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The impacts of embryo development and asynchronous hatching on morphology, growth, and development of larval burbot (Lota lota).

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THE IMPACTS OF EMBRYO DEVELOPMENT AND ASYNCHRONOUS HATCHING ON MORPHOLOGY, GROWTH, AND DEVELOPMENT OF LARVAL BURBOT (*LOTA LOTA*).

By

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ABSTRACT

THE IMPACTS OF EMBRYO DEVELOPMENT AND ASYNCHRONOUS HATCHING ON MORPHOLOGY, GROWTH, AND DEVELOPMENT OF LARVAL BURBOT (*LOTA LOTA*).

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Burbot (*Lota lota)* are native fish in Lake Superior and typically spawn during the winter under the ice or by migrating up rivers. The early life history of larval burbot, where they dispurse, how fast they develop, and what their survival rates have not been extensively studied. Asynchronous hatching is a strategy used by other cod species as a bet-hedging strategy to ensure that some larvae are hatched in more advantageous conditions in a varying environment. Asynchronous hatching has been documented in burbot, but the extent of the period and the impacts that asynchrony has on the development of burbot larvae is unknown. This study provides detailed information on the impacts of asynchronous hatching on the morphology, growth, and development of larval burbot in Lake Superior. Burbot were captured and spawned during their winter spawning migration in southern Lake Superior rivers in 2022 and 2023. Larvae were separated by their date of hatch and photographed every other day to track growth and development of the different hatch date cohorts from the asynchronous hatching period. The asynchronous hatching period ranged 20-39 days for the observed families. Initial morphology such as total length and yolk sac area, as well as growth and yolk sac utilization rates were significantly different between early, middle, and late hatching larvae within and between families of burbot ($p<0.05$). The long, variable hatching periods of burbot may be a survival strategy they have adapted to deal with the harsh and variable environmental conditions in Lake Superior.

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This thesis contains collaborative data and work with other scientists; its inclusion in this thesis does not supersede their copyrights.

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INTRODUCTION

Burbot (*Lota lota*) are cold-water fish native to the Laurentian Great Lakes with an increasing recreational importance, although they are not currently managed in Lake Superior. Burbot are benthic predators that play a substantial role in the trophic ecology of the Great Lakes, both as a top predator and as a food source for lake trout (*Salvelinus namaycush*) (Leon 2008, Cott et al. 2011). They are the only freshwater representative of the Gadidae family and exhibit some similar life history strategies to their saltwater relatives (McPhail & Paragamian 2000). Burbot can occupy inland lakes, rivers, streams, and can reach depths as great as 366 meters during the summer months in the Great Lakes (Boyer et al. 1989).

Burbot are extremely fecund, like other gadid species, and have been estimated to produce anywhere from 24,000 to 3,477,000 eggs per season, depending on the size of the fish (Roach and Evenson 1993). The sheer number of eggs likely makes up for the high mortality due to the limited resources that each egg receives (Adams 1980). Eggs are approximately 1 mm in diameter when they are released from the female and able to be fertilized. As with marine fish larvae, the large number of eggs and small egg size contribute to mass early mortality (Garrido et al. 2015). Burbot embryos can take anywhere from 25 days at 6.1°C to 128 days at close to 0°C to develop, depending on where they originate and temperature (McPhail and Paragamian 2000). Warmer water temperatures can cause a shorter developmental period, increased deformity, and increased mortality of burbot protolarvae (Ashton et al. 2021). Burbot have low sperm motility with their sperm losing all mobility within 40 seconds of activation (Lahnsteiner et al. 1997). Temperature plays an important role in spawning efficiency as well as larval development. Low temperatures during the spawning period correlated with higher egg production in a Czech stock of burbot (Slavik & Horky 2021). Males experience their highest sperm motility at low

temperatures between 2° - 4°C, although sperm velocity is the greatest at slightly higher temperatures 4°C - 6°C (Lahnsteiner & Mansour 2012). Lake Superior has the coldest annual mean temperature of the Laurentian Great Lakes at 3.6°C and temperatures from January through March, when burbot are spawning, are between 0°C and 2°C (Bennett 1978). Because Lake Superior is such a large and variable system, the different cold temperatures and variable habitat may affect spawning habits of burbot.

Burbot typically spawn in shallow rocky reefs or nearshore environments, but there are also burbot that utilize rivers for spawning habitat (McPhail & Paragamian 2000, Jude et al. 2013). There are currently four documented spawning strategies that Great Lakes region burbot exhibit: landlocked populations in inland lakes, river-resident populations, adflucial populations with spawning migrations into the rivers in the wintertime with larvae drifting to the lake, and lacustrine populations with spawning in shallow rocky areas in the main lakes (Jude et al. 2013). River migration has previously been documented in the Sturgeon River, Chassel MI with a fairly narrow spawning window from late December into early January (Ritz et al. 2020). Spawning runs have also been observed in the Chocolay River, Marquette MI, and AuTrain River, Alger MI, which show similar spawning windows (Woodworth, 2023). Burbot have been well documented in resident river populations such as the Kootenai River, Idaho (Hardy & Paragamian 2013). In the Kootenai River system, burbot spawn from the first week of January through February during their spawning migration (Paragamian et al, 2000). Larval burbot have been captured in Lake Michigan ranging from 3.0-7.5 mm from March until June, suggesting that there is a mix of nearshore and river spawning (Mansfield et al. 1983). Larval burbot have been documented in high densities, averaging 139 individuals per hectare, on Julian’s Reef, Lake Michigan (Edsall et al. 1993). This high larval density indicates that there could be large

numbers of spawning adults utilizing mid-lake reefs to yield such high densities of larvae. Jude (2013) found recently hatched, 3-4-mm larval burbot present in the Great Lakes during offshore collections in August suggesting that burbot in the main lakes can spawn later in the spring and into early summer. Other populations of resident river burbot in Alaska have been observed exhibiting spawning behavior from November through March, resulting in extreme variability in environmental conditions during their spawning and subsequent embryonic development (Breeser et al. 1988). Despite the long spawning season, female burbot have a short fertility window (Zarski et al. 2010, Boag 1989, Woodworth 2023), which raises the question of how this spawning and larval variability occurs in nature.

Burbot go through three stages during their time as ichthyoplankton: yolk sac, preflexion, and flexion (Ritz et al. 2020). After this, they are considered juveniles. Although there are a plethora of environmental conditions that can impact burbot, temperature plays an integral role on their rate of growth and development as larvae (Barron et al. 2012). Higher temperatures correlate with larvae growing at faster rates (Barron et al. 2012). When larvae hatch, the duration of the yolk sac stage depends heavily on temperature, with warmer temperatures causing faster yolk sac utilization (Kupren et al. 2013). Kupren (2013) reared larval burbot at 6°C and documented the yolk sac stage lasting 0-8 days post hatch (DPH), preflexion was 9-26 DPH, and flexion occurred from 28-34 DPH. Fischer (1999) documented wild caught larvae in Lake Constance, Germany in their yolk sac stage until 23 DPH while Ghan & Sprules (1993) state that exogenous feeding occurs as early as five DPH. Environmental conditions and food availability likely cause variability in early development. Temperature also plays a substantial role in burbot embryonic development with embryos taking anywhere from 30 days to hatch at 6°C, to 128 days to hatch at temperatures close to 0°C (McPhail & Paragamian 2000). Embryo development

and larval growth rates of burbot have not been well documented at temperatures as cold as Lake Superior, and this study investigated the impact colder natural temperatures have on their growth and development through early life stages.

Larval burbot hatch at lengths of 3 - 4 mm and can actively or passively drift, depending on the environment in which they hatch (McPhail & Paragamian 2000). Although burbot passively drift during their early stages, larvae can swim with short bursts of directed swimming (Ghan & Sprules 1991). Larval burbot have been documented to have a homogenous distribution throughout the water column during their nearshore migrations in Lake Oneida, New York, in May and June (Clady 1976). However, burbot have also been documented to be more abundant in the hypolimnion or profundal zones during their late spring migrations in Lake Constance, Germany (Fischer 1999). Larval burbot initially feed on rotifers, followed by larger copepod nauplii and cyclopoid copepods, regardless of their depth and location in the lake (Ghan & Sprules 1993). Variable otolith growth rates in larval burbot indicate that they likely encounter large environmental changes during their pelagic larval stage (Fischer 1999). Because of the variability in burbot spawning strategies in the Great Lakes and the development time when embryos and larvae can be passively drifting, little is known about when and where larvae are hatching, drifting, settling, and feeding.

Larval growth is closely correlated to temperature with larvae reared at higher temperatures growing at faster rates (Barron et al. 2012). However, survival was inversely related to temperature with burbot reared at higher temperatures experiencing lower survival rates (Barron et al. 2012). Burbot from Barron et al. 2012 were reared from 10°C to 20°C which is substantially warmer than any temperatures Lake Superior burbot would ever experience, especially during their early life stages. Larval burbot have all the typical difficulties that larval

fish experience such as high competition, food availability, environmental conditions, and predation, but burbot in Lake Superior also must deal with the extreme weather (storms, etc.) and cold temperatures. Because diet is not influenced by location or depth, survival is dependent on timing larval hatch with food availability. The need to circumvent all of the factors leading to larval mortality could have led burbot to adapt variable spawning habits to ensure that some of their offspring are hatched at more advantageous conditions.

Asynchronous hatching is a strategy used by many fish, including other cod species where embryos that are exposed to the same conditions will hatch over an extended period of time, presumably to improve the odds that some of the larvae will hatch in favorable conditions in a variable environment (Laurel et al. 2008). Temperature has a strong correlation to the duration of the asynchronous hatching period with Pacific cod, *Gadus macrocephalus*, taking six days at 8°C and 17 days at 0°C (Laurel et al. 2008). Asynchronous hatching not only impacts when larvae hatch, but it also impacts their initial morphology and growth rates (Politis et al. 2014, Laurel et al. 2008). Atlantic cod, *Gadus morhua,* larvae that hatched later in the asynchronous hatching period had longer total lengths and smaller yolk sacs than those hatched at the beginning of the period (Politis et al. 2014). Politis (2014) also found that larvae hatched earlier had faster growth rates than late hatchers. Differences in the initial morphology of burbot yolk sac larvae may also cause larvae to begin feeding exogenously at different times (Ritz et al. 2020). There are positive correlations for cod species between temperature and larval deformities, as well as temperature and growth (Politis et al. 2014). Ritz (2022) demonstrated that Lake Superior burbot hatch asynchronously over 21 days when reared at 3°C. Because burbot are close relatives to cod and they exhibit many of the same life history strategies

surrounding reproduction, this study investigated if the same patterns that larval cod experience as a result of asynchronous hatching are observable in Lake Superior burbot.

Burbot development has been explored mainly for the purposes of aquaculture, which provides limited insight into what growth patterns are occurring naturally. Burbot hatcheries generally increase the temperature at the end of the embryo incubation period to cause stress-induced synchronous hatching (Zarski et al. 2009, Ritz et al. 2020). However, the Polish population of burbot that Zarski (2009) used had extremely low mortality rates of 10% while reared at 12°C, which is the warmer end of what North American burbot would experience or tolerate. Artificially generating a synchronous hatching period masks the length of the natural hatching period and other consequences of hatching asynchronously that may be important in the wild. The duration of the embryonic and hatching periods at natural temperatures are important pieces of information in beginning to understand early life history, survival, and recruitment for Lake Superior burbot. The purpose of this study is to fill gaps in knowledge about the early development of burbot at colder natural temperatures under conditions similar to Lake Superior, to investigate the asynchronous hatching period and its impact on initial larval morphology, and to determine the impact asynchrony has on larval development.

METHODS

Fish Capture and Gamete Collection

Adult burbot were caught with hoop nets in the Chocolay River (Marquette County: 46.5001148, -87.3500539), Michigan, and the AuTrain River (Alger County: 46.4309114, -86.8370201), Michigan. Hoop nets were 2m long, 1m in diameter, made of 25mm mesh, and had a 250mm opening oriented downstream to catch fish migrating upstream. Hoop nets were set in December and checked daily until burbot were no longer migrating up the rivers in March of 2022 and 2023. Spawning condition of each burbot was assessed and fish that were actively releasing gametes were chosen for fertilization according to Neufeld et al. (2011).

Burbot were spawned in a 1:1, male:female ratio and different pairs were used for each family. Milt was collected from the male into a small container. Eggs were strip spawned directly into a 1420 mL container and the milt was immediately added in a ratio of 0.375 mL for every 50 mL of eggs. Because burbot have low sperm motility and their sperm have a short duration for motility once they are activated, the volume of milt needed was divided into three equal portions and mixed with the eggs at 0 seconds, 30 seconds, and 60 seconds to maximize fertilization rates (Kucharczyk 2016, Lahnsteiner et al. 1997). The gametes were mixed with a feather for one minute then 500 mL of filtered river water was added to the container. The fertilized embryos were allowed to rest for 30 minutes to allow for water hardening. After hardening, 3 mL of Ovadine (Syndel, Ferndale, WA) was added to disinfect the embryos for 30 minutes. Embryos were rinsed and stored in 3°C water in a temperature controlled cooler until they were transported to the Aquatics Laboratory at Northern Michigan University. Eight families of burbot were fertilized in 2022 and ten families were fertilized in 2023. Fertilization rates were only calculated for the 2023 families (Table 1).

Embryo Rearing

Each family of embryos was transferred to a 2 L conical egg jar (2L Conical Egg Jar, Pentair Aquatic Ecosystems, Florida) that sat in a temperature-controlled water bath set at approximately 3°C. Families of embryos were named by their river followed by aa number for the 2022 families, and a letter for the 2023 families (ex. Choc4, AuTrainB). The recirculating water bath systems received constant waterflow, aeration, and UV sterilization. Each egg jar received constant waterflow at a rate of 2.5 L per minute to keep the eggs agitated. Tank temperatures were monitored using temperature loggers (HOBO 64K Pendant, Onset, Massachusetts) that recorded temperature every five minutes. Light was kept at a relatively natural, constant photoperiod (spring) of 12 hours of daylight and 12 hours of night for the entirety of this experiment. Twenty embryos from each family were randomly sampled every three days following fertilization. Embryos were photographed under a WXW Digital Biological Microscope at 100X magnification with a micrometer.

In order to track fertilization rates and embryo survival of individual embryos, two microplates (96 Well U-bottom Assay Plate, Becton Dickinson Labware, Franklin Lakes NJ.) were used to incubate embryos from each family during the 2023 spawning season, using methods similar to Unuma (2004). Within six hours of fertilization, embryos were pipetted into each well on the microplates so that each 330µl well received one embryo and ~200µl of treated tank water. The microplates were stored in a refrigerator at 2°C until hatch with no extra aeration or waterflow going into the wells. Fertilization rates were calculated for each microplate based on the number of fertilized eggs after one day.

Separation of the chorion and the formation of the perivitelline space in burbot eggs and embryos was evaluated for three of the families that were fertilized from the Chocolay River

between January 7, 2023 and January 10, 2023. Family A was ~94,500 eggs, family B was ~105,000 eggs, family C was ~52,500 eggs. Egg counts were estimated based on an average number per volume of 1,050/mL (Jensen et al. 2008). Approximately 5,000 eggs from each female were placed in water without milt to compare water activated eggs to fertilized embryos. Photographs were taken of the embryos and water activated eggs every hour for the first 24 hours post fertilization, and every three days afterwards to track development (Figure 1).

Larval Rearing

Four families were observed during their asynchronous hatching period and early larval development: Choc4, ChocB, ChocH, and AuTrainB. Once hatching started, larvae were pipetted from the egg jar on the day they hatched into 400 mL incubation jars and separated by their hatch date. Incubation jars were held in floating trays in the two temperature-controlled tanks. Both 300 gal tanks were recirculating and received waterflow (Submersible Pump, JBJ Aquariums, California) at a rate of 9 L/min. The tanks were kept at 3°C using aquarium chillers (Arctica Titanium Chiller, JBJ Aquariums, California) and given constant aeration using air stones. Water was filtered using a particulate filter and a UV light filter (Lifegard Ultraviolet Sterilizer Model QL-40, Pentair Aquatics, Florida) before recirculating in the system.

Once larval burbot absorbed their yolk sac and entered their exogenous feeding stage around 55 days post fertilization (DPF), they were initially fed brackish water rotifers (*Branchionus plicatilis*) by hand three times a day at a density of approximately 1,250 rotifers per 250 mL of volume. Twenty larval burbot from each hatch date were photographed the day they hatched and every other day for their first 20 days post hatch (DPH). Larval burbot associated with hatch dates were then photographed every five days from 21 to 40 DPH. All larval photos were taken with a WXW Digital Biological Microscope at 40X magnification with

a micrometer for scale. The software SeBaView was used to analyze all larval photos and calibrated to a micrometer for each picture. The total length and yolk sac area of each larvae were measured, and the presence of a capsule and any deformities were noted. There are no data on survivorship of larvae by date due to the number of larvae observed from each family. Data collection ended 40 days post hatch because this project is focusing on the asynchronous hatching period and the early development stages of the larvae. Data on the duration of the asynchronous hatching period and the growth and development of larvae were collected for families Choc4, ChocB, ChocH, and AuTrainB (Table 3). Only data on initial morphology of hatch groups was collected for family AuTrainB.

All field sampling and lab work followed NMU IACUC 410 protocol (Appendix A)

Statistical Analysis

One-way ANOVA was used to compare the initial total length and initial yolk sac area for early, peak, and late hatchers for Choc4, ChocB, ChocH, and AuTrainB. Hatch groups were compared within families to identify similar trends across all families. Hatch groups were also compared between families to determine if family was a cause for variability in initial morphology. If the initial or final day of hatch didn't have a large enough sample size for any of the families, the next closest day was used for analysis. Regressions were run to examine the relationships between the hatch date and the initial total length and yolk sac areas for each family.

ANCOVA was used to compare the growth rates and yolk sac utilization rates between early, peak, and late hatching larvae within families. ANCOVA was also used to compare growth rates and yolk sac utilization rates between families to determine if family impacted growth and development. Binomial logistic regression was used to analyze the relationship

between hatch date, inability to escape the capsule at hatch, and the presence of deformities at hatch.

RESULTS

Embryo Development and Asynchronous Hatching

Fertilization rates varied among the families and ranged from 15.6% to 97.4% success (Table 1). Three of the families were spawned in the lab and had the lowest fertilization rates: ChocG (15.6%), ChocB (26.6%), and ChocC (46.5%). The average fertilization rate for Chocolay River families was 62.38% ±0.29% and the fertilization rate for the AuTrain family was 67.70%. Embryos from families ChocA, ChocB, and ChocC had very similar timing in early development while incubated at 3°C. Initiation of the first cleavage occurred 10 hours post fertilization with each subsequent cleavage taking 3 to 3.5 hours to complete. The embryos from ChocA, ChocB, and ChocC all followed the same timing for cell division through blastulation (Figure 1).

Once the unfertilized eggs were water activated, the perivitelline space was formed visually similarly to a fertilized embryo. Cytoplasm gathered at the animal pole of the embryo at the same rate for unfertilized and fertilized eggs. Water activated eggs were not able to be distinguished from fertilized embryos until the initialization of the first cleavage at 10 hours post fertilization (HPF). Water activated eggs continued gathering cytoplasm at the animal pole while the embryos continued with cell division.

Embryonic development periods for the Chocolay River families averaged 31 days (±1.7 days), and the embryo development period for the AuTrain River family was 27 days (Table 3). Chocolay River families ranged from 98.43 to 126.3 accumulated thermal units (ATU) to their time of first hatch while the AuTrain River family began hatching at 86.48 ATU (Table 2). Families from the Chocolay River had hatching periods that ranged from 20 to 26 days; the AuTrain River family's hatching period lasted 39 days (Figure 2).

Morphology at Hatching

The relationship between hatch date and initial total length at hatching was compared between hatch groups within families. Family ($F=62.55$, $df=3, 1779$, $p<2.2e-16$), DPH ($F=864.1$, $df=1, 1779$, $p<2.2e-16$), and the interaction between family and DPH ($F=33.76$, $df=3, 1779$, $p<2.2e-16$) all had a significant impact on the initial total length of larval burbot. When hatch early, peak, and late hatching larvae were compared within families, mean initial total length of larvae from family Choc4 was not significantly different between early, middle, and late hatchers ($F=1.168$, $df=2, 35$, $p=0.323$) (Figure 5). Mean initial total length was significantly different between early and mid-hatchers, and early and late hatchers for families ChocB, ChocH, and AuTrainB ($P<0.05$) (Figure 5). Larvae that hatched later in the hatching period were longer than early hatching larvae.

The relationship between hatch date and initial yolk sac area at hatching was compared between hatch groups within families. Family ($F=966.4$, $df=3, 1779$, $p<2.2e-16$), DPH ($F=5912$, $df=1, 1779$, $p<2.2e-16$), and the interaction between family and DPH ($F=51.65$, $df=3, 1779$, $p<2.2e-16$) had a significant impact on the overall growth rates of families of larval burbot during their yolk sac stage. When initial yolk sac area at hatching was compared between early, peak, and late hatching larvae within families, mean initial yolk sac area was significantly different between early, middle, and late hatching larvae for family Choc4 ($F=105.2$, $df=2, 35$, $p=1.58e-15$) (Figure 6). Mean initial yolk sac area was significantly different ($p<0.05$) between early and middle, and early and late hatching larvae for families ChocB, ChocH, and AuTrainB (Figure 6). Larvae hatched later in the hatching period had much smaller yolk sacs than those hatched earlier.

Growth and Development

Growth rates were compared between hatch groups within each of the Chocolay River families. Family, DPH, and the interaction between family and DPH had a significant impact on the overall growth rates of families of larval burbot during their yolk sac stage ($F=1499$, $df=5$, 16048, $p<2.2e-16$) (Figure 7). Larvae from all families that were hatched earlier in the asynchronous hatching period had higher growth rates.

Yolk sac utilization rates were compared between hatch groups within each of the Chocolay River families. Family and DPH had a significant impact on the overall yolk sac utilization rates of families during their yolk sac stage ($F=808.2$, $df=5$, 16047, $p<2.2e-16$) (Figure 8). Larvae from all families that were hatched earlier in the hatching period had faster yolk sac utilization rates and larger initial yolk sacs than those hatched later.

Deformities and Capsules

Every family had larvae that were unable to escape their chorion (capsule) when they hatched. Remaining encapsulated was not a deformity, but rather an inability to fully hatch. Because there was no way to determine which fish were able to escape from their capsule later or if they died, partial de-encapsulation was evaluated only at hatching and compared between hatch groups and families. Family ChocH had the highest percentage of larvae unable to escape their capsule at hatch (46.3% ± 20.0%) and AuTrainB had a very low percentage of encapsulated larvae at hatch (10.9% ± 25.0%). The probability that larvae were unable to escape their chorion when they hatched was significantly related to their hatch date ($P=0.0064$) (Figure 9). Larvae hatched earlier in the asynchronous hatching period had a higher likelihood of being unable to escape from their chorion.

There were various deformities that appeared in the larvae such as misshapen yolk sacs, bent notochords, and bent tails. The probability that larvae were deformed at hatch was

significantly related to their hatch date ($P<0.001$) (Figure 10). Larvae were more likely hatch with deformities the later they hatched in the asynchronous hatching period.

DISCUSSION

Egan (2014) provided a baseline for the timing of embryonic development for burbot in an aquaculture setting, but lacked the variability caused by asynchronous hatching. This study builds on the foundation of knowledge on burbot early life history by investigating the effects of natural variability of developmental at cold temperatures. Canadian burbot populations from Moyie Lake, British Columbia, have been reared at 3°C and experienced very similar timing for both embryonic development and first hatch as the families from the Chocolay and AuTrain Rivers in this study. Egan (2014) recorded cell divisions taking ~8 hours to complete which was much slower than the embryos in families ChocA, ChocB, and ChocC (Table 2). Fertilization rates varied widely between families used in this study, but the most notable trend was the decrease in fertilization rates when fish were held in the lab prior to fertilization. Low sperm motility may have been a possible cause for the low fertilization rates in lab-fertilized families. Similar observations have been seen when comparing the overall health of burbot sperm between a cultured broodstock and wild-caught burbot (Blecha et al. 2018). Blecha (2018) observed less of the broodstock producing sperm, and the sperm that the broodstock fish did produce had low motility (<5%). The lower fertilization rates and sperm count observed in lab-reared burbot may indicate that captivity stress has an impact on sperm health and fertilization rates. Ashton (2019) used wild caught burbot for lab fertilizations and saw no relationship between the age or size of the females and the survival of embryos. Even though there were timing differences in early embryonic development, Egan (2014) still saw fish beginning to hatch at 33 DPF, similar to my study. Embryonic development averaged 30 days at 3°C in this study before onset of hatching. Lake Superior and its tributaries can be much colder (as low as 0°C) throughout the spawning season, which still leaves the question of how long burbot embryos take to develop in their

natural environment. Burbot embryos can experience more variable, colder temperatures in a natural setting which further adds to the variability in hatch times within families.

This study compared the initial morphology of larval burbot throughout the asynchronous hatching period and demonstrated the consequences of asynchrony. Early hatching burbot from this study resembled yolk sac larvae as described by McPhail and Paragamian (2000) and Ritz (2020). Larvae that hatched very late in the asynchronous hatching period had very little yolk sac left, had a developed jaw, and would be considered preflexion larvae despite being newly hatched. Larvae from the same family hatched with varying morphology at different points in the asynchronous hatching period with some larvae exhibiting extended embryonic development and skipping their larval yolk sac stage altogether. Similarly, Politis (2014) found that asynchronous hatching caused differences in initial morphology of Atlantic cod larvae. Although the cod asynchronous hatching period was shorter than that of burbot, he noted that the initial total lengths were shorter, and the yolk sacs were larger in earlier hatching larvae (Politis et al. 2014). Like Atlantic cod, burbot that hatched earlier in the hatching period had larger yolk sacs and shorter total lengths than burbot hatched later in the hatching period. Asynchronous hatching caused larvae within the same family to display very different morphologies at the time of hatch, but the consequences of these differences were more evident when the growth of the burbot larvae was evaluated. When lab-reared Atlantic cod hatch at different sizes, the morphological differences in larvae at hatch diminish over time, implying initial morphology doesn't impact development of cod (Jordaan et al. 2006). The differences in initial morphology of burbot in this study had long term impacts on the growth rates and yolk sac utilization rates of larvae with outcomes that persist into the juvenile stage. These morphological effects of asynchrony also result in fish being at different stages when they first encounter the environment outside their

capsule. Some must immediately begin to feed, since they essentially lack a yolk sac, while others are well-provisioned with yolk, but have poor movement ability and little control over their orientation. These differences are likely to result in differential responses to the environment, though we have little understanding of the ecological consequences of these different experiences.

Along with the variation in developmental stage at hatching, asynchrony generates the opportunity for embryos from the same family to hatch in different locations in the natural environment because of the variability in time until hatching. Environmental factors such as temperature and current may greatly influence the dispersion of larval burbot in rivers and nearshore environments, depending on their time spent as an embryo. It is also likely, though poorly understood, that the encapsulated embryo is moved differently through the water than a hatched embryo because of shape and density differences. This results in the potential for high variability in dispersal of a family, which could potentially be advantageous for fitness in a highly variably or risky habitat. It is possible that selection acts differently on early and late hatchers from a single spawning in such a way that these differences in morphology are adaptive to the hatch location. However, not enough is known about the dispersion of larval burbot in the Great Lakes to make these connections with any clarity.

Asynchronous hatching has been observed in Atlantic Salmon (*Salmo salar*) and larvae that emerged earlier were larger and had better survival than larvae that hatched later (Einum and Fleming 2000). Larval burbot that hatched earlier in the hatching period exhibited similar trends to Atlantic Salmon in that early hatchers had faster growth rates and fewer deformities than late hatchers. The most notable trend in this study is that earlier hatching fish from all of the Chocolay River families had significantly better growth rates and less deformities than larvae

that hatched later, despite the larger size at hatching of late hatchers. Hatching at a larger size can have benefits such as better swimming capabilities and the ability to consume larger prey items (Mayer & Wahl 1997). However, some larger larval fish experience higher predation rates (Litvak & Leggett 1992). The natural predation rates on larval burbot are unknown, but the greater growth rates and decreased morphological deformity in smaller hatching larvae may be an adaptation for better dispersal and potential predator avoidance during their earliest larval stages.

The overall growth rates of families were variable throughout the yolk sac stage, creating an even wider range of phenotypes when coupled with the different growth rates exhibited by intrafamilial hatch groups. Although the Chocolay and AuTrain Rivers are both tributaries on Lake Superior, the larvae from the two rivers exhibited differences in growth rates. Ritz (2020) showed differences in growth rates in spatially separated populations of larval burbot throughout their larval stages. The differences in growth rates between spatially separated populations, between rivers connecting to the same lake, as well as within families, highlight the variability in early growth of larval burbot. These patterns substantially complicate back-calculation of hatching for wild-collected burbot larvae, and it would be advantageous to evaluate other methods of aging these young fish rather than relying on size.

A large percentage of burbot larvae were unable to escape from their capsule at hatch in this study. It is unknown whether these encapsulated larvae would have been able to escape from their capsules later during their yolk sac stage or if the capsules are a chokepoint for mortality in yolk sac larvae. We also cannot rule out that persistent encapsulation was an artifact of artificial rearing conditions. Low pH has been shown to prevent the chorion from completely degrading in trout species, leading to hatching problems and the inability to escape the chorion (Kugel et al.

1990), although the pH of our rearing water was 8.6. Other researchers have suggested that persistent encapsulation may be linked to cold incubation (Ken Cain, University of Idaho, pers. comm.), although it's unclear how this may intersect with population differences. Nonetheless, if larvae are unable to escape from their capsule before they absorb their yolk sac and need to start exogenous feeding, the capsule would prohibit feeding, leading to death. If it occurs in nature, inability to escape the chorion may be a cause of early mortality in larval burbot during the early yolk sac stage.

Prior studies have shown that when burbot embryos from the Kootenai River, Idaho, encounter temperatures above 4°C within the first week of development, they experience higher rates of mortality and deformity in prolarvae (Ashton et al. 2021). Burbot embryos experienced the best survival rates and the lowest amount of deformity at lower temperatures around 2°C (Ashton et al. 2019). None of the observed larval families in this study were kept at temperatures as low as 2°C, which could have led to an overall increase in larval deformity. However, if this is the case then family Choc4 would have been expected to have the highest deformity rates due to higher tank temperatures, yet ChocB and ChocH had higher rates of deformity.

Deformed larvae showed a wide range of total length regardless of their hatch date. However, due to the increased number of deformed fish hatching later in the hatching period, the increased variability may have lessened the differences in total length between early and late hatching larvae. Because larvae were randomly sampled from each hatch group every other day, no data on individuality was able to be collected to see if deformity was a cause of mortality in yolk sac larvae. The rates of larval deformity in wild populations of burbot are unknown, and we do not know if deformity in larval burbot is an artifact of lab rearing. Developing a better

understanding of what deformities larval burbot display and the rate at which they express them would add to our understanding of recruitment barriers.

In conclusion, burbot in Lake Superior have multiple spawning strategies that range in timing from late January through March with evidence suggesting they can continue spawning into April (McPhail 1997, Woodworth 2023). The families of burbot that were observed in this study had embryonic development periods (prehatching incubations) up to 32 days with hatching periods as long as 39 days. The extended duration of early development and slow growth rates in this species, tied to low embryonic and larval temperature conditions and a planktonic life history, may result in highly variable environmental conditions at hatching combined with variable dispersal. Differences in initial morphology and growth rates between families, locations, and within families add additional variability into the complex early life history of larval burbot. Given the importance of selection on embryos and larvae in a highly fecund species, greater understanding of the complexities of early life history in this species is warranted to appreciate population dynamics and species recruitment. Embryonic and larval response to environmental change seems likely to also be critical to the resiliency of the species to ongoing habitat and climate change.

Family	Fertilization Date	Fertilization Rate	Fertilization Location	Size of Female (mm)	Size of Male (mm)
Choc4	Jan 14, 2022	Unknown	Field	551	551
ChocA	Jan 7, 2023	86.46%	Field	510	429
ChocB	Jan 10, 2023	26.56%	Lab	407	452
ChocC	Jan 10, 2023	46.35%	Lab	384	452
ChocD	Jan 12, 2023	55.21%	Field	674	468
ChocE	Jan 13, 2023	97.40%	Field	537	477
ChocF	Jan 14, 2023	71.35%	Field	-	429
ChocG*	Jan 16, 2023	15.62%	Lab	521	477
ChocH	Jan 18, 2023	92.71%	Field	-	-
AuTrainA	Jan 26, 2023	67.71%	Field	549	478
AuTrainB	Feb 7, 2023	Unknown	Field	612	420

Table 1. Summary of the metrics important to each family of embryos: Fertilization Date, Fertilization Rate, location each family was fertilized, and the total length of the parents. *Family ChocG was fertilized in the lab, but the sperm used was kept on ice for 30 minutes and transported from the field to the lab.

Family	Rearing Temperature	Accumulated Thermal units to first Hatch	Hatch Period	Total Larvae
Choc4	3°C	107.84	26 days	5965
ChocB	3°C	126.29	20 days	4140
ChocH	3°C	98.43	22 days	5784
AuTrainB	3°C	86.48	39 days	-

Table 2: A summary of the fertilization dates, fertilization rates, temperature during the embryo development period in degree days, length of the asynchronous hatching period, and the total larvae hatched for each Family. Only families Choc4, ChocB, and ChocH were used to track larval growth and development; AuTrainB was only used in the initial morphology comparisons.

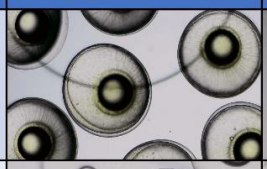
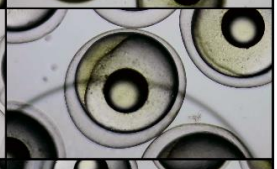
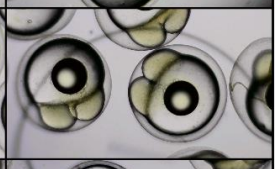
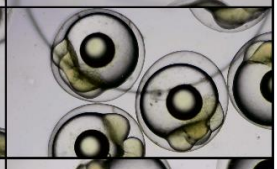
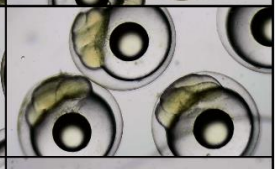
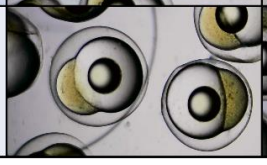
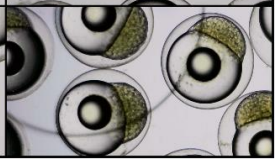
Hours Post Fert	Water Activated Eggs ChocB	Fertilized Embryos ChocB
1		
6		
10		
14		
17		
21		
24		
48		

Figure 1: Images of embryos from Chocolay River family B every hour for the first 48 hours post fertilization. Images included in the table are representative of important events during early embryo development: buildup of cytoplasm at the animal pole, beginning of first cleavage, two cell stage, four cell stage, eight cell stage, and blastulation. Unfertilized, water activated eggs from family ChocB were compared to determine when unfertilized eggs could be distinguished from fertilized embryos.

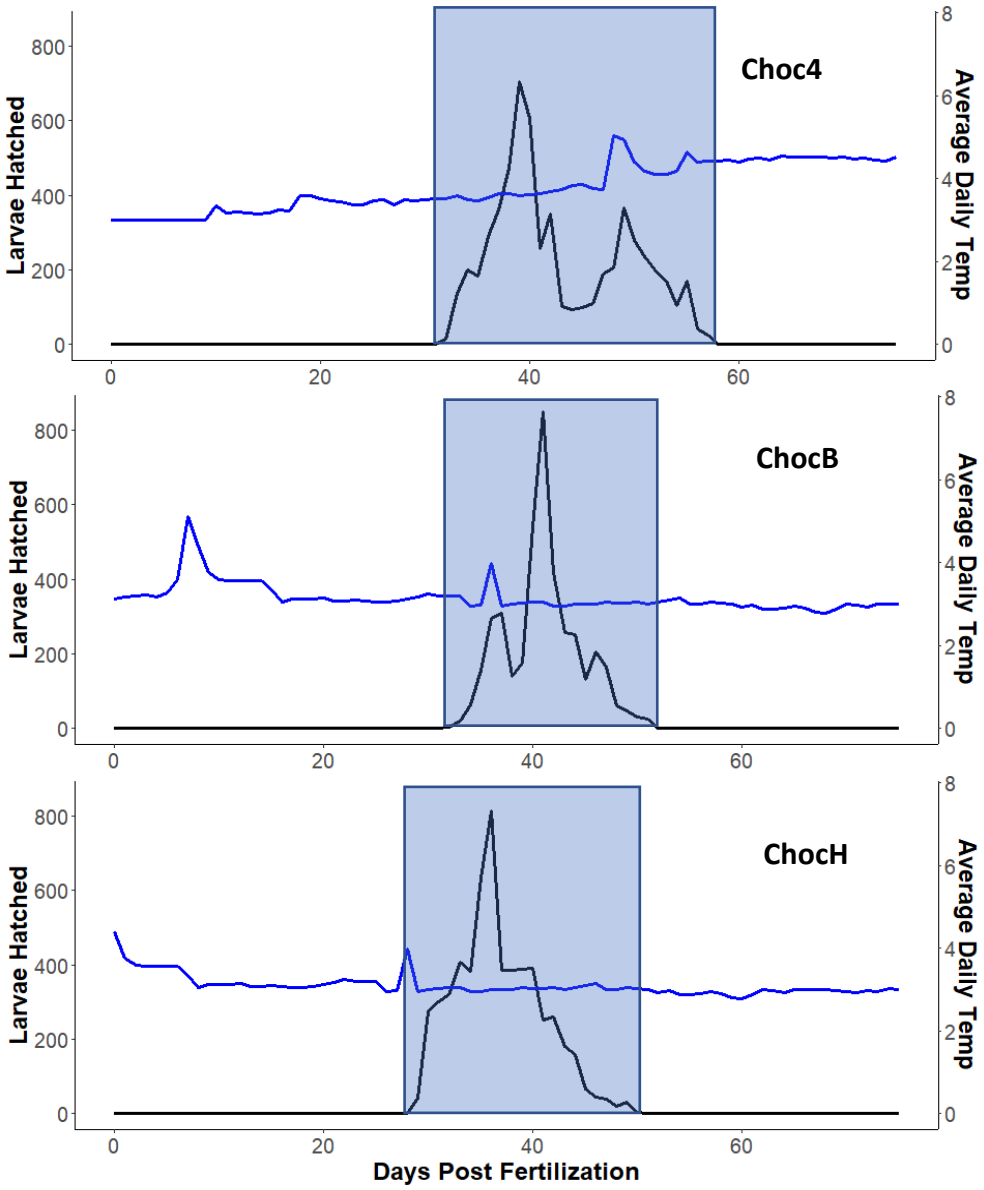


Figure 2: Larval hatch period for each family (in black) overlaid with the tank temperature (in blue). Blue boxes represent the duration of the hatch period for each family. The asynchronous hatching period for Choc4 was 26 days, ChocB was 20 days, and ChocH was 22 days.

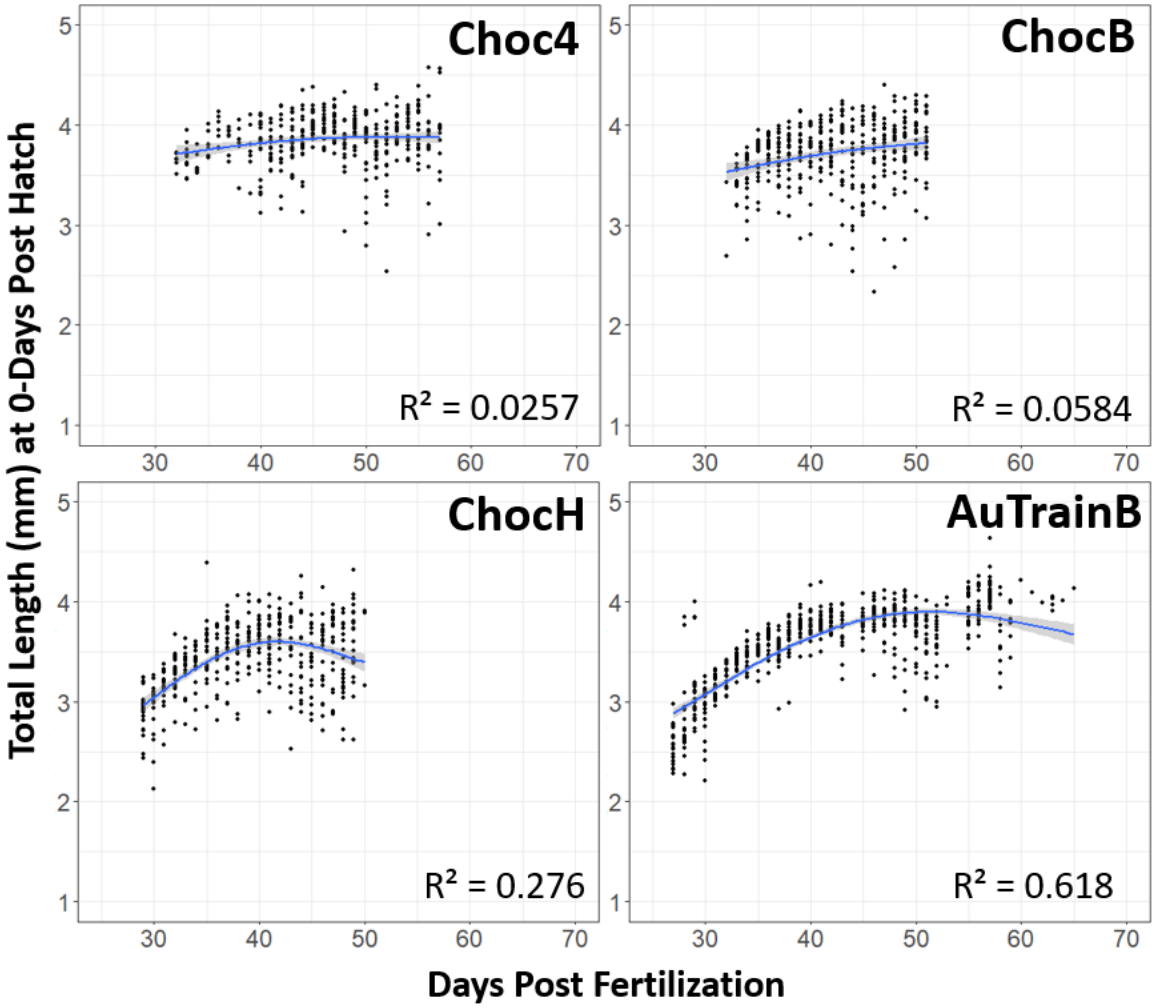


Figure 3: Total length of larvae on their initial day of hatch (0DPH) for each of the observed families. Families Choc4 (F=5.076, df=1, 407, P=0.00442), ChocB (F=11.73, df=1, 370, P=3.13e-5), ChocH (F=81.02, df=1, 418, P<2.2e-16), and AuTrainB (F=475.6, df=1, 584, p<2.2e-16) were compared.

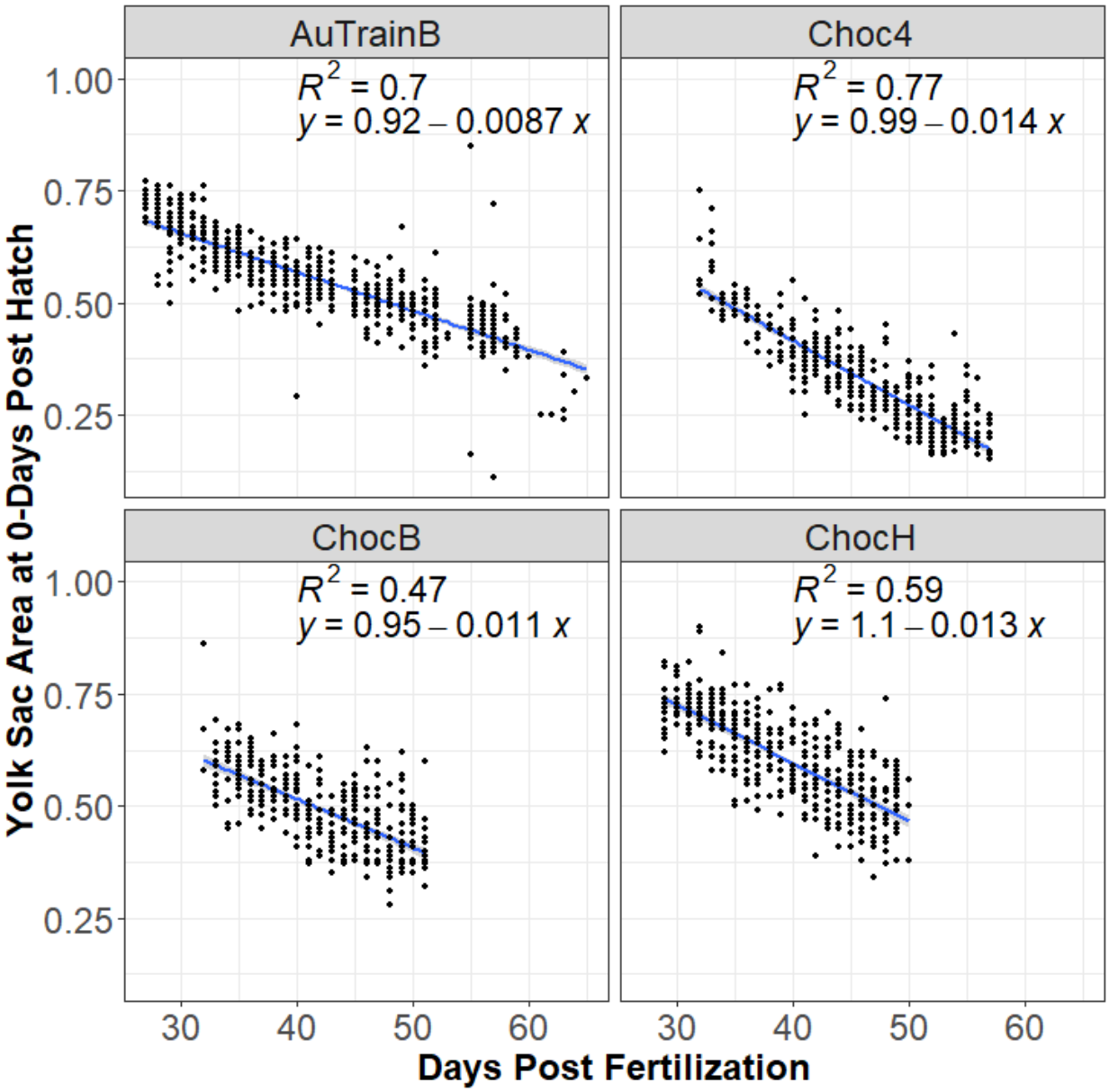


Figure 4: Yolk sac area of larvae on their initial day of hatch (0DPH) for each of the observed families. Families Choc4 (F=1345, df=1, 407, P=<2.2e-16), ChocB (F=327.8, df=1, 370, p<2.2e-16), ChocH (F=596.9, df=1, 418, p<2.2e-16), and AuTrainB ((F=1375, df=1, 584, p<2.2e-16) were compared. R^2 values shown represent how much of the variation in initial yolk sac area is explained by the hatch date.

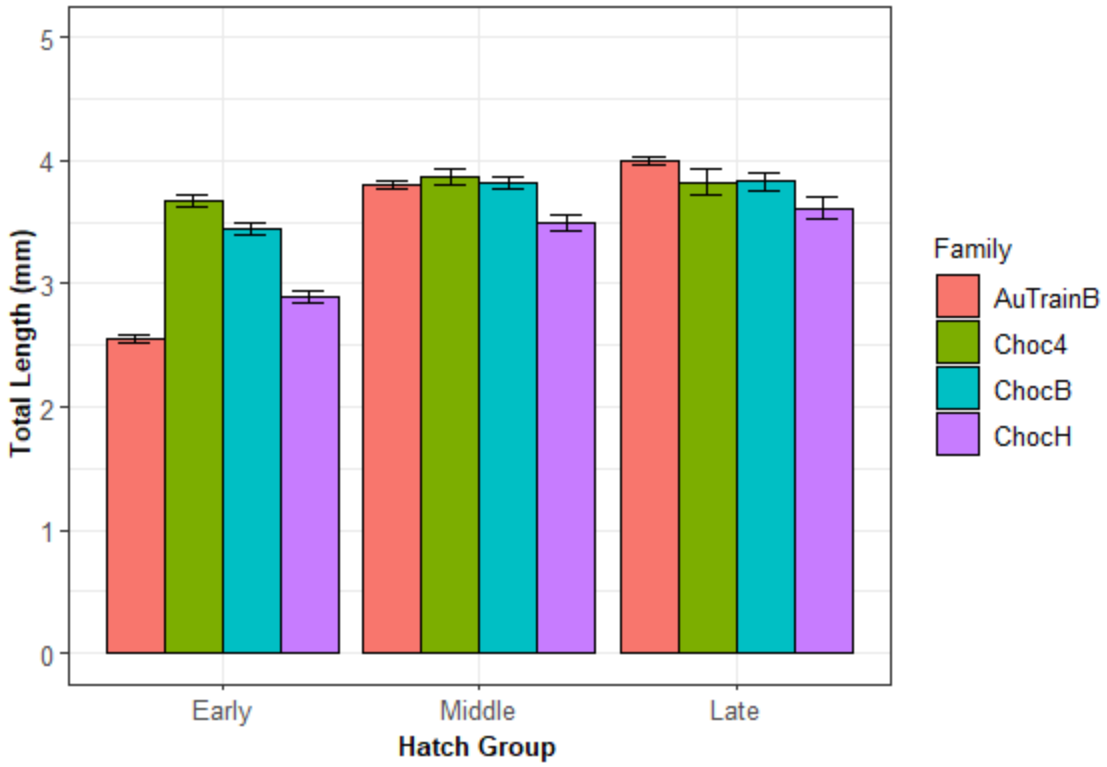


Figure 5: Mean initial total length for early, middle, and late hatchers from representative hatch groups from each family. Mean initial total length was significantly different between early and mid, and early and late hatchers for families ChocB, ChocH, and AuTrainB ($P<0.05$).

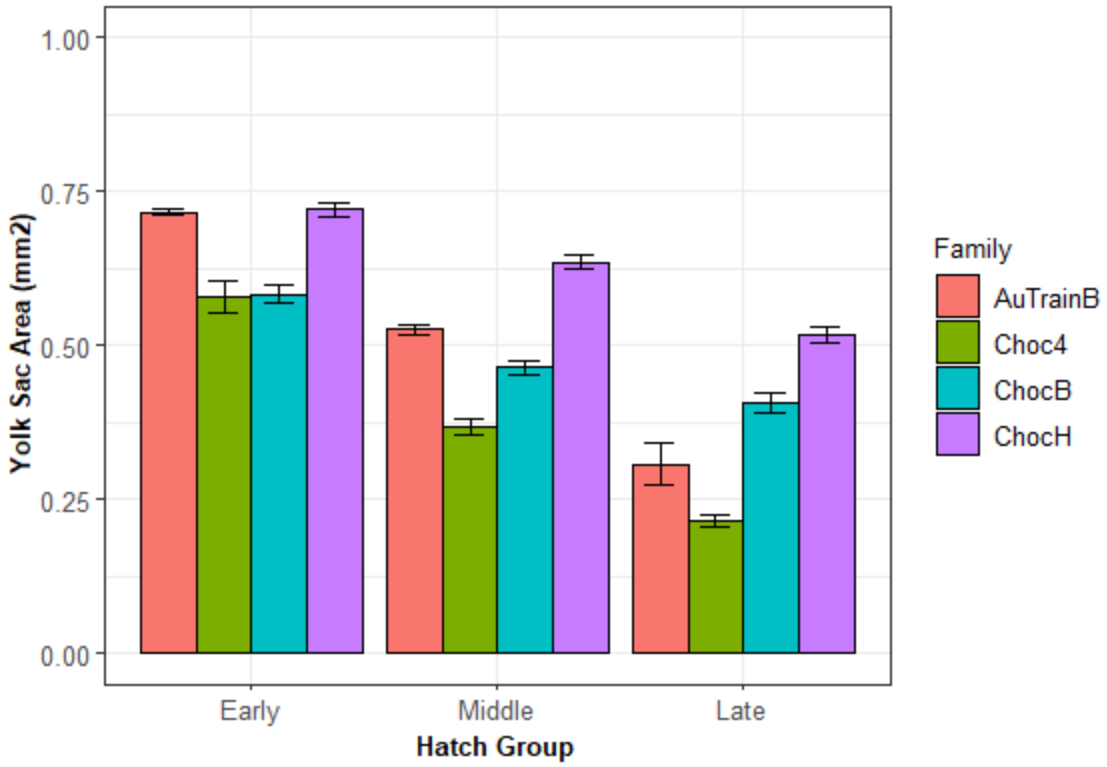


Figure 6: Mean initial yolk sac area for early, middle, and late hatchers from representative hatch groups from each family. Mean initial yolk sac area was significantly different between early, middle, and late hatching larvae for family Choc4 ($F=105.2$, $df=2, 35$, $p=1.58e-15$). Mean initial yolk sac area was significantly different ($p<0.05$) between early and middle, and early and late hatching larvae for families ChocB, ChocH, and AuTrainB.

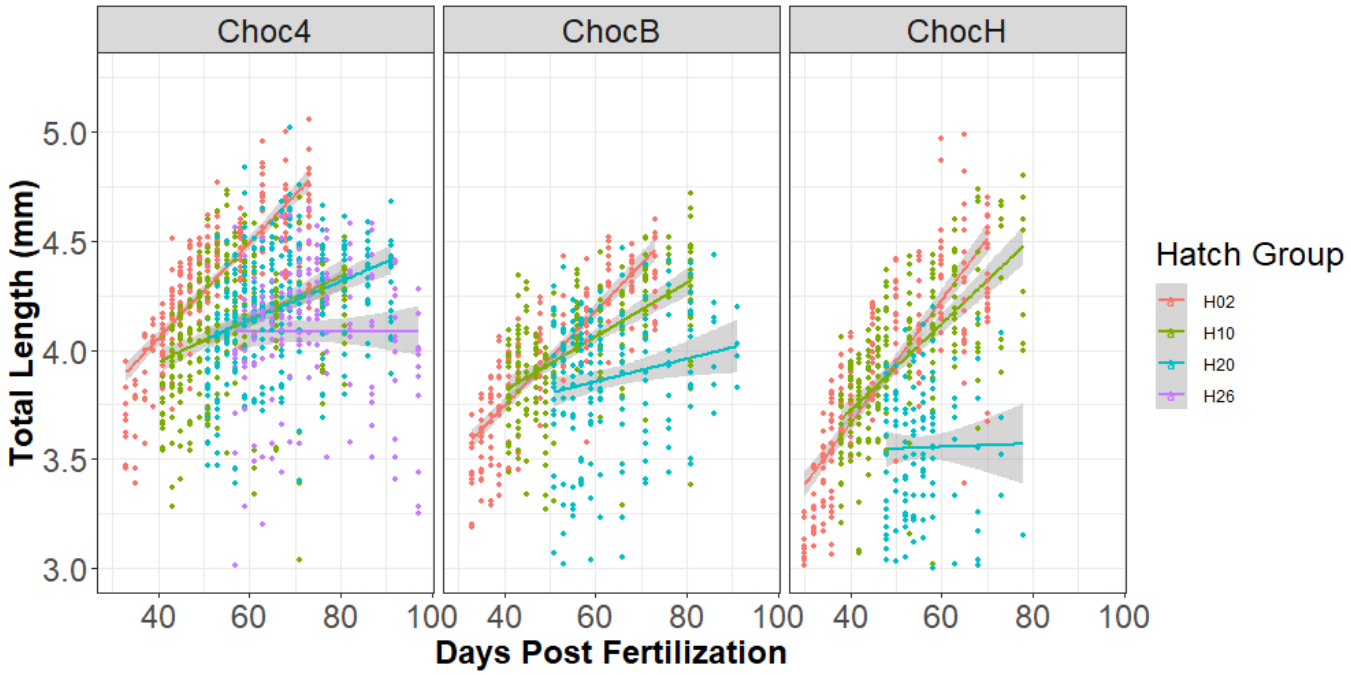


Figure 7: Change in total length over time as a measure of growth for representative hatch groups of early, middle, and late hatching larvae from each family. Regression lines show the growth rate for the selected hatch groups. Confidence intervals for regression lines are shaded in gray. Family, DPH, and the interaction between family and DPH had a significant impact on the overall growth rates of families of larval burbot during their yolk sac stage ($F=1499$, $df=5, 16048$, $p<2.2e-16$).

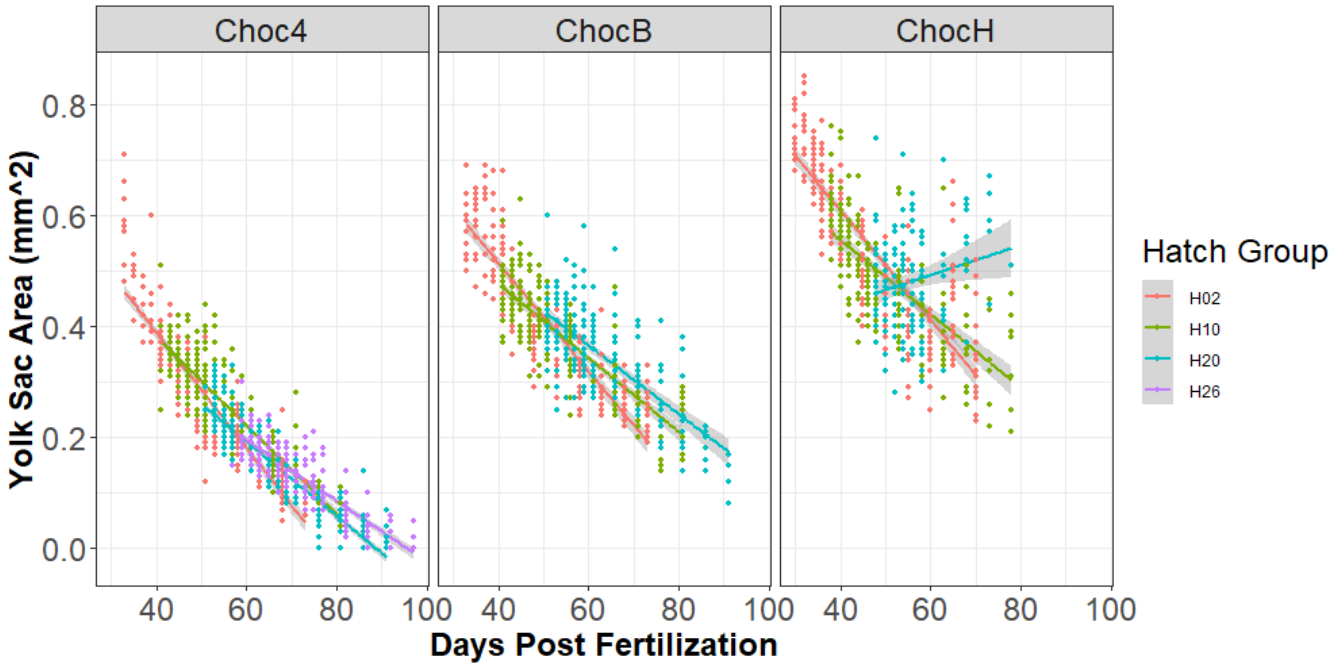


Figure 8: Change in yolk sac area through the first 40 DPH for representative hatch groups of early, middle, and late hatching larvae from each family. Regression lines show the yolk sac utilization rates for the selected hatch groups. Confidence intervals for regression lines are shaded in gray. Family and DPH had a significant impact on the overall yolk sac utilization rates of families during their yolk sac stage ($F=808.2$, $df=5, 16047$, $p<2.2e-16$).

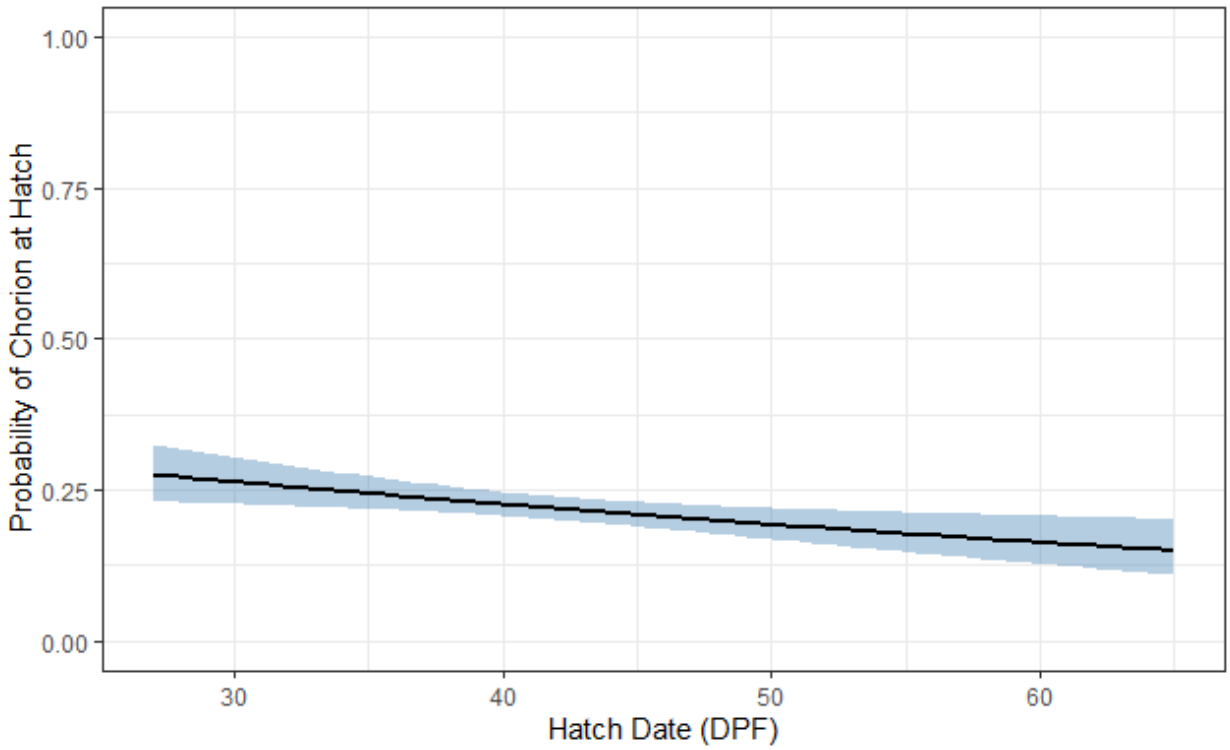


Figure 9: Binomial logistic regression for the relationship between hatch date and the likelihood that larvae were unable to escape their chorion at hatch. Likelihood of larvae escaping their chorion at hatch was significantly related to hatch date (p=0.0064).

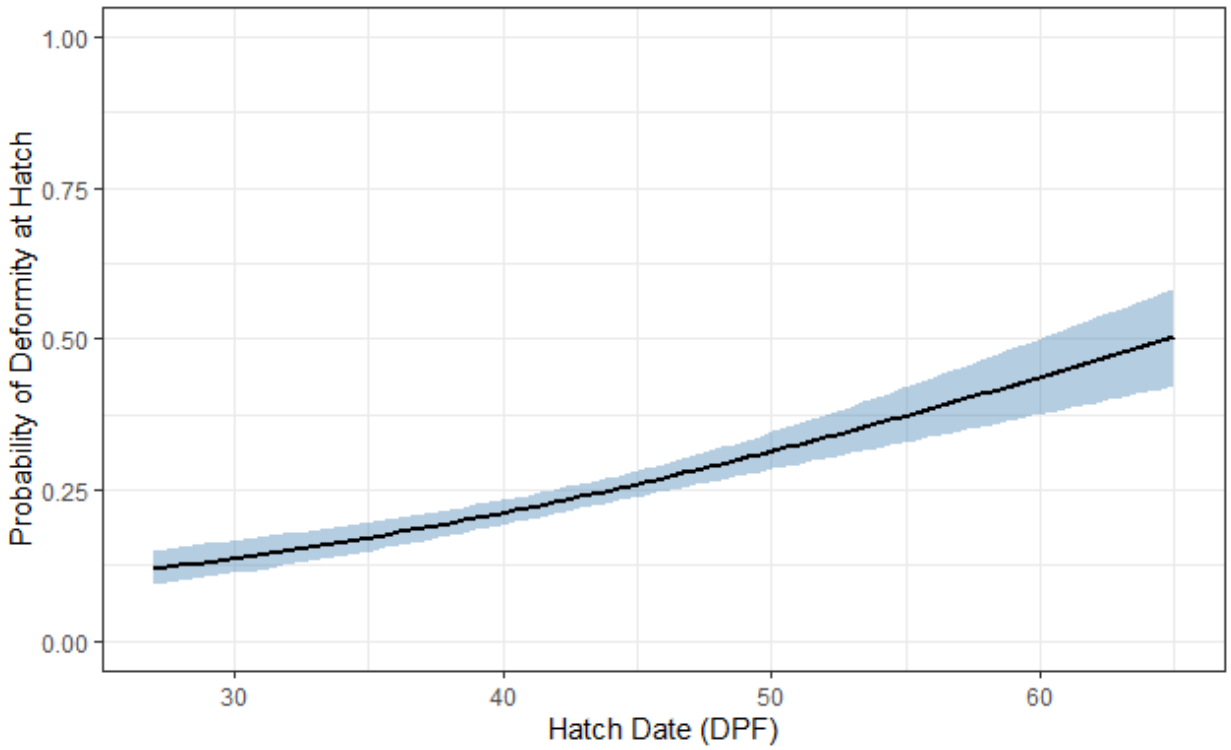


Figure 10: Binomial logistic regression for the relationship between hatch date and the likelihood of deformities present at hatch. Likelihood of deformity was significantly related to date (p=7.7e-13).

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APPENDIX A

Application to Use Vertebrate Animals in Research, Testing or Instruction

NORTHERN MICHIGAN UNIVERSITY

Project Title (If using external funds, enter the title used on the grant application): Site selection, sexual maturation and larval development of southern Lake Superior burbot (*Lota lota*)

Shaded area for IACUC use only.

Application Number: 410 Modification 2
Date Application Received: 11/28/2022
☒ Approved ☐ Denied on December 23, 2022

General Instructions
Please check the IACUC website to ensure you are using the current version of the form. All parts of this form *must be submitted electronically* and Use Committee (email: to the Institutional Animal Care IACUC@nmu.edu) and the relevant Department Head or other departmental designee. Review of this application will commence upon receiving the electronic application, but the project may not begin until all required approval signatures are obtained via Right Signature. Please contact the IACUC chair (email: IACUCChr@nmu.edu) if you have any questions.

Review Dates:
Designated Member Review of applications (appropriate for USDA Use Categories B and C) will be completed within two weeks after receipt of the electronic application.

Full Committee Review of applications will take place on the last Friday of every month. Applications for Full Committee Review must be electronically received by the first Friday of the month. Full Committee Review is required for applications that fall under USDA Use Categories D and E. Applications that fall under USDA Use Categories B and C will receive Full Committee Review if requested by an IACUC member. Detailed procedures on the IACUC review processes are located at the IACUC website.

I. Principal Investigator (Must be a faculty member or Department Head): Dr. Jill Leonard

Co- Investigator: Michael Woodworth, Andrew Shapiro & Alexis Pupo

Department: Department of Biology

Phone number: 906-227-1619

II. Funding Sources/Course Information and Dates **If the proposed work is for a course, please include the number of the course and title of the course**

Funding Sources (External & Internal, if applicable) Internal

Additional Funding Pending (click on the correct box)? ☐Yes ☒No

Project/Course Start Date: January 1, 2022 **End Date (three year maximum):** January 1, 2025

This application is (check one) ☐New ☒ Modification of an application currently approved by the Institutional Animal Care and Use Committee (a **new** protocol must be submitted after three years)