormone analysis were collected throughout the earliest stages of Grayling development to aid in identifying life stages in which age-0 Grayling are most likely to be imprinting. This included key life history changes such as eyed-egg, hatch, and swim-up followed by bi-weekly sampling (Table 1), timings that closely follow those of Tilson et al. (1994) for Kokanee Salmon. As eggs were transported from Fairbanks, AK to East Lansing, MI, the earliest collection occurred only once they have reached the eyed-embryo, or eyed-egg stage. Ten replicates were collected during each sampling event. Each replicate consisted of at least 0.1g of tissue. Fish were humanely euthanized (following Animal Use protocol accepted by Michigan State University), length and weight recorded, placed in labeled centrifuge tube, and quickly transferred to an -80C freezer.

Methods for plasma thyroid hormone extraction closely followed those set forth in Tilson et al. (1994) and modified based on initial examination of 2018 samples. The 2018 samples were specifically used to determine the methodology to be used in analyzing subsequent years and considered "test" samples not for final analysis. Thyroid hormone analyses were done in collaboration with Drs. Weiming Lin, Sonam Tamrakar, and Tyler Buchinger at Michigan State University and concentrations (ng/g tissue) reported back for final analysis. Thyroid hormones, T4, T3, and rT3, in Grayling tissues were extracted and quantified according to a previously described method (Tamrakar et al. 2023) with slight modifications. In brief, Grayling tissues were homogenized in a bead mill homogenizer at 5-7 m/s for 30 s. For samples weighing more than 500 mg, the tissues were divided into two or more portions and homogenized separately, then were combined before extractions. The samples were then digested overnight with pronase enzyme (from *Streptomyces griseus*, Roche Diagnostics) at 37°C and 100 rpm. A 100 μL portion of antioxidant solution (6 mg each of citric acid, L-ascorbic acid, and DL-dithiothreitol in 1 mL deionized water) and 10 μ L of internal standard mix (100 ng/mL each of T4-¹³C₆, T3-¹³C₆, and rT3-¹³C₆) were added to the samples. Sample cleanup prior to LC-MS/MS analysis included a protein precipitation step with ice cold methanol and centrifugation (3000 rpm) for 10 minutes. The supernatant was adjusted to 1:1 (methanol: water v/v) and subjected to liquid-liquid extraction using 1 mL chloroform. Sample matrix from the top layer containing the thyroid hormones was then cleaned further using SampliQ OPT cartridges for solid phase extraction (SPE). The eluent from the SPE step was freeze dried under vacuum and reconstituted in 1 mL of 50% methanol in water for LC-MS/MS analysis.

The samples were analyzed in an ACQUITY H-Class UPLCTM connected to a Xevo TQ-S triple quadrupole mass spectrometer (Waters Corporation, Milford, MA). A 10 μ L portion of the sample was injected into a BEH C18 column (2.1 x 100 mm, 1.7 μ m particle size; Waters Corporation). Deionized water containing 0.1% formic acid and methanol were used as mobile phase A and B respectively. The LC program constituted of an 8-minute gradient starting at 50% B (0 – 0.2 min), followed by 99% B (4 – 5.5 min) and re-equilibration at 50% B (6 -8 min). The mass spectra were acquired by electrospray ionization in the positive mode using multiple reaction monitoring (MRM). MassLynx 4.2 was used for data acquisition and Targetlynx XS was used for data processing.

Statistical Analysis

A generalized linear model with gaussian distribution and an identity link function was performed to examine if T4 concentrations (*Bpt*) changed over time based on life stage. The model included Grayling life stage (Ls), a surrogate for time, as the main effect:

$$Bpt_j = Ls_j + e_j$$

where j = life stage. Mean T4 concentrations for each life stage were plotted to visualize changes in concentration over time.

Methods: Water Choice Trials

2018

Water choice trials during the 2018 season closely followed procedures used by Greg Byford (unpublished data) for Lake Sturgeon Acipenser fulvescens at the Black River Sturgeon Facility in Cheboygan County, Michigan, in 2017. In summary, this consisted of a 5-foot section of 4" PVC cut in half and capped at each end. The midpoint was marked to indicate the location at which fish were added as well as dividing the apparatus into two equal sections. Each side was divided into five equal sections for a total of 10 equal zones, labeled Z01-Z10, with Z01 being located the furthest from midpoint in side A and Z10 being located furthest from the midpoint in side B. The apparatus was filled with fresh chilled water (a mixture of 50% reverse osmosis water and 50% fish water supplied by the containment center). Each burette was filled with either tank water or fish water. The burette assignment (A or B) of the water type was chosen randomly. An individual fish was placed into the apparatus at the midpoint and allowed to acclimate for a period of time ranging from 5-30 minutes prior to commencing the trial. Upon acclimation, water was added to the trough from the burettes at a rate of 20 mL per minute, equating to a fast drip. The zone location of the fish was recorded every minute for 10 minutes. At the completion of each trial, the fish was removed, and apparatus rinsed and cleaned. The burettes were rinsed after each trial. Several attempts were made to use this method; however, fish were consistently observed jumping out of the apparatus leading to the requirement of different protocols for 2019.

2019

Methodology and water choice equipment were updated for the 2019 experimental season based on the difficulties encountered during the 2018 season. Methods and water choice flume design (Figure 1) were loosely based on Keefe and Winn (1991). To determine if fish were imprinting to their tank water and not simply an amino acid present in the water through the addition of food or other organisms, exposure to a synthetic chemical (morpholine) was used in the 2019 trials (ROFM water). Since morpholine is not found in natural waters, is chemically stable, highly water soluble, and show to be safe to fish, it is an ideal substance to use in examining imprinting (Scholz et al. 1975). Exposing fish to water that has been inoculated with morpholine at various life stages has been found to be useful in the study of imprinting behavior, primarily when testing with water from various sources without the substance and water with morpholine to allow for water recognition based on this chemical (Tilson et al. 1994). Two water types were used for trials: ROFL water which consisted of prechilled (16°C) reverse osmosis (RO) water with trace mineral supplement Flourish® and ROFM water which consisted of ROFL + Morpholine.

A dye test was performed to determine the amount of time for solution from the two dosing buckets to reach the end of each channel, closest to the choice arena. Dye diffused to the choice arena within one minute. For each water choice trial, the flume was thoroughly rinsed and filled with pre-chilled ROFL water. Test water buckets were then filled with prechilled 1000 mL ROFM or CONS and ROFL water corresponding to the channel to be dispensed (bucket A flows into channel A, bucket B flows into channel B). Flow rates were calibrated so that each bucket dispensed solution at 1L/min.

Trials were recorded remotely using a GoPro HERO 7[®] camera mounted above the water choice flume such that the choice arena and both arms of the flume were within the frame.
Recording trials remotely allowed for time spent in various locations of the flume and behavior (stationary, active, direction, etc.) to be examined. This remote recording decreased the influence of the experimenter that may alter the behavior of the fish (close proximity, shadowing, etc.). Further, as the GoPro camera system allowed for WiFi connection via the GoPro App for smartphones, the experimenter was able to observe a live feed of the trial to ensure that the flume gate fully lifted without disruption of the trial and to track duration of the trial via the timer. This also allowed for later analysis to determine behavior and time spent in each arm of the flume for each trial (Jutfelt et al. 2017). The water choice flume was isolated from the laboratory area by placing it within an empty holding tank and surrounding the section with black-out curtain lined with mesh fabric to mute the white coloration of the black-out curtain. Light was provided to the area by a full-spectrum LED light with filter and vegetative materials (artificial vine) to reduce glare.

Acclimation time was determined a priori using pilot trials in which time to "acclimation" was recorded. It is recognized, as described by Pickering et al. (1982) that stress due to minimal handling, two minutes, can result in stress that may last up to 2 weeks. However, practicality of completion of experimentation must also be acknowledged thus, acclimation was determined to occur when the fish had sampled each arm of the water choice flume (Jutfelt et al. 2017). Recent studies utilizing water choice flumes to determine preference provide 2-5 minutes of acclimation and short trials of 9-15 minutes (Jutfelt et al. 2017). Twenty acclimation pilot trials were performed; the time for an individual fish to transition from holding stationary to sampling each arm of the flume ranged from 8-10 minutes. Therefore, I set the acclimation period as 10 minutes for 2019 water choice trials.

For the water choice trials, an individual fish was randomly netted from the rearing tank and immediately placed in the choice arena of the flume with the gate down and allowed to acclimate for 10 minutes. The gate (clear plexiglass with mesh screen to allow water flow) slides into two slots on either side of the water choice flume to separate the choice arena from each arm of the flume (not shown in Figure 1). Valves were opened on both buckets at minute 10 of the trial followed by lifting the gate at minute 11. The one-minute gap between opening of valves and lifting the gate allowed for the treatment water to reach the choice arena as confirmed a priori via dye testing. An additional 1 minute of acclimation was allotted due to disturbance caused from lifting the gate. The choice trial began at minute 12, during which the fish was exposed to treatment water choices with the gate fully lifted and lasted a total of 10 minutes. The total time to complete an entire replicate from placing fish into the choice arena to removal at the end of the choice trial was 22 minutes. At the end of each trial, the fish was removed, humanely euthanized, and total length and weight were recorded. All equipment was thoroughly rinsed between each trial and at the beginning of each new trial day.

2021

The methods for water choice trials were further refined for the 2021 laboratory season, driven primarily to behavioral concerns observed with fish during the 2019 trials. In short, fish were commonly observed remaining stationary within an arm of the water choice flume during the 2019 trials. To address this, three fish were to be placed within the choice arena of the water choice flume and the gate remained down during the entirety of the trial. Consistent with findings of Jutfelt et al. (2017), in which providing 2-5 minutes of acclimation and short trials of 9-15 minutes were deemed appropriate, the 2021 acclimation time was reduced to 5 minutes and the total trial time to 11 minutes. Additionally, test water types were limited to ROFM (as described in 2019

methods) and TANK. TANK water was water taken directly from the rearing tank within the recirculating system. This was deemed a suitable test water as a natural stream system would be a complex collection of odorants including conspecifics, substrate, excrement, food particles, etc., and TANK water would, therefore, represent a viable option.

For the water choice trials, the flume was filled with prechilled ROFL and test water added to the system using a Stenner® Econ VX series variable speed peristaltic metering pump set to deliver 19.4 mL/min (set on highest setting) during the test portion of the trial. The GoPro camera used in 2019 was set above the flume to record the entirety of the trial and allow for remote monitoring of the trial without disturbance. Placement of the flume and lighting was the same as described in the 2019 methods. Test water was introduced either to arm A or B of the flume. Three fish were randomly netted from the rearing tank and immediately placed in the choice arena of the flume with the gate down and allowed to acclimate for 5 minutes. At minute 5, the peristaltic pump was turned on high to begin adding the test water (ROFM or TANK) to the selected arm of the flume. A one-minute acclimation period was again provided, similar to 2019, to allow the test water to reach the choice arena. The test portion of the trial started at minute 6 and concluded at minute 11. The total time to complete an entire replicate from placing fish into the choice arena to removal at the end of the choice trial was 11 minutes. At the end of each trial, the fish were removed, humanely euthanized, and total length and weight recorded. All equipment was thoroughly rinsed between each trial and at the beginning of each new trial day.

Statistical Analysis: Water Choice 2019

Video recordings of the trials were reviewed, and total time spent in each arm of the flume quantified. The proportion of time spent in the test water arm of the flume was recorded and used for analysis. Visual examination of the data using a histogram and Q-Q plot indicated the data was left-skewed and may not follow a normal distribution (Figure 2) and a Shapiro-Wilk test was utilized to determine whether data followed a normal distribution. A one-sample sign test was deemed most suitable due to the characteristics of the data to examine whether the resulting median significantly differed from the null median of 0.5. A median of 0.5 was selected as the null as it was representative of 50% of the time spent in either arm of the flume, or "no preference" to either water type.

Statistical Analysis: Water Choice 2021

Video recordings of the trials were reviewed, and time spent in each half of the choice arena was quantified. Locations of each of the three fish were recorded every 10 seconds during the test portion of the choice trial. In the event that a fish was breaking the plane of center, the direction of the snout of the fish determined which side (test or control) the fish was considered to be occupying. Thus, if the snout of the fish was within the test water side and not facing away from that half, the fish was considered to be occupying the test portion of the choice arena; if the snout of the fish was breaking the plane of center but facing in the direction of the control portion of the choice arena, the fish was considered to be occupying the control portion of the choice arena. A total of 93 data collection points were recorded for each trial (3 fish * 31 time steps = 93). The proportion of time (number of occurrences in the control/number of data points) spent in the test water side of the choice arena was quantified for analysis. Visual examination of the data using a histogram and Q-Q plot appeared to show a normal distribution (Figure 3) and a Shapiro-Wilk test was utilized to verify this observation. A one sample t-test was performed to test whether the mean of all trials was different from the null of 0.5. The null value of 0.5 was selected as it represented the "no choice preference" value with 50% of time spent in either side of the choice arena. A twosample t-test was performed to test whether the mean was different between the two types of test waters, ROFM and TANK.

Results

Results: Imprinting - Plasma Thyroid

A total of 189 fish were collected in 2018, 474 fish were collected during the 2019, and 336 fish were collected during the 2021 research season (Table 1). As described in the methods, the 2018 samples were used to determine the methodology to be used for the analysis of subsequent years. Cost constraints limited the number of samples that could be analyzed, resulting in final analysis including those collected in 2021.

Plasma T4 levels were significantly different over time. Levels differed at the eyed-egg stage (p < 0.001), at hatch (p < 0.001), and at swim-up (p < 0.001) as compared to levels at the first bi-weekly collection (Table 2). Levels did not differ at any other time when comparing all other bi-weekly collections to the first bi-weekly collection. Visual inspection of the mean T4 concentrations indicate a peak in T4 levels corresponding with eyed-egg stage followed by significantly lower concentrations at both hatch and swim-up (Figure 4) and then higher concentrations that remained relatively constant across the remainder of the sampling period. Results: Water Choice

A total of 80 trials were completed in 2019 and 80 in 2021. All 80 trials performed in 2021 were included in the final analysis. A subset of 32 trials from 2019 were analyzed. Full analysis was not performed based on observed behavior of Grayling in 2019 in which fish would swim up either arm of the water choice flume and hold position for the remainder of the trial. None of the trials from 2018 were analyzed due to lack of fish remaining in the water choice trough (i.e., the fish were jumping out) resulting in unreliable data.

In 2019, Grayling did not show a preference to test water, however the median was significantly different from the null median value of 0.5, indicating the sample median was not equal to 0.5 (p = 0.002, $\tilde{x} = 0.102$). Visual inspection of the 2019 data indicated no preference for the test water, with the majority of time spent in the control water flume arm (Figure 2).

In 2021, Grayling showed a weak preference for the test water ($\bar{x} = 0.585$, SD = 0.105) as compared to the null mean value ($\mu = 0.5$), t(79) = 7.226, p < 0.001. Visual inspection of the distribution of the proportion of time spent in the test water half of the choice area indicates a weak preference (Figure 3). There was no significant effect of test water type on proportion of time spent in the test water half of the choice arena, t(78) = -0.217, p = 0.829, with trials with TANK water having a mean of 0.587 (SD = 0.107) and trials with ROFM water having a mean of 0.582 (SD =0.103) (Figure 5).

Discussion

There is evidence of potential olfactory imprinting occurring in Grayling at the eyed-egg stage, as indicated by an elevation of plasma thyroxine levels (Table 2, Figure 4). However, the extent to which the peak plasma thyroxine level may be attributed to maternal influence remains unknown. Maternal origin of thyroxine has been documented in several species of fish (Lam 1994; Deal and Volkoff 2020). Generally, thyroxine levels decrease over time during development of fish eggs with endogenous production occurring concurrently with yolk-sac absorption (Tagawa et al. 1990; Lam 1994; Castillo et al. 2015). Not all embryonic thyroxine is of maternal origin, however, with endogenous production occurring before the development of thyroid follicles in Fathead Minnow *Pimephales promelas* and Zebrafish *Danio rerio* (Crane et al. 2004; Vergauwen et al. 2018). As the thyroid hormone complex is critical to development, it may be difficult to determine whether the peak in thyroxine is attributed to developmental needs, imprinting potential,

or both. Additional exploration would likely be able to determine the degree of maternal origin of thyroxine in the embryonic stage of development, similar to Vergauwen et al. (2018). Further exploration of blood plasma thyroxine levels of embryonic, larval, and juvenile stages of development of Grayling is warranted.

A weak preference was later exhibited to familiar water in the 2021 water choice trials (Figure 3). This is consistent with the observed success of reestablishing stream resident Grayling populations using RSIs in Montana, as identified as a critical aspect of reintroduction efforts (Kaeding and Boltz 2004). The use of RSIs during the critical period of imprinting for Grayling, the eyed-egg stage, will likely increase the chances of a successful reintroduction to Michigan streams and to reestablish Grayling populations with site fidelity. Rapid outmigration was a challenge with past reintroduction attempts (Nuhfer 1992) and was thought to be due to Grayling imprinting on hatchery water and lacking appropriate navigational cues or lack of familiar water. Similar patterns of rapid outmigration occurred in Montana reintroduction efforts prior to the use of RSIs (Kaeding and Boltz 2004). Understanding that the eyed-egg stage is likely a period of olfactory imprinting provides further explanation to these past challenges as well as points to areas where future improvements can be made.

Grayling showed a weak preference to familiar water, both TANK and ROFM, in 2021 (Figure 3). While Rehnberg et al. (1985) found the addition of morpholine did not result in increased attraction to home water, there was no difference in attraction to water inoculated only with morpholine (ROFM) compared to the complex TANK water which contained odorants from morpholine within the recirculating water system, conspecifics, substrate, food residue, and other odorants within the stream system (Figure 5). The weak preference shown to familiar water during the 2021 water choice trials may be due to two factors: the potential for altered behavior in the

laboratory environment or seasonal fluctuations in olfactory sensitivity. During non-migratory periods, olfactory sensitivity to cues may decrease and responsiveness to olfactory cues may follow a temporal pattern (Døving et al. 1980; Johnson and Li 2010). Grayling follow a migratory pattern such that they migrate to over-wintering habitat in the autumn and return to spawning and summer habitat in spring. Previous research with older European Grayling found minimal sensitivity starting in late August with increasing sensitivity starting in January (Døving et al. 1980). The temporal variation in olfactory sensitivity of sub-yearling Arctic Grayling has not been identified, however it may be possible that trials were performed during a period of lower receptiveness for olfactory cues. Further exploration into potential patterns of olfactory responsiveness would help identify temporal and seasonality in Grayling olfactory receptivity. Seasonal fluctuations in olfactory sensitivity and response to cues may closely mimic ontogenetic shifts of Grayling.

While no preference to water containing morpholine (ROFM) was identified in the 2019 trials, this may be attributed to morpholine being added to the recirculating system waters after the critical imprinting window. In 2019, morpholine was added to the system two days after swim-up. Tilson et al. (1994) found imprinting to morpholine in Kokanee Salmon occurred when fish were exposed at the eyed-egg, hatch, or swim-up stages. This may further indicate the importance of allowing exposure to home waters as early as the eyed-egg stage to facilitate time of imprinting in Grayling.

Sub-yearling Grayling may not exhibit a strong sensitivity to familiar water. Previous research has either naturally or artificially imprinted young fish to cues, allowed the cohort to reach maturity, then perform water choice trials (Brannon et al. 1984; Rottiers and Lemm 1985; Keefe and Winn 1991; Arnesen and Stabell 1992; Nevitt et al. 1994; Tilson et al. 1994; Bett and Hinch

37

2015; Jutfelt et al. 2017). Responsiveness to cues can be influenced by maturity (Johnson and Li 2010). As a result, young Grayling may not show a strong behavioral response to imprinted waters during their sub-yearling stage, sensitivity may increase throughout maturity. However, use of RSIs in reintroductions in Montana (Kaeding and Boltz 2004) in combination with evidence obtained through this study, point to the importance of early exposure to home waters.

Elevations in plasma thyroxine have been found to correspond with times of sensitivity to olfactory imprinting in salmonids (Dickhoff et al. 1978; Hasler and Scholz 1983; Dickhoff and Sullivan 1987; Hassler and Kucas 1988; Tilson et al. 1994; Dittman et al. 1996; Dittman et al. 2015; Havey et al. 2017). Identifying the potential for imprinting and olfaction of olfactory cues are critical components in understanding the complex migratory behavior of fishes. While imprinting is thought to be primarily driven by the olfactory reception of odorants in the water (Hasler et al. 1978), other aspects can guide migration including stream landmarks. The Olfactory Imprinting Hypothesis (Bett and Hinch 2016) recognized migration as a complex behavior that incorporates the imprinting of unique natal stream odors, conspecific odors, and stream landmarks and the recognition of the odors and landmarks as guides for adult fish during migration. The identification of the eyed-egg stage as a likely time in which Grayling are sensitive to imprinting is one piece in the migratory puzzle.

Understanding the time at which Grayling are sensitive to imprinting is critical to understanding when fish require exposure to familiar water. This is particularly important in reintroductions in which there is reliance on brood stock and a degree of hatchery rearing (Tilson et al. 1994). To decrease the likelihood of rapid outmigration or straying, I recommend that Grayling should be stocked into their selected site waters within the timeframe of potential sensitivity to imprinting, the eyed-egg stage.

38

TABLES

Table 1: Arctic Grayling specimen collections during 2018, 2019, and 2021 for plasma thyroid

Collection Date	# fish	Stage
07 Jun 2018	33	Pre-hatch eyed eggs
07 Jun 2018	33	Post-hatch alevin
12 Jun 2018	21	Swim-up
26 Jun 2018	22	1 st bi-week
07 Jul 2018	20	2 nd bi-week
23 Jul 2018	10	3 rd bi-week
05 Aug 2018	10	4 th bi-week
19 Aug 2018	10	5 th bi-week
04 Sep 2018	10	6 th bi-week
17 Sep 2018	10	7 th bi-week
03 Oct 2018	10	8 th bi-week
26 May 2019	180	Eyed eggs
28 May 2019	66	Pre-hatch eyed eggs
29 May 2019	58	Post-hatch alevin
02 Jun 2019	58	Swim-up
18 Jun 2019	20	1 st bi-week
01 Jul 2019	12	2 nd bi-week
15 Jul 2019	10	3 rd bi-week
30 Jul 2019	10	4 th bi-week
12 Aug 2019	10	5 th bi-week
26 Aug 2019	10	6 th bi-week
09 Sep 2019	10	7 th bi-week
23 Sep 2019	10	8 th bi-week
07 Oct 2019	10	9 th bi-week
21 Oct 2019	10	10 th bi-week
28 May 2021	100	Pre-hatch eyed eggs
02 Jun 2021	80	Post-hatch alevin
06 Jun 2021	66	Swim-up
21 Jun 2021	20	1 st bi-week
04 Jul 2021	10	2 nd bi-week
17 Jul 2021	10	3 rd bi-week
01 Aug 2021	10	4 th bi-week
16 Aug 2021	10	5 th bi-week
29 Aug 2021	10	6 th bi-week
13 Sep 2021	10	7 th bi-week
27 Sep 2021	10	8 th bi-week

hormone (T4) extraction.

Table 2: Results of general linear model analysis of plasma thyroxine (T4) concentrations by life stage (time). Overall test statistics were $R^2 = 0.646$, F(10, 81) = 14.76, p = < 0.001. Significant values ($\alpha < 0.05$) are in bold.

Main Effect	Estimate	β Coefficients	S. E.	<i>t</i> value	<i>p</i> value
Intercept	90.931	-	7.104	12.799	< 0.001
Eyed-egg	53.913	0.345	12.305	4.381	< 0.001
Hatch	-79.693	-0.510	12.305	-6.476	< 0.001
Swim-up	-73.707	-0.472	12.305	-5.990	< 0.001
Bi-week B	15.969	0.120	11.071	1.442	0.153
Bi-week C	-10.223	-0.090	10.047	-1.018	0.312
Bi-week D	-7.357	-0.065	10.047	-0.732	0.466
Bi-week E	-17.284	-0.152	10.047	-1.720	0.089
Bi-week F	-10.307	-0.091	10.047	-1.026	0.308
Bi-week G	5.523	0.049	10.047	0.550	0.584
Bi-week H	-0.304	-0.003	10.047	-0.030	0.976

FIGURES



Figure 1: Water choice flume used in 2019 and 2021. Mesh screen was located at the bottom region of the divider between the outflow and choice arena. A reinforced mesh gate was placed at the entrances of flume arms to hold fish within the choice arena. Dimensions: A) total flume length, 124.6 cm; B) total flume width, 22.7 cm; C) flume arm length, 87.3 cm; D) flume arm width, 9.4 cm; E) choice arena length, 23.0 cm; F) choice arena width and outflow width, 19.4 cm; G) outflow length, 7.5 cm; water depth, 10.2 cm.



Figure 2: Proportion of water choice test trial time spent in test water flume arm in 2019 water choice trials. Panel A) Distribution and frequency of proportional time in test water. Panel B) Quantile-Quantile plot of proportion of time spent in test water flume arm.



Figure 3: Proportion of water choice test trial time spent in test water half of choice arena in 2021 water choice trials. Panel A) Distribution and frequency of proportional time in test water; upper panel corresponds to test water type ROFM (Morpholine) and lower panel corresponds to test water type TANK (Tank water). Panel B) Quantile-Quantile plot of proportion of time spent in test water half of choice arena.



Figure 4: Mean thyroxine (T4) concentrations ± SE of Grayling by life stage (age in days). Labeled data points correspond to key ontogenetic changes: EE, eyed-egg; H, hatch; and SU, swim-up. Unlabeled data points correspond to bi-weekly collections over time.



Figure 5: Proportion of time spent in test water half of choice arena by test water type in 2021 water choice trials. Dot size corresponds to the frequency of trials with the same time (larger dot = higher frequency). Blue dots correspond to Tank water and pink dots correspond to ROFM (Morpholine) water. There was no significant effect of test water type on proportion of time spent in the test water half of the choice arena, t(78) = -0.217, p = 0.829, with TANK water having only a slightly higher preference to test water ($\mu = 0.587$, SD = 0.107) than ROFM water ($\mu = 0.582$, SD = 0.103).

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CHAPTER 2: PREDATION

Introduction

Broad ecosystem changes have occurred since Arctic Grayling Thymallus arcticus (henceforth, Grayling) occupied their historic range in the northern lower peninsula of Michigan. These changes include altered fish community structures, landscape changes, and changes in overall climate. On a global scale, impacts on native salmonid populations have been deleterious and are attributed to climate change, introduction of non-native species, habitat destruction and other anthropogenic effects (Brown 1943; Vincent 1962; Fausch and White 1981; Waters 1983; Fausch and Cummings 1985; Meffe 1985; Fausch and White 1986; Fausch 1988; Moyle et al. 1986; Miller et al. 1989; Nuhfer 1992; Wang and White 1994; Moyle and Light 1996; Nakano et al. 1998; Vander Zanden et al. 1999; Quist and Hubert 2004; Clavero and García-Berthou 2005; Fausch 2007; Zorn and Nuhfer 2007; Arismendi et al. 2009; Morita 2018; Zorn et al. 2020; Gallagher et al. 2022). Land use such as deforestation for agriculture and timber products, impoundments, transportation of goods using waterways, and terrestrial and aquatic navigation play a role in watershed scale changes over time (Vincent 1962). Climate change has potential to alter suitability of historical ranges (Seddon and Armstrong 2016). A critical component of successful reintroductions is determining contemporary factors that are expected to be the most limiting. Factors that affect the success of a reintroduction such as Grayling may or may not be under the control of a fishery manager. Further, impacts that were once limiting, such as habitat degradation from historic logging practices, may be alleviated and the ecosystem stable such that this once-limiting factor is no longer of concern. Others, such as impacts of climate change, may have little control and uncertain future impacts. However, the abiotic and biotic community structure of streams can provide managers with a suite of suitability indices, allowing for site

selectivity upon reintroduction. Knowing the potential impact of interspecies interactions, such as predation, can provide insight and guidance to the decision-making process.

Predation has the ability to limit the success of reintroductions through direct, consumptive effects or indirectly with non-consumptive effects (Noakes and Curry 1995; Lima 1998; Jachowski et al. 2016; Baker et al. 2022). The predator can directly affect the prey by consumption or through mortally wounding prey but not consuming during a predation event. In the latter case, this may occur if the prey was able to escape during handling (the time in which the predator is manipulating the prey) but was wounded during the predation event such that even upon escape, the result is death of the prey. Under the direct, consumptive effects of predation, there is a clear winner and loser in which losing comes with the ultimate cost, death (Lima and Dill 1990; Brodie III & Brodie Jr. 1999). Both consumptive and non-consumptive effects of predation come at a cost. Predator defense is often energetically costly, requiring a trade-off balance such that the defense provides a greater benefit than the energetic cost (Lima & Dill 1990; Lima 1998; Baker et al. 2022).

Predators can also indirectly affect prey organisms through increased stress of prey by creating a fearful atmosphere resulting in changes in prey behavior such as decreased foraging and foraging efficiency, shifts in habitat occupancy, and increased stress hormones (Werner et al. 1983; Lima & Dill 1990; Lima 1998; Preisser et al. 2005; Baker et al. 2022; Balaban-Feld et al. 2022; Groves et al. 2022; Ducsai et al. 2023; Gu et al. 2023;). Such non-consumptive effects of predation can play a large role in how prey interact with their environment. A fearful landscape may increase investment in anti-predator responses (Lima & Dill 1990; Lima 1998; Brodie III & Brodie Jr. 1999; Preisser et al. 2005; Billman et al. 2011; Balaban-Feld et al. 2022; Ducsai et al. 2023). Reproduction can be negatively affected for prey if the risk of predation either limits forage ability or results in energetic resources being used for predator deterrents (arms), by stress decreasing

fecundity with either poor quality eggs produced (low viability), skipped spawning, or decreased egg production as compared to times without predation stress (Magurran & Seghers 1994; Billman et al. 2011; Latli et al. 2017). The "arms race" for prey describes the adaptation of ways to defend against predation, which can come at high energetic costs (Brodie III & Brodie Jr. 1999; Harper 2006; Bom et al. 2018). However, nothing has a higher energetic cost to prey than direct consumption by a predator.

Predator recognition followed by an appropriate predator avoidance response are critical to survival. Predator avoidance measures often include increased vigilance and scanning for predators, which results in less time spent actively foraging such that total consumption of food items may decrease (Lima & Dill 1990; Lima 1998; Preisser et al. 2005; Billman et al. 2011; Baker et al. 2022; Balaban-Feld et al. 2022; Ducsai et al. 2023). This may result in avoidance of prime foraging grounds if predators are detected either visually or olfactory. Prey species may change behavior to avoid predation when a predator odorant is sensed (Wisenden et al. 2004; Nelson et al. 2013; Wisenden 2019). This can be due to the release of schreckstoff, the chemical odorant "alarm cue" released by epithelial cells when tissue is injured, or through pheromone odorants released from the predator (in fecal matter, tissue, etc.) (Brown et al. 1995; Magurran et al. 1996; Mathis et al. 1996; Brown et al. 2003; Wisenden et al. 2004; Ferrari et al. 2010; Wisenden 2019).

Conspecific alarm cue has been shown to cause behavioral shifts including shoaling behavior, increased vigilance, decreases in activity level, hiding, and other evasive tactics upon exposure (Heczko and Seghers 1981; Waldman 1982; Lawrence and Smith 1989; Chivers and Smith 1993; Houtman and Dill 1994; Chivers and Smith 1998). Behavioral plasticity under conditions of predation risk may increase survival of young fish during their early life history, when they are often the most vulnerable. Berejikian et al. (1999) demonstrated the ability of young, hatchery-reared Chinook Salmon to exhibit a learned antipredator behavior upon exposure of conspecific alarm cue associated with a predator cue. Subsequently, learned predator recognition was also demonstrated by juvenile Rainbow Trout upon in a similar fashion (Brown and Smith 1998). A fish must possess the chemical alarm substance if a response is to occur. While this substance is rather ubiquitous in ostariophysans, it is not universal (Smith 1992; Wisenden et al. 2004). Green Swordtail *Xiphophorus helleri*, for example, lack injury-induced alarm cue (Mathis and Smith 1993). Recent studies have demonstrated the presence of alarm cue in Rainbow Trout *Oncorhynchus mykiss* and Chinook Salmon *Oncorhynchus tshawytscha* (Brown and Smith 1997, Berejikian et al. 1999) however, it is unknown if Grayling possess or recognize conspecific alarm cue.

Understanding the potential impact of predation, therefore, is a critical step in early stages of reintroduction. Many species of salmonids are known to be piscivorous. For example, Fausch and White (1981) found predation likely played a role in declines of Brook Trout populations in Michigan. This was consistent with the findings of Alexander (1977) in which Brown Trout were found to heavily prey upon Brook Trout in the North Branch of the Au Sable River, Michigan. Alexander (1977) found the diet composition of large Brown Trout consisted of 61% small Brook Trout during the summer season and 42% of the diet during winter. Brown Trout and Pacific salmonids have been found to have negative impacts on Brook Trout populations in Michigan rivers (Zorn et al. 2020).

Historically, the only native stream salmonids in Michigan were Grayling and Brook Trout *Salvelinus fontinalis*. Overlap in distribution was thought to be minimal as Grayling were dominant in the northern lower Peninsula and Brook Trout dominant to the upper Peninsula of

Michigan (Vincent 1962), but the records are ambiguous and conflicting regarding potential populations of Brook Trout in the lower Peninsula (Strang 1855; Hubbard 1887; Smedley 1938; Vincent 1962; Rozich 1998; Goble et al. 2018). Both natural and anthropogenic range expansion resulted in increased Brook Trout populations in the lower Peninsula during the late 1800s (Mershon 1923; Vincent 1962; Westerman 1974). Rainbow Trout *Oncorhynchus mykiss* were introduced into the Au Sable River in approximately 1876 (Bower 1910). Brown Trout *Salmo trutta* were introduced into the Pere Marquette River in 1884 with broad introductions to other rivers commencing shortly after (Mershon 1923; Vincent 1962; Westerman 1974; Zorn et al. 2018). Introductions of nonnative species are known to be detrimental to native populations and can be a factor limiting the success of reintroduction attempts (Vincent 1962; Meffe 1985; Krueger et al. 1995; Marsh and Douglas 1997; Quist et al. 2004; Clavero et al. 2005; Mueller 2005; Fausch 2007; Zorn and Nuhfer 2007; Arismendi et al. 2009; Zorn et al. 2020).

Grayling are likely to be vulnerable to predation by young salmonids within the tributaries where they will be reintroduced (Kaya 1992). As juvenile fish numerically dominate these systems (Zorn and Nuhfer 2007; Nuhfer et al. 2017; Zorn et al. 2020), it is critical to understand potential predation interactions. Evidence of the consumption rates of fry by young fish is prone to underestimation due to the rapid digestion of such prey items (Trippel and Beamish 1987; Hansel et al. 1988; Scharf et al. 1998; Waraniak 2017). In addition to the small size of newly emerged Grayling (7-15 mm) they are poor swimmers for the first 14 days post-hatch which may further increase their vulnerability (Nelson 1954). There does not appear to be previous research regarding the vulnerability of Grayling fry to predation by Brook and Brown trout. This study aimed to fill this gap by examining the predatory interactions between young (age-1) Brook and Brown trout, and newly emerged Grayling fry and to determine whether predation differs between the two trout

species. Additionally, I explore if conspecific alarm cue alone can result in behavioral changes such as a change in spatial distribution.

Methods

Predation Trials

Fish Rearing and Field Collections

Grayling for this study were obtained annually from the Ruth Burnett Sport Fish Hatchery as eyed eggs and reared in the Michigan State University Containment facility: 5,000 in 2018; 10,000 in 2019 and 2021 (no fish in 2020 due to COVID-19 pandemic). Michigan Department of Natural Resource (MI DNR) personnel assisted Alaska Fish and Game (ADF&G) personnel in the collection of eggs from adult Grayling from the Chena River which were held in temporary ponds. I transported eggs from Fairbanks, AK to East Lansing, MI once they reached eyed-egg stage. Rearing methods closely followed protocols set forth by Travis Hyer and Charles Pratt (Ruth Burnett and William Jack Hernandez Sport Fish Hatcheries, Alaska Department of Fish and Game, respectively, personal communications). Eggs were reared in heath-style incubation trays with trout mesh screens. Fish were transferred (ponded) to a rearing tank located below the incubation trays 48 hours after 50% of the eggs have hatched. The rearing tank was a low-flow, shallow depth tank equipped with a fine-mesh screen on a bottom-drawn outflow standpipe. The ponding time of 48 hours was selected as it represented a hybridization of the rearing techniques used at both Alaska hatchery facilities. Flow rate and depth of water was increased corresponding with fry growth and development and based upon the recommendations of Travis Hyer and Charles Pratt, ADF&G (personal communications).

A 24hr Artemia solution drip was immediately started upon ponding with newly hatched alevin keying in on the feeding drip within 24 hours (personal observation). Six additional feeding lines

further distribute the Artemia solution and decrease localized competition for food. Each of the additional lines were operated by dosing pumps in which set volumes (50 mL) of solution were administered every 10 minutes from a single dosing line. Each line dosed solution once every hour; with six lines to provide additional feed every 10 minutes. Transitioning from Artemia to Bio-Oregon trout mash began 10 days after ponding with full transition from Artemia to 100% trout mash at 14 days post-ponding. Changes in dry feed size and amount fed corresponded to increases in growth of fry and was based upon the recommendations of Bio-Oregon[®] feeding guides for trout and salmon (https://www.bio-oregon.com/assets/FeedingGuidelines.pdf). Dry feed was administered via Eheim[®] automatic fish feeders. Each feeder was programed to dispense a measured amount of food 1-4 times per day, dependent on the daily feeding requirements of the fish. Placement of the feeders as well as timing of food dispension rotated daily. Fresh food was placed in each feeder daily. Feeding amounts were regularly monitored to decrease the likelihood of overconsumption by Grayling as they are prone to feeding beyond satiation to the point of rupture (Kindschi and Barrows 1990; Travis Hyer, personal communication).

Age-1 Brook and Brown trout were collected using backpack electrofishing in 2018, 2019, and 2021 from Sweetwater Creek and Sanborn Creek (Michigan). Both streams are tributaries of the Pere Marquette River and sustain wild populations of resident trout. Sweetwater Creek sustains a plentiful population of Brook Trout and other non-game fish while Sanborn Creek sustains a diverse population of Brown Trout and other migratory salmonids including Rainbow Trout *Oncorhynchus mykiss*, Chinook Salmon *Oncorhynchus tshawytscha* and Coho Salmon *Oncorhynchus kisutch*, as well as other non-game fish. These tributaries are readily sampled in a single day of field work and approximately a 15-minute drive apart. This close proximity lessens the degree of stress on fish during holding and transportation. Fish were immediately brought back to the containment lab at Michigan State University. Brook and Brown trout were held in a divided holding tank with Brook Trout and Brown Trout separated by a fine mesh screen barrier. The holding tank was equipped with enrichment items such as gravel/cobble substrate, freely floating artificial plants, submersed artificial plants, woody debris and covered hiding areas. Predator fish were fed daily in holding tanks using a live blackworm dispenser and thawed bloodworms. The holding tank was cleaned as needed to decrease excess food and/or feces build-up within the substrate of the tank.

In 2018, a total of 15 of each species of predator were collected on 25 May 2018 and allowed to acclimate in the holding tanks at the containment facility until starting predation trials. Predators gradually began to show signs of stress (weight loss, poor fin condition, mortality) resulting in a change in predator collections in 2019 and 2021. Overcrowding in the Brook and Brown trout holding tanks was thought to be a contributor to stress, in addition to length of holding time. Predator collections occurred one day prior to beginning predation trials and on three occasions during 2019 and 2021 in which only five individuals of each species were collected at a time. This allowed for lower densities in holding tanks and short overall lengths of time in captivity. Brook and Brown trout appeared to exhibit less signs of stress by following the updated collection and holding techniques. This also allowed for the capture of natural growth in predators seen in the natural environment; Fausch and White (1986) found young Brook and Brown trout tend to grow at a slower rate in laboratory conditions than conspecifics in the field.

Experimental Stream Channels

Experimental stream channels were constructed utilizing fiberglass tanks that were 0.25 m (10.00 in) deep, 0.61 m (2.00 ft) wide, and 3.05 m (10.00 ft) long (Figure 6). Three replicate experimental stream channels were constructed. Streambeds consisted of varying diameters of

substrate, woody debris, artificial vegetation, and artificial undercut bank structures. Varying diameters of "river rock", gravel, and pebbles were used to build a sinuous stream channel within the tank. Substrate diameter generally decreased from upstream (largest material) to downstream (finest material). Two "point bars" were constructed with one in the upstream reach and one midchannel. A deeper downstream region was maintained in the lower third of the stream, nearest the outflow. Air stones were integrated into the substrate to oxygenate the water within the stream channels and create moderate turbulence. Each stream had a 60 cm tall netting lined with camouflage fabric to prevent any fish from potentially jumping out of the streams and to further isolate streams from each other. A full spectrum aquarium grade LED light strip filtered by a semitransparent section of plexiglass to decrease the light intensity and with artificial leafy vining underneath to mimic tree canopy was installed above each stream. Streams were covered with black-out fabric lined with camouflage mesh fabric to isolate the streams from the rest of the laboratory area. Cameras were installed in both the inflow and outflow areas of the streams and angled to allow 100% visual coverage when viewed on the live-stream monitor and in recordings. **Predation** Trials

Predation trials were conducted from 26 Jun – 09 Aug 2018, 19 Jun – 30 Jul 2019, and 26 Jun – 03 Aug 2021. These dates provided age ranges for the Grayling of 19 - 63 days old in 2018, 21 - 58 days old in 2019, and 22 to 60 days old in 2021. These ages were separated into fry ageclasses with age-class one ranging from 19 - 36 days, age-class two ranging from 38 - 49 days, and age-class three ranging from 50 - 63 days. All trials were recorded remotely using a camera system installed above experimental streams. Cameras were mounted to ensure full view of the stream system. The literature provided little insight into detailed options previously used in the design of similar predation experiments. As such, the overall experimental design was adaptive in nature as described below.

2018 Predation Trials

The preliminary experimental season of 2018 incorporated multiple treatments using an adaptive design. A total of 83 predation trials were conducted from 26 Jun – 09 Aug 2018. I varied the duration of trials (one or two hours), the number of predators per experimental stream channel (one, two, or three), and light conditions (light or dark) in order to determine how these choices affected the outcome of predation trials and to "fine tune" the experimental set-up for the subsequent years (Table A 1). For example, I wanted to avoid the situation where all Grayling were consumed before the end of the trial as this would artificially truncate predation rates, hence the exploration of one- and two-hour trials.

Ten of the predation trials were control trials in which 20 Grayling fry were added to an artificial stream with no predator present and collected at the end of the trial (one- or two- hour trial length). This was performed to ensure that all Grayling fry were able to be consistently recaptured as to avoid artificially increasing apparent mortality.

For each experimental trial, predator fish were acclimated to the stream for a minimum of 8 hours and fed thawed frozen bloodworms and live blackworms. After predator acclimation, 20 Grayling fry were randomly selected from the holding tank and added to the stream in various locations. No human presence occurred during trials by closing experimental stream isolation blackout curtains. At the end of the trial, predator fish were removed from the stream and placed in the predator holding tanks, surviving Grayling fry were recaptured, enumerated, and placed back into the Grayling rearing tank. The stream channel was checked repeatedly for a total of two hours as needed to ensure all surviving Grayling were recaptured. In the event a deceased Grayling

fry was found in stream at the end of the trial with an injury likely resulting from a predation attempt, it was counted as a mortality due to predation. The total mortalities were calculated as: $f_a - f_b = M$, where f_a represents the number of Grayling added at the start of the trial, f_b represents the number of Grayling collected at the end of the trial, and *M* represents mortality due to predation.

At the end of the 2018 predation trials, it was determined that the use of multiple predators per trial may confound the results due to the potential for intraspecific competition between predators within the trial. Thus, it was determined that future trials would utilize a single predator per experimental stream per trial. This resulted in a predator density of 0.0625/ft² which is within the range documented in Michigan streams: 0.0042/ft², 0.0001/ft², 0.0823/ft², mean, minimum, maximum, respectively (Troy Zorn, personal communication). Acclimation time provided to the predators limited the number of trials that were performed, and it was determined that a one-hour acclimation time was sufficient for subsequent years.

2019 and 2021 Predation Trials

Refinements to protocols for the 2019 and 2021 experimental seasons included the updated predator collection protocols as described previously to decrease predator stress, the number of predators in each experimental stream was limited to one, predators were acclimated for one-hour prior to the start of the trials, number of Grayling prey was either 20 or 40, and additional trial lengths were included.

From 19 Jun – 30 Jul 2019, a total of 133 predation trials were performed (Table A 2). The following conditions were varied: duration of trial (1-, 2-, 3-, 4-, and 24-hours); number of prey (n = 20 or 40) and light conditions (light or dark). From 26 Jun – 03 Aug 2021, a total of 106 predation trials were performed under the following conditions: duration of trial (2- or 24-hours); number of

Grayling prey (n = 20), and light conditions were either light (2-hour trials) or diel (24-hour trials) (Table A 3). Lights were on automatic timers for 24-hour trials that followed the natural diel light cycle for East Lansing, Michigan. Initial exploration of the data from 2018 and 2019 determined that light was not a significant factor in predation, therefore, all 2-hour trials in 2021 were performed in light conditions while 24-hour trials remained on a natural diel light cycle. Trials were conducted following the fry age-classes categories described previously. Within each of the fry age-classes, a minimum of two replicates were performed of all trial types with naïve Grayling and a randomly selected age-1 predator. Trials were recorded remotely using a camera system installed above experimental streams. Cameras were mounted to ensure full view of the stream system.

For each trial, predator fish were acclimated to the stream for 1 hour. After predator acclimation, Grayling fry were randomly netted from the holding tank, photographed, and added to the stream in a random location. No human presence occurred during trials. At the end of the trial, predator fish were removed from the stream, photographed, and placed in the predator holding tanks. Surviving Grayling fry were recaptured, enumerated, photographed, and placed back into an isolation area of the Grayling rearing tank to separate naïve fish from those used in a predation trial. Grayling were only used once in predation trials; thus, all individuals were naïve to predators. The stream channel was checked repeatedly for a total of two hours as needed to ensure all surviving Grayling were recaptured. In the event a deceased Grayling fry was found in stream at the end of the trial with an injury likely resulting from a predation attempt, it was counted as a mortality due to predation. The total mortalities were calculated as: $f_a - f_b = M$, where f_a represents the number of Grayling added at the start of the trial, f_b represents the number of Grayling collected at the end of the trial, and M represents mortality due to predation.

Statistical Analysis

Data included for final analysis was survival outcome of each Grayling fry (1 = survive; 0 = dead); trial length (1, 2, 3, 4); age in days of Grayling fry; year; and fry age-class (a, b, c). As the 24-hour trials could be seen as having different design, all 24-hour trials were omitted from the final analysis. Predation data were summarized by predator species and fry age-class and by trial length and fry age-class to obtain sample size, minimum and maximum predation per trial type, mean predation per trial type, and standard error of the mean (Table 3, Table 8). A generalized linear model with binomial distribution and a logit link function was used to examine if species (Brook Trout or Brown Trout), time (represented by fry age-class category), or trial length (1-, 2-, 3-, 4-hours) affected survival of Grayling with an a-priori α = 0.05. An iterative, backwards stepwise process was used to identify the most parsimonious model (Venables and Ripley 2002). This type of stepwise approach to linear modeling removes variables whose elimination results in small decreases in the test measure in a sequential manner (Finos et al. 2010). The full model included predator species type (*Ps*) as the main effect with time (*Ga*, fry age-class) and trial length (*Tr*) as covariates:

$$S_{ijt} = Ps_i + Ga_j + Tr_t + Ps * Ga * Tr + e_{ijt}$$

where i = predator species type; j = fry age-class; and t = trial length. The stepwise process indicated the best model omitted trial length and species as the model with the most parsimony. Thus, the final model included fry age-class (*Ga*), a surrogate for time, as the main effect:

$$S_j = Ga_j + e_j$$

where j = fry age-class. From the final model, odds of survival was calculated as: *Odds of survival* = e^{ce} , where the *ce* is the coefficient estimate for the predictor in the model, fry age-class B and fry age-class C.

Biomass of Grayling consumed by predator species was calculated for the 2019 and 2021 predation trials. A length-weight regression was performed on fish with known length (mm) and weight (g) from the 2018 cohort of Grayling. The slope (β_1) and intercept (β_0) for the regression were then applied to the 2019 and 2021 fish to obtain the weight (g) as only the length (mm) was known. Fish for each trial in 2019 and 2021 were photographed and total length of each Grayling fry was measured using ImageJ® software (Schneider et al. 2012). Total length of the 2019 and 2021 fish was log-transformed and the log weight calculated using the formula: $lnW_i = \beta_0 + \beta_0$ $\beta_1 ln L_i$, where W is the weight and L is the length of each fish (i). The weights were backtransformed using the power function to obtain weight in grams using the formula: $W_i = e^{\beta_0} L_i^{\beta_1}$. The mean weight for each fry age-class (A, B, and C) was calculated for all fish in the trials. Biomass was calculated using the formula: $B_j = \overline{X}_{W_j} * \overline{X}_{C_{ij}}$, where \overline{X}_W represents the mean weight, \overline{X}_{C} the mean consumption for the predator species (i) for each fry age-class (j). The standard error for biomass was calculated using the formula: SE = $\sqrt{\overline{W}_{ij}^2 S E_{\overline{C}_{ij}}^2 + \overline{C}_{ij}^2 S E_{\overline{W}_{ij}}^2 + S E_{\overline{W}_{ij}}^2 S E_{\overline{C}_{ij}}^2}$, where \overline{W} is the mean and $S E_{\overline{W}}$ the standard error of Grayling weights, \overline{C} is the mean and $SE_{\overline{C}}$ the standard error of consumption, for each predator species (i) and fry age-class (j). A multiple linear regression model was used to examine if predator species (Brook Trout or Brown Trout) or time (represented by fry age-class category) affected the amount of Grayling biomass consumed (B) with an a-priori $\alpha = 0.05$. The model included predator species type (Ps) as the main effect with time (Ga, fry age-class) as an additive effect:

$$B_{ij} = Ps_i + Ga_j + e_{ij}$$

Where i = predator species type and j = fry age-class. A simplified linear regression model was used to examine if time (represented by fry age-class category) affected the amount of Grayling

biomass consumed (*B*) with an a-priori $\alpha = 0.05$. The model included time (*Ga*, fry age-class) as the main effect:

$$B_{ij} = Ga_j + e_{ij}$$

Where i = predator species type and j = fry age-class.

Alarm Cue

The methodology of the alarm cue trials closely follows that of Wisenden et al. (2004). Grayling used for alarm cue trials were from the same population reared for predation trials (see previous rearing methods). Grayling used in alarm cue trials were naïve to predators. Trials occurred from 29 Jun 2019 through 10 Jul 2019; Grayling were 31 – 42 days old. The test aquarium was 37 L with a 5 cm x 5 cm grid on the front (long side); all remaining sides were blocked with opaque white board. The top was covered with a clear acrylic sheet. An air-powered sponge filter was installed in the rear, left end (viewing from the front) of the test aquarium and the bottom was covered in light, natural colored gravel. A water line was installed into the lift tube of the sponge filter to distribute the treatment waters (Figure 10). Treatment water was either distilled water (control) or alarm cue rinsate. The alarm cue rinsate was prepared immediately before each trial to ensure it was metabolically viable. Fish were humanely euthanized using cervical dislocation in accordance with Michigan State University's IACUC guidelines, and the right flank tissue lacerated 5 times using a sterile small scalpel. The post-mortem lacerated area was flushed with 10 mL distilled water with the rinsate collected in a 10 mL glass beaker.

At the start of each trial, pre-chilled trace mineral and nutrient supplemented reverse osmosis water (RO water with Seachem Flourish[®]; ROFL) was added to the experimental aquarium. A random subset of 10 naïve Grayling fry (not exposed to predation trials) were collected from the population of Grayling in the rearing tank and added to the experimental
aquarium. Trials were recorded using a GoPro HERO Black 7[®]. Recording started immediately upon adding Grayling fry to the experimental aquarium. An initial acclimation period of 1-minute was followed by a 5-minute monitoring period. At minute 5, the olfactory stimulus (either 10 mL distilled water or 10 mL alarm cue rinsate) was slowly infused via the water line followed by a 100 mL flush of ROFM water. Immediately upon flushing the water line, monitoring commenced. At minute 11, the trial ended. A total of 14 trials were conducted: 7 control (distilled water) and 7 alarm cue trials all consisting of 10 fish each. At the end of each trial, the water was drained from the experimental aquarium and rinsed with RO water.

Statistical analysis

The spatial position of each fish was recorded at minutes 2, 3, 6, and 7. Minutes 2 and 3 were representative of the pre-olfactory stimulus and minutes 6 and 7 representative of postolfactory stimulus. Position was recorded as occupancy coordinates (horizontal x, vertical y) corresponding to spatial positioning within the experimental aquarium grid system. In the event that an individual transected a line in the grid, the grid cell in which the head of the fish was contained was assigned to that fish. X values represented the horizontal position from 1 - 10 with 1 being closest to the sponge filter (left side of aquarium). Y values represented the vertical position from 1 - 6 with 1 being at the bottom and 6 nearest the top of the aquarium. The mean X and Y values for all fish within a trial at each time step analyzed were recorded as the mean horizontal score (*hz*) and mean vertical score (*vt*) for the trial, pre- and post-treatment. The occupancy score (O_{pre} and O_{post}) was calculated using the mean horizontal and vertical scores as follows:

$$O_{pre} = \frac{((hz_2 + vt_2) + (hz_3 + vt_3))}{2}$$

$$O_{post} = \frac{((hz_6 + vt_6) + (hz_7 + vt_7))}{2}$$

Occupancy scores, O_{pre} and O_{post} , were analyzed for both the control samples and alarm cue treatment samples. A one-way ANOVA was used to compare the means of the treatment groups (distilled, alarm cue) using the aov function in RStudio to determine whether the means for O_{pre} and O_{post} were significantly different. Spatial positioning for each fish in each trial was entered into surrogate experimental tank figures for visualization using the package ggplot2 in RStudio. Data points were weighted based on the number of fish within the square of the grid such that the more fish within a specific grid square would equate to a larger dot within the figure.

Results

Predation Trials

The full generalized linear model had a significant intercept (p < 0.001), however neither the main effect, the covariates, nor the interactions were found to have a significant effect on Grayling predation (Table 4). The full model had weak explanatory power ($R^2 = 0.017$). Iterative backwards stepwise analysis of the full model resulted in a final model that included fry age-class as the sole predictor as the model with most parsimony (Table 5). The final model also had a significant intercept (p < 0.001) with weak explanatory power ($R^2 = 0.013$, Table 6). Predation differed between fry age-class C as compared to fry age-class A (p < 0.001, Table 6). Predation did not differ between between fry age-class A and B (p = 0.226, Table 6). Grayling in fry ageclass B had 1.141 times better odds of survival as compared to those in fry age-class A and Grayling in fry age-class C had 1.963 times better odds of survival as compared to those in fry age-class A.

Mean predation by Brook Trout was 5.67, 2.54, and 1.31 (Table 3) with the mean proportion of Grayling dead 0.13, 0.09, and 0.07 (Table 7), in fry age-classes A, B, and C,

respectively, from apparent predation. Mean predation by Brown Trout was 4.49, 2.65, and 1.27 (Table 3) with the mean proportion of Grayling dead 0.10, 0.12, and 0.05 (Table), in fry ageclasses A, B, and C, respectively, from apparent predation.

The number of Grayling preyed upon per trial decreased over time, as represented by fry age-class (Figure 7). Little difference was seen between predator species with both Brook Trout and Brown Trout preying upon Grayling similarly (Figure 7). Predation decreased over time from mean = 5.67 (fry age-class A) to mean = 1.31 (fry age-class C) for Brook Trout and from mean = 4.49 (fry age-class A) to mean = 1.27 (fry age-class C) for Brown Trout (Table 3). Similar trends occurred when comparing Grayling predation by trial length and fry age-class with the highest predation occurring in fry age-class A (maximum = 19, Table 8). The mean predation also decreased over time for all trial lengths in fry age-class A ranged from 2.76 - 10.12; from 1.25 - 3.53 in fry age-class B; and from 0.63 - 1.56 in fry age-class C (Table 8).

Fry age-class A (19 - 36 days old) had the highest mortality for both Brook Trout (mean = 5.67) and Brown Trout (mean = 4.49) per trial (Table 3). The maximum number of Grayling preyed upon by Brown Trout during any predation trial was 19 and in fry age-class A (Table 3). The maximum number of Grayling preyed upon by Brook Trout during any predation trial was 17 also in fry age-class A (Table 3). The distribution of predation was widest for fry age-class A, ranging from 0 - 19 and the most constrained in fry age-class C, ranging from 0 - 5 (Table 3).

The mean weight of Grayling was 0.149 ± 0.002 , 0.338 ± 0.005 , and 0.805 ± 0.014 g for fry age-classes A, B, and C, respectively (Table 9). The biomass consumed by Brook Trout was 0.85 ± 0.61 , 0.86 ± 0.37 , 1.05 ± 0.18 g for fry age-class A, B, and C, respectively (Table 10, Figure 8). The biomass consumed by Brown Trout was 0.67 ± 0.75 , 0.90 ± 0.43 , 1.02 ± 0.22 g for fry ageclass A, B, and C, respectively (Table 10, Figure 8). The full linear model for Grayling biomass consumed by predator species and fry age-class was not statistically significant ($R^2 = 0.877$, F(3, 2) = 4.742, p = 0.179, Table 11), and neither the main effect, predator species, or the covariate, fry age-class, were found to have a significant effect on Grayling predation (Table 11). Predator species was determined as the least significant factor and dropped from the subsequent simplified linear model. The simple linear model was not statistically significant ($R^2 = 0.826$, F(2, 3) = 7.119, p = 0.073, Table 12), however, the model had a significant intercept (p = 0.001) and the biomass consumed differed between fry age-class C as compared to fry age-class A (p = 0.033, Table 12). The biomass consumed did not differ between between fry age-class A and B (p = 0.204, Table 12).

Alarm Cue

A one-way analysis of variance showed that the effect of the treatment type (distilled, alarm) was significant (F(1, 12) = 11.17, p = 0.006, 95% CI [0.12, 1.00]). A change in spatial distribution occurred in the alarm trials once the alarm cue olfactory stimulus was flushed into the experimental tank (Figure 12) with mean occupancy scores of $O_{pre} = 9.64 \pm 0.283$ and $O_{post} = 6.71 \pm 0.493$ (Table 14). Post-olfactory stimulus spatial distribution was similar to pre-stimulus in the control trials (Figure 11) with mean occupancy scores of $O_{pre} = 9.86 \pm 0.459$ SE and $O_{post} = 9.29 \pm 0.586$ SE (Table 14). Spatial distribution occupancy plots identify fish dispersed throughout the experimental aquarium during the pre-olfactory (O_{pre}) stimulus portion of the trials (Figure 11, Figure 12), with larger occupancy scores corresponding to a larger spatial distributions (Table 13). Occasional shoaling occurred but was limited (Figure 11, AC01; Figure 12, AC24).

Discussion

Both Brook and Brown trout preyed upon young Grayling, with no significant difference in the amount of predation occurring by predator species, neither numerically (number of Grayling consumed) or by biomass (mean grams Grayling consumed) for the full linear regression. There was a difference in biomass consumed in the simple linear model comparing consumption with fry age-class A and C. Vulnerability of Grayling to predation decreased over time with the highest predation occurring with fry age-class A (age = 19 - 36 days) and the lowest occurring with fry age-class C (age = 50 - 63 days) (Figure 7). This may be attributed to ontogenetic changes of Grayling fry with age such as increased musculature and fin development (Figure 9) resulting in potential increased swimming ability and predator avoidance movements. As this development progresses, Grayling fry appear to be better equipped to avoid predation. The decrease in vulnerability over time may also be attributed to the mean size of an individual Grayling increasing over time, thus increasing the overall biomass of an individual prey item (Grayling) corresponding to a lower number of Grayling contributing to predator satiation over time. As biomass of an individual Grayling increases, the predator consumes less individual fish to result in a similar amount biomass of prey over time. This indicates a higher probability of predation occurring at the earliest life-stage, shortly after entering the water column with a gradual decrease over time. This is consistent with Montana reintroduction efforts in which Grayling were held after emerging from RSIs in reintroduction streams until further developed helped increase survival (Kaeding and Boltz 2004).

Fry age-class A was associated with the earliest development stage of Grayling used in this study and had the highest predation in number of Grayling consumed (Table 3, Table 4, Figure 7). This is consistent with previous research finding predation is negatively associated with size of

fish with predation decreasing as size of fish increases, thus growth relates to a lower risk of predation (Jones et al. 2003; Duffy and Beauchamp 2008; Dermond et al. 2019). During this early life stage, Grayling are likely more susceptible to predation due to poor development of fins and musculature affecting swimming and causing a decreased ability to perform burst swimming motions or to take other actions to avoid predation (Figure 9). An innate, primary defense against predation during this stage is likely shoaling behavior as poor muscle and fin development would likely limit mobility. The ability to perform a fast-start escape response, an innate response requiring rapid muscle contraction followed by a strong propulsive tail stroke (Bisazza and Brown 2011), would be unlikely during this period of development. Through the live camera feeds during active predation trials, Grayling were observed exhibiting shoaling behavior, hiding in substrate, and exhibiting rapid movements. The changes in behavior appeared to correspond with changes in ontogeny (Figure 9) with shoaling behavior commonly seen at the end of trials in fry age-class A, rapid response movements with fry age-class B, and combinations of responses including fast response movements and hiding in interstitial spaces of substrate with fry age-class C.

Predation is a major contributor to mortality in juvenile fishes and both predator recognition and escape responses are contributors to predator avoidance. Predation events follow a common sequence: encounter, detection, recognition, approach, and evasion or attack (Brown et al. 2011). Innate responses which are naturally occurring and learned mechanisms can decrease the chance of a lethal predation sequence for prey. Experience can allow prey to fine tune their response to match the magnitude of the stimulus, thus ensuring increased energetic efficiency. An appropriate response from Grayling to a predation event is critical as failure to avoid predation dramatically reduces future fitness of an individual (Lima and Dill 1990).

Juvenile Grayling recognized conspecific alarm cue and changed behavior by decreasing spatial distribution upon olfactory detection of the cue (Table 13, Table 14, Figure 11, Figure 12). The ability to recognize and appropriately respond to conspecific alarm cue has potential to provide an early warning of potential danger such as predation (Wisenden et al. 2004). Innate predator recognition plays a critical role in elicitation of appropriate responses to stimuli. This alarm cue elicits an innate antipredator response that can include increased shoaling, burst swimming, freezescan behavior, increased vigilance, and increased use of cover (Brown et al. 1995). Response to chemical odorants can allow fish to take appropriate behaviors based on situational awareness and cue intensity by changing behavior such that individuals increase vigilance while foraging (Brown et al. 2011). Berejikian et al. (2003) found predator-naïve juvenile Chinook Salmon initiated a fright response when exposed to Northern Pikeminnow odor. Predator feces can also act as a deterrent when the predator has consumed conspecifics and, in some cases, heterospecifics. Fathead Minnows were found to avoid regions in which the feces of Northern Pike who had consumed conspecifics was present (Brown et al. 1995). This, however, can result in behavior shifts in the predator such that the predator does not defecate near their foraging areas (Brown et al. 1995), exemplifying the intricacies of the predator-prey interactions. Efficiency in the recognition of predator odorants is critical to the fitness of the prey species. The prey must react appropriate to the level of stimulus that is present at the current time with appropriate monitoring for changes such that the prey individuals are able to function: foraging, spawning, migratory, rest, and other behaviors. The extent to which conspecific alarm cue recognition and response will benefit young Grayling in natural, lotic systems remains unknown but it will likely aid in predator recognition and response.

A deeper understanding of predation interactions in early Grayling life stages will be useful to management decisions regarding selection of Grayling reintroduction locations which are most likely to be successful and levels of stocking that might be needed to achieve success. Knowledge of potential levels of Grayling predation associated with different sizes and densities of resident trout will help in selection of suitable tributaries based on resident stream populations. Understanding hatch and early life survival rates of Grayling at different predator densities will aid in estimating the number of eggs that might need to be reared in RSIs on a given stream reach to overcome anticipated predation by resident trout and achieve desired densities of young Grayling. This information is critical for successful reintroduction of Grayling in Michigan streams and will further assist managers in their decision making of what streams are best suited to Grayling reintroduction based on existing population dynamics and to understand the potential impacts on the current populations. Considerations of the complex system dynamics occurring within the fish community will further increase the understanding of Grayling reintroduction and provide a platform for the scientific understanding of the consequences of intraguild predation. Young Grayling appear to possess the innate building blocks of predator recognition with appropriate responses. Understanding times at which the population is the most vulnerable can assist in decision making and potential reintroduction success.

TABLES

Table 3: Summary of predation mortalities by fry age-class for each predator species in all single predator trials (trials that included more than one predator have been omitted).

Predator	Fry age-class	Max	Min	Mean	n	S.E.
Brook Trout	А	17	0.00	5.67	67	0.61
Brook Trout	В	12	0.00	2.54	54	0.37
Brook Trout	С	5	0.00	1.31	54	0.18
Brown Trout	А	19	0.00	4.49	45	0.75
Brown Trout	В	15	0.00	2.65	46	0.43
Brown Trout	С	4	0.00	1.27	37	0.22

Table 4: Results of the full generalized linear model analysis of predation. Overall test statistics were χ^2 (11) = 38.727, n = 5041, p = < 0.001, AIC = 3119.382. Significant values ($\alpha < 0.05$) are in bold.

			Z	
Main Effect and Interactions	Estimate	S. E.	value	<i>p</i> value
Intercept	2.043	0.261	7.842	0.000
Brown	0.098	0.392	0.251	0.802
Age-class B	0.484	0.453	1.067	0.286
Age-class C	0.654	0.506	1.291	0.197
Trial length	-0.030	0.098	-0.307	0.759
Brown:Age-class B	-1.125	0.649	-1.734	0.083
Brown:Age-class C	-0.412	0.784	-0.526	0.599
Brown:Trial length	0.042	0.146	0.288	0.773
Age-class B:Trial length	-0.067	0.187	-0.357	0.721
Age-class C:Trial length	0.012	0.213	0.055	0.956
Brown:Age-class B:Trial length	0.322	0.271	1.187	0.235
Brown:Age-class C:Trial length	0.197	0.329	0.599	0.549

Table 5: Comparison table for all predation models using iterative backwards step-wise regression analysis with AIC values. The response variable was binomial with the outcome of a Grayling either alive or dead. Species = predator species; age = fry age-class; duration = trial length; K is the number of parameters in the model.

Predictor Variables				Relative	AICc
(Response = Predation)	Κ	AIC _c	ΔAIC_{c}	Likelihood	weight
Age	3	3110.74	0.00	1.00	0.39
Species + Age + Species:Age	6	3111.69	0.95	0.62	0.24
Species + Age	4	3112.61	1.87	0.39	0.15
Species + Age + Duration + Species: Age	7	3113.43	2.69	0.26	0.10
Species + Age + Duration + Species:Age + Species:Duration	8	3113.57	2.83	0.24	0.09
Species + Age + Duration + Species:Age + Species:Duration + Age:Duration	10	3116.94	6.20	0.04	0.02
Species * Age * Duration	12	3119.44	8.70	0.01	0.00
Null model	1	3136.11	25.37	0.00	0.00
Species	2	3138.11	27.37	0.00	0.00

Table 6: Results of the generalized linear model analysis of predation following stepwise deletion of non-significant factors. Overall test statistics were χ^2 (2) = 29.375, n = 5041, *p* = < 0.001, AIC = 3110.735. Significant values ($\alpha < 0.05$) are in bold.

Main Effect and Interactions	Estimate	S . E.	z value	<i>p</i> value
Intercept	2.060	0.074	27.838	< 0.001
Age-class B	0.131	0.109	1.202	0.226
Age-class C	0.675	0.132	5.114	< 0.001

Predator	Fry		Mean Proportion	
Species	Age-Class	n Trials	Grayling Dead	S.E.
Brook Trout	А	41	0.13	0.02
Brook Trout	В	41	0.09	0.02
Brook Trout	С	45	0.07	0.01
Brown Trout	А	35	0.10	0.02
Brown Trout	В	38	0.12	0.03
Brown Trout	С	28	0.05	0.01

Table 7: Summary of the mean proportion Grayling dead by predator species in each fry ageclass.

Table 8: Summary of predation mortalities by fry age-class for each trial length in all single

Trial length	Em aga alaga	Mox	Min	Moon	n triols	SЕ
(hours)	Fly age-class	Iviax	IVIIII	Ivicali	II UTAIS	5.E.
1	А	9	0	2.76	17	0.72
2	А	19	0	5.26	54	0.78
3	А	7	1	3.25	8	0.70
4	А	5	0	3.31	16	0.46
24	А	17	4	10.12	17	0.93
1	В	6	0	1.25	8	0.77
2	В	15	0	2.78	59	0.38
3	В	7	0	1.88	8	0.81
4	В	4	0	1.25	8	0.62
24	В	11	0	3.53	17	0.73
1	С	2	0	0.75	8	0.37
2	С	5	0	1.39	49	0.19
3	С	4	0	1.38	8	0.63
4	С	1	0	0.63	8	0.18
24	С	4	0	1.56	18	0.30

predator trials (trials that included more than one predator have been omitted).

Fry Age-Class	n Grayling	Mean weight (g)	S.E.
А	1699	0.149	0.002
В	1279	0.338	0.005
С	960	0.805	0.014

Table 9: Summary of weight data for Grayling fry by age-class.

Table 10: Summary of the biomass consumed by predator species in each fry age-class.

	Fry	Biomass	
Predator Species	Age-Class	Consumed	S.E.
Brook Trout	А	0.85	0.90
Brook Trout	В	0.86	0.13
Brook Trout	С	1.05	0.15
Brown Trout	А	0.67	0.11
Brown Trout	В	0.90	0.15
Brown Trout	С	1.02	0.18

Table 11: Results of the full linear model analysis of predation examining biomass consumed and the effect of predator species and fry-age class. Overall test statistics were $R^2 = 0.877$, F(3, 2) =4.742, p = 0.179. Significant values ($\alpha < 0.05$) are in bold.

Main Effect and Interactions	Estimate	S. E.	<i>t</i> value	<i>p</i> value
Intercept	0.786	0.063	12.520	0.006
Species	-0.057	0.063	-0.908	0.460
Age-class B	0.121	0.077	1.571	0.257
Age-class C	0.281	0.077	3.649	0.068

Table 12: Results of the simple linear model analysis of predation examining biomass consumed and the effect of fry-age class. Overall test statistics were R2 = 0.826, F(2, 3) = 7.119, p = 0.073. Significant values ($\alpha < 0.05$) are in bold.

Main Effect and Interactions	Estimate	S . E.	<i>t</i> value	<i>p</i> value
Intercept	0.758	0.053	14.362	0.001
Age-class B	0.121	0.075	1.619	0.204
Age-class C	0.281	0.075	3.761	0.033

Table 13: Alarm cue trial mean horizontal (hz), vertical (vt), and occupancy (O_{pre} , O_{post}) scores. Mean horizontal position (hz) and mean vertical positions (vt) were calculated at time steps 2, 3, 6, and 7, based on individual fish positions for each trial.

	Pre- olfactory stimulus					Pos	st- ol:	factor	y stir	nulus
Treatment	hz ₂	vt ₂	hz ₃	vt ₃	Opre	hz ₆	vt ₆	hz7	vt7	Opost
Control	5	6	5	4	10.00	4	3	3	3	6.50
Control	7	2	6	3	9.00	4	4	5	4	8.50
Control	6	4	5	3	9.00	7	4	7	4	11.00
Control	4	5	4	3	8.00	6	3	5	3	8.50
Control	7	5	5	5	11.00	6	5	6	4	10.50
Control	8	4	7	3	11.00	7	4	6	3	10.00
Control	8	4	7	3	11.00	7	4	6	3	10.00
Alarm	7	3	5	4	9.50	4	3	3	2	6.00
Alarm	5	5	6	5	10.50	5	4	7	3	9.50
Alarm	6	5	5	5	10.50	5	3	3	2	6.50
Alarm	5	2	7	3	8.50	3	3	4	2	6.00
Alarm	6	4	5	5	10.00	4	2	5	3	7.00
Alarm	6	4	5	4	9.50	3	3	4	3	6.50
Alarm	6	4	4	4	9.00	4	1	4	2	5.50

Table 14: Pre- and post-stimulus summary statistics for occupancy scores in alarm cue trials by

treatment type.

	Control			_	Alarm C	Cue
Group	n	Mean	SE	n	Mean	SE
Pre-stimulus (Opre)	7	9.857	0.459	7	9.643	0.283
Post-stimulus (<i>O</i> _{post})	7	9.286	0.586	7	6.714	0.498

FIGURES



Figure 6: Experimental stream channel used in predation and competition trials. In top image, water flow is from right to left; in bottom image, water is flowing from top of image to bottom. Each stream channel was surrounded by fine mesh netting to prevent fish from potentially escaping; camouflage fabric was added over the white mesh netting and leaves under lighting to reduce glare on water surface at the beginning of the 2019 experimental season (bottom image).



Figure 7: Mean \pm SE proportion of Grayling dead by fry age-class and predator species in predation trials. Pink circles and solid line represents mortalities associated with Brook Trout; blue squares and dashed line represents mortalities associated with Brown Trout.



Figure 8: Mean \pm SE biomass of Grayling consumed by fry age-class and predator species in predation trials. Pink circles and the solid line represents biomass consumed by Brook Trout; blue squares and dashed line represents biomass consumed by Brown Trout.



Figure 9: Scaled photographs of Grayling fry representative of fry age-class A (19-36 days, panel A), fry age-class B (38-49 days, panel B), and fry age-class C (50-63 days, panel C).



Figure 10: Schematic of alarm cue experimental aquarium. Grid cells were 5cm x 5cm across the front, long side of the aquarium; uplifting sponge filter is represented by the grey shape in the left corner of the aquaria; the secondary water line used to infuse the olfactory stimuli is represented by the black dashed line. The sides and back of the aquarium were covered with opaque film.



Figure 11: Spatial distribution of young Grayling during time steps of Control trials (distilled water as olfactory stimulus). Each trial (A - G) consists of a column of four observed time steps represented by figure cells (aquaria): (a) two minute, (b) three minute, (c) six minute, (d) seven

Figure 11 (cont'd)

minute. A vertical position (y-axis) of 6 is nearest the top of the aquaria; a horizontal position (x-axis) of 10 is nearest the right side. Blue dots represent time steps before the infusion of olfactory stimulus; pink represents time steps after infusion. The size of the dot corresponds to the number of fish observed within an individual grid cell (smallest dot = 1 fish). Ten fish were present in each trial/time step.



Figure 12: Spatial distribution of young Grayling during time steps of Alarm trials (conspecific alarm cue as olfactory stimulus). Each trial (A - G) consists of a column of four observed time steps represented by a figure cells (aquaria): (a) two minute, (b) three minute, (c) six minute, (d) seven minute. A vertical position (y-axis) of 6 is nearest the top of the aquaria; a horizontal position

Figure 12 (cont'd)

(x-axis) of 10 is nearest the right side. Blue dots represent time steps before the infusion of olfactory stimulus; pink represents time steps after infusion. The size of the dot corresponds to the number of fish observed within an individual grid cell (smallest dot = 1 fish). 10 fish were present in each trial/time step.

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APPENDIX A: CHAPTER 2

Predator	Fry	Trial	Light	Number	Initial number	
	age-class	length	condition	predator(s)	prey	11
Brook Trout	А	1	light	3	20	5
Brook Trout	А	2	light	1	20	6
Brook Trout	А	2	light	2	20	6
Brook Trout	А	2	light	3	20	6
Brook Trout	В	2	light	1	20	8
Brook Trout	В	2	dark	1	20	4
Brook Trout	В	2	dark	2	20	2
Brook Trout	В	2	dark	3	20	2
Brook Trout	С	2	light	1	20	6
Brook Trout	С	2	dark	1	20	14
Brown Trout	А	2	light	2	20	2
Brown Trout	В	2	light	1	20	6
Brown Trout	В	2	dark	1	20	3
Brown Trout	С	2	dark	1	20	3
Control	А	1	light	0	20	3
Control	А	2	light	0	20	7
				Total	number of trials	83

Table A 1: Description of 2018 predation trial types and sample size for each type.

Table A 2: Description of 2019 predation trial types and sample size for each type. Diel light condition represents a 24-hour time period in which fish experienced both light and darkness during the duration of the trial that was matched with current geographical conditions.

	Fry	Trial	Light	Number	Initial	
Predator	age-class	length	condition	predator(s)	number prey	n
Brook Trout	А	1	light	1	20	2
Brook Trout	А	1	dark	1	20	4
Brook Trout	А	2	light	1	20	2
Brook Trout	А	2	light	1	40	2
Brook Trout	А	2	dark	1	20	2
Brook Trout	А	2	dark	1	40	2
Brook Trout	А	3	light	1	20	2
Brook Trout	А	3	dark	1	20	2
Brook Trout	А	4	light	1	20	2
Brook Trout	А	4	light	1	40	2
Brook Trout	А	4	dark	1	20	2
Brook Trout	А	4	dark	1	40	2
Brook Trout	В	1	light	1	20	2
Brook Trout	В	1	dark	1	20	2
Brook Trout	В	2	light	1	20	2
Brook Trout	В	2	light	1	40	2
Brook Trout	В	2	dark	1	20	2
Brook Trout	В	2	dark	1	40	2
Brook Trout	В	3	light	1	20	2
Brook Trout	В	3	dark	1	20	2
Brook Trout	В	4	light	1	20	2
Brook Trout	В	4	dark	1	20	2
Brook Trout	В	24	diel	1	20	2
Brook Trout	С	1	light	1	20	2

Table A 2 (cont'd)

Brook Trout	С	1	dark	1	20	2
Brook Trout	С	2	light	1	20	2
Brook Trout	С	2	dark	1	20	2
Brook Trout	С	3	light	1	20	2
Brook Trout	С	3	dark	1	20	2
Brook Trout	С	4	light	1	20	2
Brook Trout	С	4	dark	1	20	2
Brook Trout	С	24	diel	1	20	4
Brown Trout	А	1	light	1	20	2
Brown Trout	А	1	dark	1	20	4
Brown Trout	А	2	light	1	20	2
Brown Trout	А	2	light	1	40	2
Brown Trout	А	2	dark	1	20	2
Brown Trout	А	2	dark	1	40	2
Brown Trout	А	3	light	1	20	2
Brown Trout	А	3	dark	1	20	2
Brown Trout	А	4	light	1	20	2
Brown Trout	А	4	light	1	40	2
Brown Trout	А	4	dark	1	20	2
Brown Trout	А	4	dark	1	40	2
Brown Trout	В	1	light	1	20	2
Brown Trout	В	1	dark	1	20	2
Brown Trout	В	2	light	1	20	2
Brown Trout	В	2	light	1	40	2
Brown Trout	В	2	dark	1	20	2
Brown Trout	В	2	dark	1	40	2
Brown Trout	В	3	light	1	20	2
Brown Trout	В	3	dark	1	20	2
Brown Trout	В	4	light	1	20	2

Table A 2 (cont'	d)					
Brown Trout	В	4	dark	1	20	2
Brown Trout	С	1	light	1	20	2
Brown Trout	С	1	dark	1	20	2
Brown Trout	С	2	light	1	20	2
Brown Trout	С	2	dark	1	20	2
Brown Trout	С	3	light	1	20	2
Brown Trout	С	3	dark	1	20	2
Brown Trout	С	4	light	1	20	2
Brown Trout	С	4	dark	1	20	2
Brown Trout	С	24	diel	1	20	3
				Total n	umber of trials	133

Table A 3: Description of 2021 predation trial types and sample size for each type. Diel light condition represents a 24-hour time period in which fish experienced both light and darkness during the duration of the trial that was matched with current geographical conditions.

Predator	Fry	Trial	Light	Number	Initial	n	
	age-class	length	condition	predator(s)	number prey		
Brook Trout	А	2	light	1	20	9	
Brook Trout	А	24	diel	1	20	9	
Brook Trout	В	2	light	1	20	9	
Brook Trout	В	24	diel	1	20	9	
Brook Trout	С	2	light	1	20	9	
Brook Trout	С	24	diel	1	20	9	
Brown Trout	А	2	light	1	20	9	
Brown Trout	А	24	diel	1	20	8	
Brown Trout	В	2	light	1	20	9	
Brown Trout	В	24	diel	1	20	8	
Brown Trout	С	2	light	1	20	9	
Brown Trout	С	24	diel	1	20	9	
				Total n	Total number of trials		

CHAPTER 3: COMPETITION

Introduction

Native salmonid populations have been declining globally due to climate change, introduction of non-native species, habitat destruction and other anthropogenic effects (Brown 1943; Vincent 1962; Fausch and White 1981; Waters 1983; Fausch and Cummings 1985; Meffe 1985; Fausch and White 1986; Moyle et al. 1986; Miller et al. 1989; Nuhfer 1992; Wang and White 1994; Moyle and Light 1996; Nakano et al. 1998; Vander Zanden et al. 1999; Quist and Hubert 2004; Clavero and García-Berthou 2005; Fausch 2007; Zorn and Nuhfer 2007; Arismendi et al. 2009; Morita 2018; Zorn et al. 2020; Gallagher et al. 2022). Reintroductions of extirpated species into their historic range can play important ecological and cultural roles with potential to counter declining populations (Williams et al. 1988; Converse et al. 2013a). Arctic Grayling *Thymallus arcticus*, henceforth Grayling, hold both cultural and historic significance to Indigenous communities and to the State of Michigan and current efforts aim to reestablish self-sustaining populations in their historic range in the region. As many ecological changes have occurred, it is important to understand how such changes, in particular to the fish community structure, potentially limit the success of reintroduction efforts.

While primary causes of the extirpation of Grayling from Michigan have been cited as habitat loss and overfishing (Vincent 1962; Nuhfer 1992), previous research has identified competition with non-native fish as one of the primary causes of declines in native fish populations (Brown 1943; Vincent 1962; Fausch and White 1981; Waters 1983; Fausch and Cummings 1985; Meffe 1985; Fausch and White 1986; Moyle et al. 1986; Nuhfer 1992; Wang and White 1994; Moyle and Light 1996; Nakano et al. 1998; Zorn and Nuhfer 2007; Zorn et al. 2020). Two of these factors have been addressed as fishing for Grayling in Michigan is currently prohibited and habitat has largely been restored with logging and forestry activities no longer being practiced as in the late 1800's and early 1900's. The introduction of non-native species to the historic range of Grayling began in the late 1800's primarily with Brown Trout *Salmo trutta* being introduced from Europe and both natural and anthropogenic range expansion of Brook Trout *Salvelinus fontinalis* (Mershon 1923; Vincent 1962; Westerman 1974; Zorn et al. 2018). The establishment of these two species since extirpation of Grayling in 1936 may complicate reintroduction efforts (Goble et al. 2018) and necessitates an understanding of complex competitive interactions that may occur between Grayling and the contemporary fish community. Understanding interspecies interactions such as competition is a critical step to successful reintroduction.

Early life stage Grayling are likely to be particularly vulnerable to competition with young resident salmonids within the tributaries where they will be reintroduced. As juvenile fish numerically dominate these systems (Zorn and Nuhfer 2007; Nuhfer et al. 2017; Zorn et al. 2020), it is critical to understand interspecies interactions, including potential predation rates and competitive effects, at this life stage. Non-native Brown Trout are widely known to be deleterious to native fishes (Alexander 1977; Fausch and White 1981; Fausch and White 1986; Zorn et al. 2020). Previous research from Montana has shown a negative association between densities of Brown Trout 250 mm and larger and Grayling (McCullough 2017). However, there is little evidence of negative association between Grayling and Brook Trout between 100-250 mm (Byorth and Magee 1998). The effect their presence will pose on young (age-0) Grayling is largely unknown and warrants further examination as most studies have explored age-1 or older year classes.

Size advantages early in life can lead to competitive advantages. Fausch and White (1986) found negative associations between age-0 Coho Salmon *Oncorhynchus kisutch* and both Brook
and Brown trout. Coho Salmon fry emerge approximately two weeks earlier than both Brook and Brown trout and at a relatively larger size (Fausch and White 1986). Relative total lengths at emergence for Grayling, Brook Trout, and Brown Trout are 7.00-15.00 mm, 28.45-30.55 mm, and 27.24-29.21 mm, respectively potentially contributing to a competitive advantage for these trout species (Fausch and White 1986; Northcote 1995). The approximate time of emergence for Brook and Brown trout in Michigan streams is between early April through early May (Zorn and Nuhfer 2007). Time of emergence for Grayling in Michigan is expected to be early spring, with historic documentation providing a spawning range from March to May (Metcalf 1961; Vincent 1962). Thus, competition between age-0 resident trout, potentially having a size advantage early in the growing season, could adversely affect Grayling survival through aggressive interactions, resulting in young Grayling being pushed into less profitable microhabitats, but the effects of this are unknown.

Competition is a complex of interactions, manifesting in many ways. Reductions in growth can serve as an indicator of competition as the presence of a competitor would be expected to reduce food abundance or push fish into less profitable feeding habitats as they compete for space. At an extreme end, mortality can serve as an indicator of stress (isolation from valuable resources) and as a proxy for competition. Biomass integrates both growth and mortality into a single measure, thus providing an additional proxy for comparing complex interactions between species such as competition and can be used to quantify the degree of competition (Mittlebach 1988; Goldberg et al. 1999; Moustakas et al. 2013; Hart et al. 2017). Fish may change behavior in the presence of dominant or aggressive fish by avoiding profitable positions, altering distribution, and other avoidance measures (Alexander 1977; Fausch and White 1981; Fausch and White 1986; Hakoyama and Iguchi 2001; Taniguchi et al., 2002). Fin damage can arise from aggressive behavior with other fish and lack of adequate food and can thus act as a measure of competition (Abbott and Dill 1985; Turnbull et al. 1998; Latremouille 2003).

The goal of this study is to examine competitive interactions between age-0 Grayling and Brook and Brown trout of the same year class in experimental streams. My specific objectives were to test the hypothesis that the presence of either resident trout species will result in reductions in growth and/or biomass, increased mortality, changes in behavior, and visible evidence of physical damage.

Methods

Fish

Grayling for this study were obtained annually from the Ruth Burnett Sport Fish Hatchery, Fairbanks, Alaska, as eyed eggs and reared in the Michigan State University Containment facility: 5,000 in 2018; 10,000 in 2019 and 2021 (no fish in 2020 due to COVID-19 pandemic). Michigan Department of Natural Resource (MI DNR) personnel assisted Alaska Fish and Game (ADF&G) personnel in the collection of eggs from adult Grayling from the Chena River which were held in temporary ponds. I transported eggs from Fairbanks, AK to East Lansing, MI once they reached eyed-egg stage. Rearing methods closely followed protocols set forth by Travis Hyer and Charles Pratt (Ruth Burnett and William Jack Hernandez Sport Fish Hatcheries, Alaska Department of Fish and Game, respectively, personal communications). Eggs were reared in heath-style incubation trays with trout mesh screens. Fish were transferred (ponded) to a rearing tank located below the incubation trays 48 hours after 50% of the eggs have hatched. The rearing tank was a low-flow, shallow depth tank equipped with a fine-mesh screen on a bottom-drawn outflow standpipe. The ponding time of 48 hours was selected as it represented a hybridization of the rearing techniques used at both Alaska hatchery facilities. Flow rate and depth of water was increased corresponding with fry growth and development and based upon the recommendations of Travis Hyer and Charles Pratt, ADF&G (personal communications).

A 24-hr Artemia solution drip was immediately started upon ponding with newly hatched alevin keying in on the feeding drip within 24 hours (personal observation). Six additional feeding lines further distributed the Artemia solution and decreased localized competition for food. Each of the additional lines were operated by dosing pumps in which set volumes (50 mL) of solution were administered every 10 minutes from a single dosing line. Each line dosed solution once every hour; with six lines to provide additional feed every 10 minutes. Transitioning from Artemia to Bio-Oregon trout mash began 10 days after ponding with full transition off Artemia and on to 100% trout mash at 14 days post-ponding. Changes in dry feed size and amount fed corresponded to increases in growth of fry and was based upon the recommendations of Bio-Oregon[®] feeding guides for trout and salmon (https://www.bio-oregon.com/assets/FeedingGuidelines.pdf). Dry feed was administered via Eheim[®] automatic fish feeders. Each feeder was programed to dispense a measured amount of food 1-4 times per day, dependent on the daily feeding requirements of the fish. Placement of the feeders as well as timing of food dispension rotated daily. Fresh food was placed in each feeder daily. Feeding amounts were regularly monitored to decrease the likelihood of overconsumption by Grayling as they are prone to feeding beyond satiation to the point of rupture (Kindschi and Barrows 1990; Travis Hyer, personal communication). Additional food items including thawed bloodworms, mysis, and diporeia were supplemented into the diet as growth of Grayling allowed. This supplementation was increased in frequency and amount prior to transitioning to competition trials, which typically began in August. This was done to allow the young Grayling to recognize these items as food sources as no trout mash was offered in competition trial experimental streams, only in Grayling rearing tanks. Based on laboratory

observations, Grayling are opportunistic feeders and easily switched from dry, hatchery type feed to "natural" food items. One week prior to starting competition trials, 50 Grayling were randomly collected from the rearing tank and held in a separate holding tank. From this population, Grayling were randomly collected to be placed into the experimental streams at the start of the competition trials.

Age-0 Brook and Brown trout were collected using backpack electrofishing in 2018, 2019, and 2021 from Sweetwater Creek and Sanborn Creek (Michigan), respectively, one week prior to the start of competition trials. Both are tributaries of the Pere Marquette River and sustain wild populations of resident trout. Sweetwater Creek sustains a plentiful population of Brook Trout and other non-game fish while Sanborn Creek sustains a diverse population of Brown Trout and other migratory salmonids including Rainbow Trout *Oncorhynchus mykiss*, Chinook Salmon *Oncorhynchus tshawytscha* and Coho Salmon *Oncorhynchus kisutch*, as well as other non-game fish. These tributaries are readily sampled in a single day of field work and approximately a 15minute drive apart. This close proximity lessened the degree of stress on fish during holding and transportation. Fish were immediately brought back to the containment lab at Michigan State University to acclimate to the experimental stream channels for one week prior to commencement of competition trials.

Experimental Stream & Competition Trial Methods

Competition trials started on 12 Aug 2018, 02 Sep 2019, and 16 Aug 2021 and lasted a total of eight weeks: 21 Aug – 16 Oct 2018, 02 Sep – 28 Oct 2019, and 16 Aug – 11 Oct 2021. The trials consisted of a control and two treatments: the control consisting of 20 age-0 Grayling in stream channel 1; the Brook Trout treatment consisting of 10 age-0 Grayling with 10 age-0 Brook Trout in stream channel 2; and the Brown Trout treatment consisting of 10 age-0 Grayling with 10

age-0 Brown Trout in stream channel 3. Each stream had an equal number of fish (n = 20) per stream at the beginning of the trial. Initial populations of n = 20 fish per stream allowed for the examination of interspecific competition above that observed through intraspecific competition in the control and allowed for initial stream densities to be equivalent across treatments.

Experimental stream channels used were the same as those constructed for predation trials (Figure 13). Briefly, three replicate experimental stream channels were constructed. Streams were covered with black-out fabric lined with camouflage mesh fabric to isolate the streams from the rest of the laboratory area. Each stream had a 60 cm tall netting lined with camouflage fabric to prevent any fish from potentially jumping out of the streams and to further isolate streams from each other. A full spectrum aquarium grade LED light strip filtered by a semi-transparent section of plexiglass to decrease the light intensity and with artificial leafy vining underneath to mimic tree canopy was installed above each stream. Streambeds consisted of varying diameters of substrate, woody debris, artificial vegetation, and artificial undercut bank structures. Cameras were installed in both the inflow and outflow areas of the streams and angled to allow 100% visual coverage when viewed on the live-stream monitor and in recordings.

After a one-week acclimation period, all Brook and Brown trout were removed from the experimental streams and placed in temporary holding containers equipped with air pumps. Ten fish of each species were randomly selected for trials, photographed in a photarium, weighed, and placed back into the designated experimental stream channel. Grayling were selected randomly from the isolation area of the holding tank, photographed in a photarium, weighed, and placed into the experimental stream channels. The competition trials started once all fish were placed into the corresponding stream channels.

In 2019, all fish were tagged with a visible implant elastomer (VIE) tag at the anterior base of dorsal fin, two days prior to commencing the competition trials. All Brook and Brown trout received red colored VIE tags and all Grayling, received yellow. VIE tagging was implemented with the hope that this would allow species identification to be more successful in video recordings. VIE tags have been successfully implemented in previous studies and have shown not to affect behavior (Olsen and Vøllestad 2001; Josephson et al. 2008; Skalski et al. 2009; Leblanc and Noakes 2012; Kozłowski et al. 2017; Jungwirth et al. 2019). Unfortunately, I found the VIE tags were not visible in camera live-feeds nor in the video recordings of the competition trials, resulting in no further tagging to be performed for the 2021 competition trials.

Throughout the trial period, human presence was kept to a minimum. Stream channels were checked once per day, cleaned as needed and food changed during daily check. Fish were fed automatically, without human presence. Two food types were offered within each stream channel with the same schedules: drift and surficial. The drift-feed system consisted of thawed frozen bloodworms and Mysis. This feed was dispensed using an overflow system in the middle region (with respect to length of the stream) of each stream channel, placed in the same location for each stream. Feed was circulated using an air stone in a small bottle with an outflow tube. Water was pumped automatically using dosing pumps into the bottle to create an overflow pushing food items down outflow tube and into the stream where it was pushed downstream using a low-flow submersible pump. The surficial feed system consisted of freeze-dried Mysis and freeze-dried bloodworms. Feed was dispensed automatically using an automatic feeder placed in the same upstream reach of each stream channel.

Fish were captured, photographed, and weighed bi-weekly through the entirety of the trials. For all weigh-ins, all fish were removed from an experimental stream and held in a temporary holding container equipped with an air pump. To obtain photographs, a photarium was used to decrease handling of fish in an effort to minimize stress. Each individual fish was placed into a photarium with a metric ruler adhered to the outside, photographed, then placed into the weighing container. To obtain each weight, a water-filled container was placed on a scale and tared. The first fish was then placed into the container of water to obtain the weight to the nearest hundredth of a gram. Each fish of the same species and treatment was then added to the container with the scaled tared between each fish until all were weighed. Once all fish were photographed and weighed from a single treatment stream, all individuals were placed back into the stream from which they were removed. When recording weights, the measurement was verified by two individuals to ensure accuracy and data quality.

Photographs were intended for two purposes: 1) to be used as a non-intrusive way of measuring fork length; and 2) to be used to track individual growth using unique spotting patterning of individuals following methods used in Bachman (1984). Unfortunately, I found that the spotting pattern in young Grayling was not an effective way to track individuals, and as such, growth could only be evaluated for surviving fish as a group. In 2018, all fish were pooled by species to obtain one mean weight for week 0 (start of trials) then individually weighed for all subsequent weigh-ins. In 2019 and 2021, all fish were weighed individually for all weigh-ins, including week 0.

Fork length was found to be the most consistent method of length measure due to live fish being measured using the photarium. Total length was not a viable option as it requires the caudal fin to be compressed such that the furthest tip of the tail is used in measurement and compression of the caudal fin was not possible in the photarium. Each length photograph was analyzed using ImageJ[®] (Abramoff 2004). To ensure high accuracy and to maintain QA/QC, technicians were

trained to use the imaging software and correct measuring techniques for each species of fish. Photos were uploaded and analyzed after the trials reached completion each experimental season. For each photograph, the scale of the image was set using the metric ruler adhered to the photarium and visible in each image, then fork length measured of the fish. Fork length was measured to the nearest hundredth of a millimeter. All length measurements were verified by a second reader for accuracy and data quality.

Fin damage was documented using the biweekly photographs. Damage was assessed on dorsal and caudal fins of fish in 2019 and 2021. Seven categories were created and scored (score in parentheses with description) that included: MiF, minor fray or split between fin rays less than $\frac{1}{2}$ depth of affected fin and \leq five frays/splits with the majority of the fin undamaged (score = 2); MaF, any major fray or split that is more than $\frac{1}{2}$ the depth of the affected fin or > five minor fray/splits, damage affecting most of the fin (score = 3); MiM, edge of the fin is missing, less than $\frac{1}{4}$ of the depth of affected fin and only in one area (score = 4); MoM, edge of fin missing, less than $\frac{1}{4}$ of the depth of affected fin and more than one area affected (score = 5); MaM, any large portion of fin missing, more than $\frac{1}{4}$ of the depth of affected fin (score = 6); NA, photo blurry, poor quality, fin not visible, fish not photographed (score = NA); ND, no damage (score = 1). The total score was the summation of the scores for dorsal and caudal fins for each fish. Examples of fin damage can be found in Figure 21, where panel A shows a Grayling with no damage, panel B with minor fraying in both dorsal and caudal fins (MiF), and panel C with major frays on the dorsal fin (MaF) and large portions of the caudal fin missing (MaM).

Behavioral observations were qualitatively made during daily maintenance of the experimental streams during 2018 and 2019. In 2021, a small observation "window" was cut out of the blackout fabric surrounding the experimental streams that could be opened and closed to

allow observation of competition trials without being seen by fish. The window was at the downstream end of each stream and set at a height in which I could easily view the entire stream inconspicuously. Observations were made at various times of the day across each stream, in addition to the daily routine observation during maintenance. Observations lasted five minutes per experimental streams with each stream observed sequentially. Notes were logged into laboratory notebook with a focus on the general distribution of the fish, swimming behavior, and intra- and interspecies interactions. Due to the size and development of the fish and unique dorsal mottling patterns between Grayling and competitors, the species of fish was identifiable, however individual fish within the species were indistinguishable.

Statistical analyses

Growth of fish was examined bi-weekly throughout the duration of the competition trials, following the procedures described in the bi-weekly weighing paragraph. After each bi-weekly weigh-in, data were entered into a database and analyzed in RStudio[®] and visualized for trends in the data through generation of a dot plot with linear trends using the tidyverse package (Wickham et al. 2019). Data were summarized to obtain sample size, minimum and maximum weights, mean weight, and standard error of the mean. This was performed each year and for each treatment group. A general linear model with gaussian distribution and an identity link function was used to examine if treatment type (Control, Brook, Brown) or time (week, year) affected growth of Grayling with an a-priori $\alpha = 0.05$. A stepwise process using the stepAIC function in the MASS package in RStudio (Venables and Ripley 2002) was used to identify the most parsimonious model. This type of stepwise approach to linear modeling removes variables whose elimination results in small decreases in the test measure in a sequential manner (Finos et al. 2010). The full model included treatment type as the main effect with week as a covariate and year as a replicate:

 $Growth_{ijy} = Treatment_i + Week_j + Year_y + Treatment * Week * Year + e_{ijy}$

where i = Treatment; j = week; and y = year. Iterative backward step AIC analysis confirmed the full model as the model with the most parsimony.

Mortality was analyzed using a Pearson's Chi-Square Test for Homogeneity. This was performed using RStudio[®] to determine whether the distribution of the categorical variable (alive) was the same for each treatment type population (Control, Brook, Brown) with an a-priori $\alpha = 0.05$. Each treatment population consisted of 30 fish for which the categorical variable was assessed. The hypothesis examined was that the proportion of fish alive at the end of the experiment was the same in all treatments:

$$H_0$$
: $alive_{ctl} = alive_{bkt} = alive_{bnt}$

Biomass was calculated bi-weekly using mean weight (g) and number of fish present in the treatment at that specific weighing event. The equations used for the control (equation 1), in which there were 10 Grayling with 10 Grayling when accounting for all three years of trials, and for the Brook and Brown trout treatment, in which there were 10 Grayling with either 10 Brook or Brown trout in each annual trial, (equation 2) were calculated as:

$$B_{tx} = \left(\frac{n_g}{2}\right) * \mu_{tx} \quad \text{Equation 1}$$
$$B_{tx} = n_g * \mu_{tx} \quad \text{Equation 2}$$

where B_{tx} is the biomass of the treatment, n_g is the total number of Grayling in the treatment, and μ_{tx} is the mean weight of the Grayling in the treatment (Granfeldt 1979; Harvey et al. 2003; Li et al. 2020). Equation 1 differed from equation 2 so that the biomass was equivalent between treatments, thus n_g was divided by two in order to account for the treatment having a total of 60 Grayling. A general linear model with gaussian distribution and an identity link function was used to examine if treatment type (Control, Brook, Brown) or time (week, year) affected total biomass of Grayling with an a-priori $\alpha = 0.05$. A stepwise process using the stepAIC function in the MASS package in RStudio (Venables and Ripley 2002) was used to identify the most parsimonious model. The full model included treatment type as the main effect with week as a covariate and year as a replicate:

 $Biomass_{ijv} = Treatment_i + Week_j + Year_v + Treatment * Week * Year + e_{ijv}$

where i = Treatment; j = week; and y = year. Iterative backward step AIC analysis resulted in the model with the most parsimony. For the final model, the response variable was biomass, and the explanatory variable was treatment type (Control, Brook, Brown) as the main effect with week as a covariate that potentially interacted with the main effect:

$$Biomass_{ijy} = Treatment_i + Week_j + Treatment * Week + e_{ijy}$$

where year was dropped from the model during stepAIC model examination.

Fin damage was analyzed using a Fisher's Exact Test due to two categories having less than 5 observations. This was performed using RStudio[®] to determine whether the distribution of the categorical variable (damage) was the same for each treatment type population (Control, Brook, Brown) with an a-priori $\alpha = 0.05$. Due to low sample sizes for some categories, fin damage was combined for caudal and dorsal fins and grouped into major (MaM and MaF), minor (MiF, MiM, and MoM), and no damage. The hypothesis examined was that the proportion of fish with fin damage was the same in all treatments:

$$H_0$$
: fin. damage_{ctl} = fin. damage_{bkt} = fin. damage_{bnt}

Results

Grayling growth, survival and biomass were the most similar with positive changes through time in the Control and Brook Trout treatment. Suppressed growth, high mortality, and lower biomass were observed in the Brown Trout treatment. The most dramatic differences in behavior and fin condition were observed in the Brown Trout treatment as compared to other treatments. Results: Growth

Grayling growth showed a significant increase over time by week (p = 0.046) but there was no significant difference in growth by year (p = 0.652). Growth of Grayling was significantly different when considering the interaction of the Brown Trout treatment and week (p = 0.021) and when considering the interaction of the Brown Trout treatment, week, and year (p = 0.021). No significant differences were apparent when comparing treatments for surviving fish in the Brook Trout treatment (p = 0.378) or in the Brown Trout treatment (p = 0.538) as compared to the control (Table 15). The stepwise model approach confirmed the full model as the model with the most parsimony. Growth in the control treatment increased from a mean of 2.17 g to 4.10 g across all years, from a mean of 2.14 g to 4.38 g in the Brook Trout treatment, and from a mean of 2.52 g to 3.14 g in the Brown Trout treatment (Table A 4).

In 2018, Grayling in the control and Brook Trout treatment exhibited positive growth over time (Figure 14, Table A 5). Surviving fish in the Brown Trout treatment also exhibited positive growth over time, but to a lesser degree than in the control or Brook Trout treatment. The mean weights \pm SD for Grayling at the end of the experiment in the control, Brook Trout treatment, and Brown Trout treatment were 4.00 ± 1.33 g, 4.35 ± 0.66 g, 2.37 ± 0.54 g, respectively (Table A 5). The mean weight of all Brook and Brown trout increased throughout the trial, however the deviation from the mean increased over time (Table A 5). In 2019, Grayling in the control and Brook Trout treatment exhibited positive growth over time while growth in the presence of Brown Trout was negative (Figure 14). The mean weights \pm SD for Grayling at week 8 representing the end of the experiment in the control, Brook Trout treatment, and Brown Trout treatment (at week 6) were 4.86 ± 1.08 g, 4.43 ± 1.24 g, 2.12 ± 0.00 g (only one Grayling remaining in week 6), respectively (Table A 6). The mean weight of all Brook and Brown trout increased throughout the trial; the deviation from the mean also increased over time (Table A 6). The two deceased Grayling in this treatment occurred on 20 and 23 Oct; the trial ended 28 Oct.

In 2021, Grayling in the control and Brook Trout treatment exhibited positive growth over time. Surviving fish in the Brown Trout treatment also exhibited positive growth over time (Figure 14). The mean weights \pm SD for Grayling at the end of the experiment in the control, Brook Trout treatment, and Brown Trout treatment were 3.41 ± 0.82 g, 4.38 ± 1.11 g, 3.91 ± 1.75 g, respectively (Table A 7). The mean weight of all Brook and Brown trout increased throughout the trial, as did deviation from the mean (Table A 7).

Results: Mortality

A Pearson's Chi-square test for homogeneity was performed to examine the distribution of the categorical variable "alive" for each of the three treatment groups. The relationship between the groups was found to be significant, $\chi^2 = 67.149$ (df = 2, n = 120), p < 0.001, indicating a difference in the proportion of fish alive by treatment group, thus rejecting the null hypothesis (equal proportions among groups) (Table 16). Mortality in the control was low, ranging from 0% in 2018 and 2019 to 5% in 2021 (Figure 15, Table A2), with an overall mortality rate across years of 1.7%. Similarly, mortality was low in the Brook Trout treatment, ranging from 0% in 2018 and 2019 with an overall mortality rate of 6.7%. Conversely, mortality was consistently

high in the Brown Trout treatment, ranging from 60% in 2018 and 2021 to 100% in 2019 with an overall mortality rate of 73.3%. Mortality of Brook Trout was 0% in all years. Brown Trout mortality was consistently low ranging from 0% in 2021 to 10% in 2018 and 2019.

Results: Biomass

Grayling biomass showed an increase over time in the control and in the Brook Trout treatment with no significant difference in the rate of increase (p = 0.729). Biomass in the control treatment increased from a mean of 21.67 g to 40.33 g across all years, and from a mean of 21.43 g to 40.92 g in the Brook Trout treatment. Biomass of Grayling in the Brown Trout treatment declined over time and was significantly different (p < 0.001) than the control and Brook Trout treatment (Table 17). Biomass of Grayling in the Brown Trout treatment declined from an average of 25.17 g at week 0 to 12.54 g at week 8 across all years (Figure 16, Figure 17).

Results: Behavior

In 2021, twenty-two five minute behavioral observation sessions occurred in the control and Brook Trout treatments and twenty-three sessions occurred in the Brown Trout treatment. Observation sessions occurred under varying conditions: at time of feeding, immediately before feeding, and between feedings. Results of the behavioral observations are presented qualitatively.

In the control, loose shoaling behavior was noted overall with Grayling positioned in the upper half of the water column throughout the upper and middle regions of the stream and away from the periphery of the channel (Figure 18, Figure 19). One chase was observed at a moderate increase in swimming speed as compared to the normal rate of swimming. Two nudges, where one Grayling used its body to push another individual, were observed and one nip event (Table 18). Position changes were frequently observed and occurred without increases in swimming speed, bodily contact, or flaring of fins. Grayling readily fed without aggressive behavior.

In the Brook Trout treatment, loose shoaling behavior was noted overall with Grayling positioned in the upper half of the water column throughout the upper and middle regions of the stream and away from the periphery of the channel (Figure 18, Figure 19). Brook Trout primarily occupied the lower half of the water column in the vicinity of cover such as the grass or woody debris in the middle reach of the experimental stream. Twelve total chases were observed: four with Brook Trout chasing Grayling; seven with Brook Trout chasing a conspecific; one with a Brook Trout chasing an unknown species (either Brook Trout or Grayling); and one in which neither species was identifiable (Table 18). Chases occurred at a moderate increase in swimming speed. No other agnostic behavior was observed. Position changes were noted but these occurred without increases in swimming speed, bodily contact, or flaring of fins. Brook Trout and Grayling readily fed together without aggressive behavior.

In the Brown Trout treatment, a lack of swimming activity was noted with Grayling primarily positioned at the periphery of the stream channel (against the edges of the tank) and at the surface of the water column (Figure 18, Figure 19). Brown Trout remained close to the substrate and within or under cover (woody debris, grasses). Grayling were located at the edges of the stream and high in the water column, often just beneath the surface of the water. When observations occurred during feeding, fish were observed making rapid swim paths to capture a food item then quickly returning to the original position. After 42 trial days, the largest Grayling was observed holding position in the center of the stream channel, just downstream of the woody debris and observed holding position when larger Brown Trout emerged to feed. At trial day 46, two Grayling were observed holding position in the upper portion of the water column in the midstream/mid-channel section of the stream, just downstream of the woody debris with the largest Grayling upstream of the smaller. These positions were observed throughout the remainder of the

trial. All remaining Grayling were isolated to the periphery of the stream channel. Ten total chase events were observed: Eight with Brown Trout chasing Grayling and two with Grayling chasing a conspecific (Table 18). All chases were noted as quick acceleration in swimming speed and faster than chases than observed in either the control or Brook Trout treatment. Four nip events were noted in which a Brown Trout nipped a Grayling. All chase and nip events occurred when a Grayling moved away from the periphery of the experimental stream. Agonistic behavior was observed by Brown Trout towards Grayling when Grayling moved to occupy habitat within the stream channel (away from edges) regardless of the presence or absence of food. Observed feeding by Grayling was isolated to food items drifting past their occupied location along the periphery of the stream channel that did not require much movement. Brown Trout were observed quickly darting out from occupied habitat to capture food items then returning to their previous location. Results: Fin Damage

Fin damage was apparent at week 2 and increased in occurrence and degree through the remainder of the trial (Figure 20). A Fisher's Exact Test was performed to examine the distribution of the categorical variable "fin damage" for each of the three treatment groups. Significant differences in fin damage was observed among treatment groups (p < 0.001, Table 19), with higher total fin damage scores in the Brown Trout treatment (Figure 20, Figure 21).

Discussion

Based on results of these experiments, Grayling reintroduction is likely to be limited by competition with Brown Trout, whereas coexistence with Brook Trout seems more probable. Growth and biomass of Grayling showed an increase over time in the control and Brook Trout treatments with no significant difference in the rate of biomass increase (Table 15, Table 17). Conversely, growth was significantly lower (Table 15) and biomass of Grayling in the Brown Trout treatment declined over the short span of these experiments (Table 17). These findings were consistent across all experimental seasons. Mortality was low in the both the control (3.3%) and Brook Trout treatment (6.7%) but high in the Brown Trout treatment (73.3%). There was consistency in the trends for Grayling biomass: positive in the control and Brook Trout treatment; negative in the Brown Trout treatment (Figure 17). There were also differences in behavior and amount of fin damage in the presence of Brown Trout, indicating that agonistic behavior for position in the stream leading to competition for space is a possible mechanism for poor growth and survival of Grayling.

Competition can have both direct and indirect effects to individual and communities of fish. Here, both were observed. Brown Trout were observed directly interacting, often in an aggressive manner, with Grayling. Grayling were also observed occupying different regions of the streams in the presence of Brown Trout than with Brook Trout or conspecifics. This type of indirect effect of competition may lead to decreased availability of resources. Although a high degree of fin damage was documented in the presence of Brown Trout, this may not have been completely due to attacks. Stressful environments and limited nutrition have the potential to degrade the overall health of a fish which may exhibit as fin damage (Latremouille 2003). Direct impacts through direct interactions with competitors often resulted in aggressive behaviors in the presence of Brown Trout. As a result, such interactions may lead to a landscape of fear. This landscape of fear can result in increased stress in perceived prey or subordinate competitors, increased vigilance, neophobia, and decreased health due to indirect effects associated with less resource availability and increased stress (Lima and Dill 1990; Brown et al. 1999; Crane et al. 2015; Bleicher 2017; Gaynor et al. 2019)

While climate change and other factors such as habitat destruction are factors in declines of native salmonid populations, the presence of non-native fishes can limit success in reintroductions and restorations of native species (Brown 1943; Vincent 1962; Fausch and White 1981; Waters 1983; Fausch and Cummings 1985; Meffe 1985; Fausch and White 1986; Moyle et al. 1986; Miller et al. 1989; Nuhfer 1992; Wang and White 1994; Moyle and Light 1996; Nakano et al. 1998; Vander Zanden et al. 1999; Quist and Hubert 2004; Clavero and García-Berthou 2005; Fausch 2007; Zorn and Nuhfer 2007; Arismendi et al. 2009; Morita 2018; Zorn et al. 2020; Gallagher et al. 2022). Habitat loss and overfishing were important factors contributing to the extirpation of historic Grayling populations in Michigan (Vincent 1962; Kaya 1992b; Nuhfer 1992). This research demonstrates that competition likely played a role as well. Competition has potential to alter habitat and stream distribution with changes in preference observed between the Brown Trout treatment and that observed in both the control and Brook Trout treatments (Fausch 1988). Displacement of Grayling likely occurred with historic populations resulting in displacement to less profitable habitat. Understanding contemporary fish communities, careful consideration of stream habitat availability, suitability, and population densities of resident salmonids are important components of stream selection for Grayling reintroduction.

Grayling growth and biomass accumulation in the presence of Brook Trout was comparable to that in the control population where Brook Trout did not occur (Figure 16, Figure 17). This is consistent with results seen by Byorth and Magee (1998) in which age-1 Grayling showed little evidence of negative association with Brook Trout of a similar size class. Thus, the competitive effect of age-0 Brook Trout on Grayling appears minimal. There was a negative effect of Brown Trout on Grayling. Similarly, McCullough (2017) found negative association between larger Brown Trout and Rainbow Trout *Oncorhynchus mykiss* and age-0 Grayling and a positive relationship between yearling and older Grayling and Brook Trout in field studies in Montana. Grayling growth was poor and mortality high (60-100%) when in the presence of age-0 Brown Trout. Our results show that the effects of Brown Trout on Grayling begin at early life stages and may have the potential to continue through maturity. All years followed similar trends, which is indicative of repeatable methodologies across the study years.

Non-native Brown Trout are known to limit native fishes in Michigan streams (Alexander 1977; Fausch and White 1981; Fausch and White 1986; Zorn et al. 2020). The most dramatic differences between treatments in this study were observed in the Brown Trout treatments. Behavior and position within experimental streams differed in the Brown Trout treatment relative to the control and Brook Trout treatment. Behavior and position within experimental streams were similar in the control and Brook Trout treatment with Grayling often occupying the mid- and upstream regions of the stream, away from the periphery of the stream channel and actively swimming with little to no agonistic behaviors observed (chasing, nipping, nudging) (Figure 18, Figure 19). In both the control and Brook Trout treatments, Grayling were observed in loose shoals and occupied the upper half of the water column. Interspecies shoaling was observed in the Brook Trout treatment with Grayling in the upper half of the water column and Brook Trout in the lower half of the water column, primarily during feeding (Figure 19). In the presence of Brown Trout, Grayling were observed in tight shoals at the surface of the water column against the edges of the experimental streams (Figure 18, Figure 19). Feeding behavior was different in the presence of Brown Trout with Grayling quickly darting out for food before returning to their position at the edge of the stream channel or relying on food items that drifted into their position. This isolation to less profitable habitat was a likely cause of suppressed growth, increased mortality, and resultant decrease in biomass.

Fin damage is often the result of aggressive attacks (Abbott and Dill 1985; Turnbull et al. 1998 Latremouille 2003). Damage was primarily observed on the dorsal and caudal fins and ranged from minor rips/tears between fin rays to missing sections of caudal fin lobes and major splits between fin rays (Figure 21). Some fin degradation was observed in all treatments, with the least damage in the control and the most damage in the Brown Trout treatment. This is likely due to the higher degree of aggressive attacks seen in association with Brown Trout on Grayling during the trials.

While both Brook and Brown trout were commonly larger in size than the Grayling in the study, detrimental effects of competition were only seen in the presence of Brown Trout. The association between size advantage and competitive advantage may have been a factor in the Brown Trout treatment, however this does not seem to affect Grayling in sympatry with Brook Trout. It is important to note that this size difference may vary due to different emergence phenology for Grayling upon natural spawning in Michigan streams with water temperatures in Michigan streams warmer than those experienced in the source population of the Chena River in Fairbanks, Alaska. As such, Grayling are likely to emerge earlier in Michigan streams which may reduce the differences in size if Grayling are able to grow quickly enough to overcome the difference by the time Brown Trout fry emerge.

It is important to acknowledge limitations of this study occurring within experimental stream channels. The stream channels were closed systems with no escape from the pressures of competition. This may have resulted in an overestimation of the extent to which Brown Trout may deleteriously affect Grayling. However, as several previous studies have confirmed the deleterious nature of Brown Trout presence in natural systems (Holland 1921; Fausch and White 1981; Waters 1983; Wang and White 1994; Essington et al., 1998; Grant et al., 2002; Zorn and Wiley 2010; van

Zwol et al., 2012a; Van Zwol et al., 2012b; McCullough 2017; Zorn et al., 2018; Hoxmeier and Dieterman 2020; Zorn et al., 2020), results within my experimental system are likely indicative of the type of interactions that would be expected in nature. Availability of suitable and bioenergetically profitable habitat that is not occupied by Brown Trout will be critical to the success of Grayling in streams which Brown Trout are present. Feeding occurred in a predictable manner which fish may have been able to learn (Bachman 1984). This may have resulted in the establishment of dominance regions. Naturally occurring food sources in streams are likely to be more widely distributed which may lessen the degree of dominance regions. However, competition for highly profitable habitat is likely in natural stream systems.

Continuous video monitoring of the streams was attempted in a non-intrusive manner by utilizing overhead cameras. The goal was to provide insight and documentation of agonistic behavior, microhabitat use, and position choice for each treatment. Due to the inability to consistently distinguish species of fish in the videos, this was omitted from the final analysis. Complications included glare on the water surface, low image quality, surface disturbance due to air stones within the stream channels, and the inability to see VIE tags during remote live-feed and playback of the recordings. Attempts to utilize spot analysis in tracking individuals through the studies also failed due to changing parr marks and the development of spotting (dark pigmented "v" marks on the lateral sides of the Grayling) throughout the trials. The changing phenotypic characteristics during this early life stage prevented the tracking of individuals through the length of the trials. This resulted in the inability to track the growth of individual fish; thus growth is seen as "apparent growth". This also prevented individual-specific analyses of interactions as sizespecific interactions may have been masked by this lack of individual fish tracking. For example, I was unable to determine with certainty if smaller individuals within a cohort fared more poorly than larger individuals and vice versa. Although these limitations occurred, the consistency observed in each year of the competition trials provided confidence in the results.

Understanding early-life competitive interactions between these species will assist in the selection of suitable habitat for reintroduction based on factors including fish assemblage and competitor density. There is a lack of data focused on competitive interactions of early-life history fish, namely Grayling, Brook Trout, and Brown Trout. This research seeks to fill this gap. A companion study explores potential predation impacts of resident stream salmonids, age-1 Brook and Brown trout, on age-0 Grayling. Early-life predation is believed to potentially have significant impacts on young Grayling populations. These data will provide guidance in the determination and prioritization of suitable fish communities based on the strength of interspecific competition found to occur during the critical first year of growth. The consistency in the trends of the data indicate that coexistence with Brook Trout seems most likely. Consistent with previous research focused on a diverse suite of salmonids, Brown Trout are likely to have deleterious impacts on Grayling and are likely to pose a limiting factor in suitable reintroduction locations.

TABLES

Table 15: Results of general linear model analysis of Grayling growth. Overall test statistics were

$R^2 = 0.315, F(11, 525)$) = 21.980, p = <	0.001. Significant values ($\alpha < 0.05$) are in bold.
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Main Effect and Interactions	Estimate	β Coefficients	S. E.	t value	<i>p</i> value
Intercept	79.667	-	171.670	0.464	0.643
Brook Tx	-261.984	-92.544	297.047	-0.882	0.378
Brown Tx	-193.457	-57.627	314.135	-0.616	0.538
Week	70.621	157.260	35.254	2.003	0.046
Year	-0.038	-0.038	0.085	-0.452	0.652
Brook:Week	-20.184	-36.677	60.791	-0.332	0.740
Brown:Week	-169.142	-197.356	73.067	-2.315	0.021
Brook:Year	0.130	92.470	0.147	0.881	0.379
Brown:Year	0.096	57.726	0.156	0.617	0.538
Week:Year	-0.035	-156.745	0.017	-1.997	0.046
Brook:Week:Year	0.010	36.776	0.030	0.333	0.739
Brown:Week:Year	0.084	197.138	0.036	2.312	0.021

Table 16: Summary of Grayling survival across 2018, 2019 and 2021. Pearson's Chi-square test for homogeneity indicated a significant difference in survival among treatments ($\chi^2 = 67.149$ (df = 2, n = 120), *p* < 0.001.).

	Alive (observed)		
Treatment Type	Yes	No	
Control	59	1	
Brook	28	2	
Brown	8	22	

Table 17: Results of general linear model analysis of Grayling biomass. Overall test statistics were

$R^2 = 0.603 E(5.38)$) = 11544 $n = <$	0.001 Significan	t values $(\alpha < 0)$	(5) are in hold
$\Lambda = 0.005, \Gamma(5, 50)$) – 11.J ++ , μ – <	0.001. Significan	u = 0.0	

Main Effect and	Estimate	β Coefficients	S . E.	<i>t</i> value	<i>p</i> value
Interactions					
Intercept	20.834	-	3.438	6.059	< 0.001
Brook Tx	-1.537	-0.064	4.863	-0.316	0.754
Brown Tx	3.014	0.124	4.896	0.616	0.542
Week	2.230	0.550	0.702	3.177	0.003
Brook:Week	0.347	0.077	0.993	0.349	0.729
Brown:Week	-4.177	-0.850	1.033	-4.043	< 0.001

Table 18: Observed behavior of Grayling in competition trials by treatment type and aggressive encounters. Chase and nip events are described with the initiator species on the left side of the arrow and the receiving species on the right.

Treatment	Swimming activity	Chase events	Nudge events	Nip events
Control	Loose shoaling, active swimming	1	2	1
Brook	Loose shoaling, active swimming	12 total 4 Brook → Grayling 7 Brook → Brook 1 Brook → Unknown	0	0
Brown	Little activity, hiding	10 total 8 Brown → Grayling 2 Grayling → Grayling	0	4 Brown \rightarrow Grayling

Table 19: Summary of Grayling fin damage by treatment type. Values are numbers of fish for each treatment and damage category. Fisher's Exact Test indicated a significant difference in fin damage among treatments (p < 0.001.).

	Damage Category			
Treatment	Major	Minor	No Damage	
Control	1	18	295	
Brook	4	36	106	
Brown	28	19	33	

FIGURES



Figure 13: Experimental stream channel used in predation and competition trials. In top image, water flow is from right to left; in bottom image, water is flowing from top of image to bottom. Each stream channel was surrounded by fine mesh netting to prevent fish from potentially escaping; camouflage fabric was added over the white mesh netting and leaves under lighting to reduce glare on water surface at the beginning of the 2019 experimental season (bottom image).



Figure 14: Mean weight ± SE of Grayling by treatment: A) Control, B) Brook Trout treatment, C) Brown Trout treatment; and year: 2018 (red), 2019 (green), 2021 (blue). All Grayling in the Brown Trout treatment (panel C) during 2019 were deceased by week 8. Week 0 values for 2018 were a pooled weight to obtain mean weight, therefore no standard deviation was calculated.



Figure 15: Number of Grayling mortalities by treatment type and week of competition trial. Red represents the control, green the Brook Trout treatment, and blue the Brown Trout treatment. Total mortalities for the duration of the competition trials were 1 (control), 2 (Brook Trout treatment), and 22 (Brown Trout treatment).



Figure 16: Biomass of Grayling showed an increase over time in the control (red line, circles) and Brook Trout (green line, triangles) treatments with no significant difference in the rate of increase (p = 0.729). Biomass of Grayling in the Brown Trout (blue line, squares) treatment declined over time and was significantly different (p < 0.001). Each point represents an experimental season for the week of trial. The regression line represents the average biomass across all three experimental seasons for each treatment. Biomass significantly changed over time (p = 0.003).



Figure 17: Biomass of Grayling in A) 2018, B) 2019, and C) 2021. Biomass consistently showed an increase over time in the control (red) and Brook Trout (green) treatments with no significant difference in the rate of increase (p = 0.729). Biomass of Grayling in the Brown Trout (blue) declined over time and was significantly different (p < 0.001). Biomass significantly changed over time (p = 0.003).



Figure 18: Cross-section schematic of the experimental streams showing vertical positioning of fish. Grayling are depicted as red fish; Brook Trout as green; Brown Trout as blue. Fish and stream components are not to scale and only a subset of fish (5 each) are used to illustrate position. A) Grayling in control treatment; B) Grayling and Brook Trout in the Brook Trout treatment; and C) Grayling and Brown Trout in the Brown Trout treatment. Substrate is shown at bottom of each cross-section using oval shapes.



Figure 19: Top-view of the experimental streams showing positioning of fish. Grayling are depicted as red fish; Brook Trout as green; Brown Trout as blue. Fish and stream components are not to scale. A) Description of schematic; B) Grayling in control treatment; B) Grayling and Brook Trout in the Brook Trout treatment; and C) Grayling and Brown Trout in the Brown Trout treatment.



Figure 20: Grayling fin damage scores by treatment type: Control (red), Brook Trout (green), Brown Trout (blue). The higher the total score, the more severe the fin damage. Total score is the sum of the values assigned to dorsal and caudal fin damage using the assessment scale.



Figure 21: Photographs of year 2019 Grayling during week 6 of competition trial showing fin damage. A) fish classified as no damage on dorsal and caudal fins; B) fish classified as minor fray (MiF) for dorsal fin and no damage on caudal fin; C) fish classified as major fray (MaF) on dorsal fin and large portions of fin missing (MaM), more than ¹/₄ fin depth for caudal fin.

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APPENDIX B: CHAPTER 3

Table A 4: Summary of combined (2018, 2019, and 2021) competition trial weight data. Weights for Brook and Brown trout were for those Brook and Brown trout co-occurring with Grayling in the corresponding treatment.

Species	Bi-week	Treatment	n	Mean (g)	SD	Min (g)	Max (g)	% Mortality
Grayling	0	Control	60	2.17	1.11	0.90	6.50	0.00
Grayling	2	Control	60	2.57	1.02	0.76	5.65	0.00
Grayling	4	Control	59	2.86	1.02	0.57	6.26	1.70
Grayling	6	Control	59	3.35	1.10	1.24	5.98	1.70
Grayling	8	Control	59	4.10	1.24	1.66	7.63	1.70
Grayling	0	Brook	30	2.14	1.06	1.07	4.74	0.00
Grayling	2	Brook	30	2.31	0.75	1.33	4.38	0.00
Grayling	4	Brook	30	2.69	0.82	1.31	4.47	0.00
Grayling	6	Brook	30	3.57	0.88	1.69	5.15	0.00
Grayling	8	Brook	28	4.38	0.98	3.00	6.52	6.70
Grayling	0	Brown	30	2.52	1.61	1.07	7.78	0.00
Grayling	2	Brown	24	2.45	1.18	0.60	6.03	20.00
Grayling	4	Brown	20	2.34	0.74	1.31	3.49	33.33
Grayling	6	Brown	10	2.64	1.15	1.32	5.34	66.67
Grayling	8	Brown	8	3.14	1.45	1.86	6.47	73.33
Brook	0	Brook	30	2.74	0.59	2.07	4.50	0.00
Brook	2	Brook	30	3.22	0.88	2.12	5.73	0.00
Brook	4	Brook	30	3.44	0.92	2.06	5.90	0.00
Brook	6	Brook	30	4.15	1.46	1.94	7.59	0.00
Brook	8	Brook	30	5.18	2.21	2.07	10.80	0.00
Brown	0	Brown	30	3.45	1.01	2.49	6.11	0.00
Brown	2	Brown	30	3.56	0.92	2.18	5.52	0.00
Brown	4	Brown	30	4.14	1.55	2.34	9.06	0.00
Brown	6	Brown	30	4.88	2.18	2.22	11.44	0.00
Brown	8	Brown	28	6.14	3.04	2.40	15.40	6.70

Table A 5: Summary of 2018 competition trial weight data. A "pooled" sample size represents all fish (20 in control, 10 in all other treatments) weighed together to obtain an estimated weight; this only occurred for the first week of trials. Weights for Brook and Brown trout were for those Brook and Brown trout co-occurring with Grayling in the corresponding treatment.

Species	Bi-week	Treatment	n	Mean (g)	SD	Min (g)	Max (g)
Grayling	0	Control	pooled	1.19	_	—	_
Grayling	2	Control	20	2.35	0.88	0.89	4.55
Grayling	4	Control	19	2.72	0.90	1.50	4.51
Grayling	6	Control	20	3.33	1.10	1.66	5.37
Grayling	8	Control	20	4.00	1.33	2.11	6.48
Grayling	0	Brook	pooled	1.07	_	_	_
Grayling	2	Brook	10	2.00	0.37	1.45	2.70
Grayling	4	Brook	10	2.66	0.62	1.91	3.89
Grayling	6	Brook	10	3.82	0.56	2.78	4.58
Grayling	8	Brook	10	4.35	0.65	3.48	5.70
Grayling	0	Brown	pooled	1.07	_	_	_
Grayling	2	Brown	7	1.50	0.71	0.60	2.85
Grayling	4	Brown	6	1.86	0.56	1.34	2.74
Grayling	6	Brown	5	2.02	0.72	1.32	3.09
Grayling	8	Brown	4	2.37	0.54	1.86	3.11
Brook	0	Brook	pooled	2.32	—	—	—
Brook	2	Brook	10	3.00	0.62	2.19	4.09
Brook	4	Brook	10	3.36	0.67	2.16	4.40
Brook	6	Brook	10	4.43	1.13	2.93	6.18
Brook	8	Brook	10	5.01	1.64	3.10	7.45
Brown	0	Brown	pooled	2.49	—	—	—
Brown	2	Brown	10	3.07	0.79	2.18	4.40
Brown	4	Brown	10	3.63	1.15	2.34	5.82
Brown	6	Brown	10	4.41	1.75	2.22	8.06
Brown	8	Brown	9	5.34	2.43	2.63	10.86

Species	Bi-week	Treatment	n	Mean (g)	SD	Min (g)	Max (g)
Grayling	0	Control	20	3.37	0.97	1.95	6.50
Grayling	2	Control	20	3.54	0.77	2.17	5.65
Grayling	4	Control	20	3.64	0.92	2.14	6.26
Grayling	6	Control	20	3.83	0.95	2.46	5.98
Grayling	8	Control	20	4.86	1.08	3.31	7.63
Grayling	0	Brook	10	3.16	0.96	1.50	4.74
Grayling	2	Brook	10	2.95	0.90	1.33	4.38
Grayling	4	Brook	10	3.11	1.01	1.31	4.47
Grayling	6	Brook	10	3.34	1.13	1.69	4.91
Grayling	8	Brook	8	4.43	1.24	3.02	6.28
Grayling	0	Brown	10	4.25	1.44	2.77	7.78
Grayling	2	Brown	10	3.48	1.02	2.59	6.03
Grayling	4	Brown	8	2.78	0.58	1.94	3.48
Grayling	6	Brown	1	2.12	—	—	—
Grayling	8	Brown	0	_	_	—	—
Brook	0	Brook	10	2.74	0.42	2.07	3.45
Brook	2	Brook	10	3.57	1.08	2.66	5.73
Brook	4	Brook	10	3.25	0.95	2.06	5.02
Brook	6	Brook	10	3.50	1.55	1.94	7.05
Brook	8	Brook	10	4.79	2.62	2.07	10.71
Brown	0	Brown	10	3.37	0.46	2.72	4.04
Brown	2	Brown	10	3.42	0.72	2.57	4.64
Brown	4	Brown	10	3.59	0.99	2.36	5.19
Brown	6	Brown	10	3.75	1.41	2.27	6.45
Brown	8	Brown	9	4.87	2.31	2.40	9.68

Table A 6: Summary of 2019 competition trial weight data. Weights for Brook and Brown trout were for those Brook and Brown trout co-occurring with Grayling in the corresponding treatment.

Species	Bi-week	Treatment	n	Mean (g)	SD	Min (g)	Max (g)
Grayling	0	Control	20	1.94	0.53	0.90	3.26
Grayling	2	Control	20	1.82	0.52	0.76	2.77
Grayling	4	Control	20	2.21	0.69	0.57	3.15
Grayling	6	Control	19	2.87	0.73	1.24	3.91
Grayling	8	Control	19	3.41	0.82	1.66	4.61
Grayling	0	Brook	10	2.20	0.50	1.55	2.98
Grayling	2	Brook	10	1.98	0.44	1.48	2.63
Grayling	4	Brook	10	2.31	0.65	1.52	3.59
Grayling	6	Brook	10	3.53	0.88	2.41	5.15
Grayling	8	Brook	10	4.38	1.11	3.00	6.52
Grayling	0	Brown	10	2.23	0.70	1.08	3.08
Grayling	2	Brown	7	1.94	0.40	1.38	2.49
Grayling	4	Brown	6	2.25	0.83	1.31	3.49
Grayling	6	Brown	4	3.53	1.23	2.70	5.34
Grayling	8	Brown	4	3.91	1.75	2.71	6.47
Brook	0	Brook	10	3.18	0.74	2.17	4.50
Brook	2	Brook	9	3.07	0.86	2.12	4.63
Brook	4	Brook	10	3.72	1.12	2.35	5.90
Brook	6	Brook	10	4.53	1.59	2.33	7.59
Brook	8	Brook	10	5.73	2.38	2.69	10.80
Brown	0	Brown	10	4.48	0.94	3.07	6.11
Brown	2	Brown	10	4.26	0.90	2.90	5.52
Brown	4	Brown	10	5.31	1.90	3.40	9.06
Brown	6	Brown	10	6.65	2.36	4.15	11.44
Brown	8	Brown	10	7.91	3.41	4.26	15.40

Table A 7: Summary of 2021 competition trial weight data. Weights for Brook and Brown trout were for those Brook and Brown trout co-occurring with Grayling in the corresponding treatment.

CONCLUSION

The renewed interest in the restoration of self-sustaining populations of Arctic Grayling to Michigan streams inspired this research. The failures of previous restoration attempts identified three potential impediments to success: (1) lack of early life imprinting to receiving waters; (2) predation by other cold-water fishes such as Brook Trout and Brown Trout; and (3) competition between Grayling and other cold-water fishes.

Rapid outmigration was a common problem with previous reintroduction attempts in Michigan. The success in Montana's Grayling restoration suggested that early life imprinting could be achieved using remote site incubators. It was unknown, however, if this success was due to capturing a key timing of imprinting to stream waters or due to other factors. My research explored the potential timing of imprinting by quantifying whole-body thyroxine levels at various stages of development. While a peak in thyroxine levels was seen at the eyed-egg stage, indicating the potential for imprinting to occur at this life stage, it is unknown if this is an artifact of maternal thyroid hormones in the embryonic phase or truly associated with potential imprinting.

Interactions with Brook Trout and Brown Trout, now established since extirpation of Grayling, have the potential to limit the success of their restoration. This research sought to determine the extent to which both predatory and competitive interactions occur with these species during early life stages. While age-1 fish of both species preyed upon young Grayling fry, my results indicate that Brook Trout and Grayling are likely to successfully coexist within the same ecological community. While age-1 Brook Trout do prey upon Grayling fry, the impacts of competition were minimal and comparable to the presence of conspecifics. The species interactions between Grayling and Brook Trout did not appear to induce a landscape of fear with

behavior and growth similar between the Brook Trout treatment and control during competition trials.

In contrast, the combined impact of Brown Trout through competition and predation is likely to limit the success of Grayling restoration in Michigan streams. Brown Trout not only consume Grayling, but the species also likely creates a landscape of fear in which Grayling are pushed to less suitable habitat with lower access to resources through a high degree of negative competitive effects. This resulted in increased fin erosion, decreased condition, and increased mortality. The direct (consumption, aggression) and indirect effects (changes in behavior) of Brown Trout on Grayling indicates that the presence of Brown Trout is likely to limit the success of Grayling restoration. It is important to recognize that even though Brown Trout provide valuable recreational opportunities, their presence threatens native species on a global scale. Thus, decisions of which species to prioritize through management may have future consequences if those prioritized negatively impact the success of native species.