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Research Article

Validation of the Use of Otoliths to Estimate Age and Growth of Larval Lake Whitefish, *Coregonus clupeaformis*

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Understanding drivers of recruitment variation in fish populations requires research conducted on early life stages. Examination of fish otoliths provides useful information for estimating hatching dates, growth, and survival rates of larvae and for investigating the relationship between early life stage phenology and variation in environmental factors such as climate and food availability. In the Laurentian (i.e., North American) Great Lakes, significant reductions in the number of young (ages 1–4 years) lake whitefish (*Coregonus clupeaformis*) recruiting into the population and commercial fishery have raised questions about factors affecting growth and survival of the larval life stage. Here, we investigate the utility of using otoliths to estimate the age and growth of larval lake whitefish. We raised offspring of wild-caught parents from Lake Simcoe (Ontario, Canada) in a hatchery environment and analyzed otoliths of these known age fish for 75 days posthatch. We further examined otoliths of wild-sampled larvae and age 0 lake whitefish from Lake Huron. We found a strong linear relationship between known age and number of postcheck increments on the otolith and between growth of the otolith and fish length. Increments formed at nearly 1 (0.9) per day beginning at day 20 after hatch. Check and subsequent increment formation was associated with disappearance of the yolk sac. Wild fish had more prominent checkmarks and grew slower than hatchery fish. Thus, otolith analysis represents a promising tool to examine dynamics of early life stages of lake whitefish, although further research is required on the effects of environmental conditions on otolith microstructure.

1. Introduction

Understanding the drivers of recruitment in fish populations remains one of the most challenging and yet important areas of research in fisheries science[1]. Examination of early life stages, in particular the larval life stage, has been viewed as a means to achieve a mechanistic understanding of the process of recruitment and to move closer to understanding the complexities of the biological and physical factors that contribute to year class strength [2, 3]. Pioneering research by Hjort [4] followed by several others [5–7], contends that the growth and survival of larval fish drive recruitment variability in fish populations, both in marine and freshwater environments.

Coregonines, known generally as ciscos and whitefishes, have a broad circumpolar distribution across the freshwaters of the Northern Hemisphere [8, 9]. Across their range, coregonines face a number of threats, most notably from eutrophication, climate change, and invasive species, which have resulted in population collapses and the loss of ecosystem services arising from these valuable fishes [10]. In the Laurentian (i.e., North American) Great Lakes, coregonines support large commercial fisheries are a historically significant component of the food web and are important to the economies, culture, and food security of Indigenous communities [11–14]. The need to understand population declines and support the recovery of coregonines, particularly in the Great Lakes, have revealed gaps in our understanding of early life stages. Large-scale monitoring programs conducted by agencies around the Great Lakes have focused primarily on later-stage juvenile and adult life stages. However, there is a growing interest in investigating early

life stage dynamics of key coregonine species and the extent to which they are important in shaping recruitment to commercial, recreational, and subsistence fisheries.

Over the past 1-2 decades, lake whitefish (*Coregonus clupeaformis*) populations in several regions of the Great Lakes have shown precipitous declines [15]. Lake whitefish support large commercial fisheries within the Great Lakes, and addressing these declines is a top priority for management agencies. In lakes Michigan and Huron, juvenile recruitment is at the lowest level since the 1960–70s when commercial harvest and sea lamprey (*Petromyzon marinus*) predation had been implicated in earlier population collapses [15, 16]. Recent research has shifted to focusing on the larval life stage of lake whitefish in an effort to determine where in the life cycle the recruitment issue is arising, and to determine which factors are causing the recent population declines [15, 17, 18]. One hypothesis being explored is that reduced food availability during the larval stage (as a result of the filter feeding activity of dreissenid mussels, *Dreissena polymorpha* and *D. bugensis*) is contributing to slower growth and reduced survival (e.g., [17, 18]). Other potential contributing factors to recruitment variation being explored include changes in ice cover, spring temperatures, and water levels [15, 17].

Research focusing on larval lake whitefish, including an examination of growth and survival, would benefit from the use of otoliths to estimate age and growth of this critical life stage. For example, aging larvae with otoliths would enable quantifying growth and survival rates for larvae of different ages throughout the spring hatching season. This would allow identifying whether growth-dependent mortality at a particular part of the larval life stage is contributing to declines in population abundance. An examination of otoliths could also be used to estimate hatch dates for larvae, which could then be related to environmental variables such as temperature. Several other studies across a variety of taxa have used otolith microstructure in similar ways to reveal how larval traits related to growth and survival affect recruitment to later life stages and fisheries, information which can be important for conservation or management [19–23].

The formation of daily growth increments on otoliths has been observed across many taxa of teleost fishes, permitting an examination of the individual growth rates and ages of larval fish [24–27]. Otoliths are the inner ear stones of fish used in hearing and balance and form prior to hatch during the embryonic stage [28, 29]. Incremental growth of the otolith occurs through the differential deposition of calcium carbonate and protein over a 24 hour period and is thought to be driven by an internal circadian rhythm under endocrinological control within the fish [30]. The daily deposition of otolith increments has been validated in several coregonines, including cisco *Coregonus artedi* [31], European whitefish *Coregonus lavaretus* [32, 33], and bloater *Coregonus hoyi* [34]; in these studies, increments were reliably used to estimate age in days of larval or postlarval stage young-of-year fish. However, exogenous factors, including temperature and food availability, can influence otolith microstructure, making the detection of daily increment formation challenging or invalid in selected populations or under certain conditions [31, 35–37]. There are several possible explanations as to why factors like food or temperature affect daily ring formation, including reduced increment contrast, changes in otolith deposition, or effects of the cessation of skeletal growth [30].

Studies validating the use of otoliths to investigate the growth patterns of larval lake whitefish would be valuable for investigating declining recruitment in the Great Lakes and for better understanding the response of the larval life stage to climate change, invasive species, and other stressors across the species range. To our knowledge, there are relatively few examples of validation of larval otoliths in lake whitefish, including populations within the Great Lakes. One study we know of by Muir et al. [38] visually examined and described the otolith microstructure of age 0 lake whitefish from Lake Michigan and noted that daily growth rings were identifiable and could be used to provide an estimate of age of the fish in days. However, given the range of conditions experienced within the Great Lakes and the recent concerns over declines in the amount of food available to support recruitment of lake whitefish, more research on larval otolith microstructure of this species would be informative.

In this study, we evaluated the utility of using otoliths for estimating the age and growth of larval lake whitefish in the Great Lakes basin. We used captive rearing, a common approach for examining the rate of increment formation in larval fishes under stable environmental conditions [39]. We sampled known aged larval lake whitefish being raised at the Ontario Ministry of Natural Resources and Forestry (MNRF) White Lake Fish Culture Station under ambient environmental conditions. The lake whitefish were the direct ($F1$) progeny of spawning adults collected in the wild from Lake Simcoe, a large lake located within the Great Lakes basin in Ontario, Canada (Figure 1). The otoliths of these known age larvae were collected and sampled routinely from the swim-up stage until 75 days posthatch and used to determine the validity of age estimation under the conditions of our study. We further compared the age and growth of larvae from the hatchery population with that of larvae from a wild population collected in Lake Huron to determine the relevance of using the hatchery population to interpret age in wild fish. The specific objectives of our study were to (1) examine whether growth of the otolith (measured by otolith diameter) was proportional to length growth of the body, (2) determine whether daily growth rings were formed on the otolith that could be used to provide an estimate of age in days of larval lake whitefish, (3) identify whether there were biases associated with the number of increments counted by different readers, and (4) compare the otolith microstructure and growth of the hatchery population with that of a wild population.

2. Methods

In the wild, lake whitefish spawn in the fall (typically November-December) on shallow (1–5 m) spawning shoals often composed of coble and smaller rocks, although other substrates can be used [40, 41]. Fertilized embryos incubate

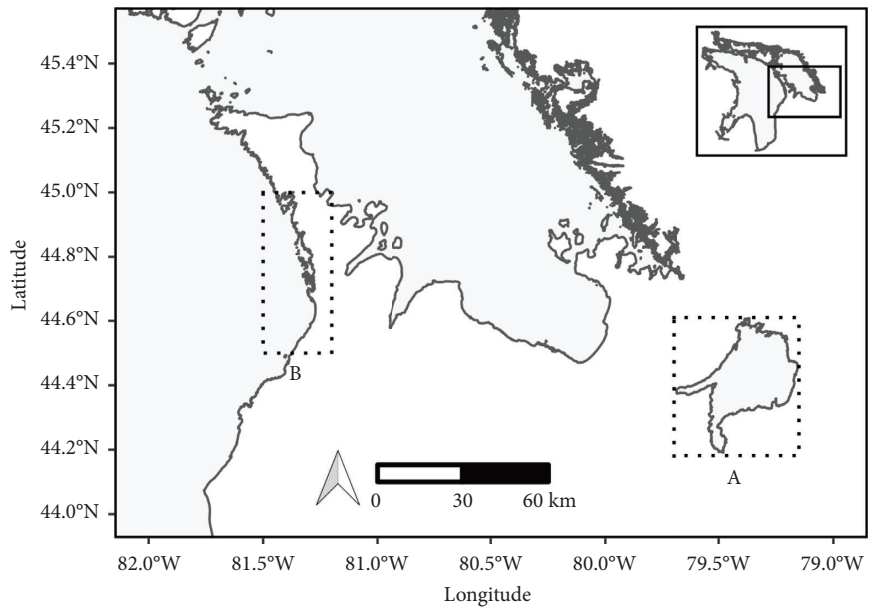

Figure 1: Sources of larval fish study populations. Lake Simcoe, study site A is the source lake for the hatchery population and the Fishing Islands, study site B is the location where the wild population of larval and age 0 fish were sampled. The box in the top right corner shows the location of the study region. Both source populations are located in Ontario, Canada, and within the Laurentian (North American) Great Lakes.

within the interstitial spaces of these rocky shoals over the winter period, hatch in the spring, and make their way to the surface [41]. The newly hatched larvae have a yolk sac that provides energy for growth and development, which is eventually absorbed as the larva makes the switch to feeding exogenously on pelagic zooplankton prey [41]. At some point during or just after the larval life stage, age 0 lake whitefish move to warmer, more productive nearshore embayments that act as nursery areas [42]. Over the course of the summer and into the fall, the age 0 juvenile fish gradually shift to a more benthic and offshore lifestyle and their diet shifts towards an increased reliance on benthic invertebrates [42]. The adult life stage begins at maturation, which can occur generally between ages 3 and 11 years depending on growth rates [43].

Larval fish used in this study were reared from fertilization at the MNRF White Lake Fish Culture Station (Sharbot Lake, Ontario, Canada). These fish were the progeny of wild-caught fish from Lake Simcoe (Ontario, Canada) and were intended for stocking as spring fingerlings to aid in population rehabilitation. The stocking program supports the Fish Community Objectives established for Lake Simcoe [44], which includes the rehabilitation of cold water fishes that experienced population declines as the result of excessive nutrients and poor water quality [45, 46]. Embryos were reared in the hatchery from fertilization of males and females captured in the wild during the fall spawning season of 2018. The larvae hatched on March 27th, 2019, and were then sampled between March 28th and June 10th, 2019. Larvae were initially reared in 500 L tanks and then moved into 15000 L tanks at 10 weeks old. Tanks were supplied with unfiltered water at ambient temperatures from White Lake in a flow-through system. Surface water from the lake was used until temperatures reached 12°C degrees after which time cold water was injected from a deep line from White Lake that pumps water into the building where it is mixed with the surface water. Daily water temperatures were monitored using Tidbit V2 Water Temperature loggers (Onset HOBO data loggers, Pocasset, MA, USA). Larvae were fed daily ad libidum beginning on April 1st, 2019 (5 days posthatch), first with GEMMA (produced by Skretting; https://www.skretting.com/en/feed-for-aquaculture/gemma-for-sole-17/) until they were ~1.5 g and then with EWOS (https://www.ewos.com/ca). Given that the water source from the lake was unfiltered, larvae could also eat zooplankton present naturally in their environment.

Ten larval individuals were randomly sampled every 1–6 days from hatch until June 10th (Table 1) and placed in labelled vials of ethanol for later analysis by researchers in the MNRF Aquatic Research Laboratory at Trent University. The sampling date was recorded for each larval fish, providing a known age in days of each individual. Standard lengths of individually collected larvae were measured using digital images captured from a camera (Leica MC170 HD) mounted to a dissecting scope (Leica S8AP0) in the open-source Java image processing program, ImageJ (core version 1.48v) [47]. The presence or absence of a yolk sac and the presence of food in the digestive tract was documented.

2.1. Otolith Preparation and Interpretation. Otoliths, the left and right sagittae and lapilli, were identified using polarized, transmitted light from a dissecting scope and removed from the larval fish using insect pins attached to a wooden dowel. Small otoliths were mounted to a microscope slide using

Table 1: Summary of hatchery lake whitefish sampled for otolith examination at each age, in number of days since hatched.

Posthatch age (days)	Mean length (mm)	Fish with otoliths extracted	Fish with otoliths examined	Fish with yolk present	Fish with checkmark	Fish with postcheck increments	Mean otolith diameter (μm)	Mean C	Mean APE	GDD_c
1	13.0	10	9	9	0	0	97.2	0	0	0.2
2	13.4	10	8	6	1	0	94.0	0	0	0.4
5	13.9	10	10	8	2	0	95.5	0	0	0.9
10	13.8	10	9	5	3	0	101.5	0	0	2.5
15	15.3	10	9	2	2	0	106.6	0	0	5.1
20	15.3	10	9	0	4	0	115.3	0	0	10.8
24	14.7	10	9	0	7	5	116.5	6.2	3.3	16.1
29	14.8	10	8	0	8	8	125.7	9.2	10.1	21.9
34	16.1	10	9	0	9	9	139.0	13.3	3.4	34.9
40	17.1	10	10	0	9	10	162.3	18.4	11.8	55.5
45	19.4	10	10	0	10	10	195.1	24.6	7.7	84.2
50	18.4	12	5	0	5	5	190.2	22.1	11.6	115.6
55	19.2	10	8	0	8	8	236.5	28.4	7.0	151.9
61	22.5	10	7	0	7	7	288.7	33.6	6.4	194.2
65	25.6	10	8	0	8	8	376.6	37.4	6.6	222.1
70	30.1	10	5	0	5	5	443.4	41.4	7.2	256.3
75	28.1	10	10	0	10	10	431.8	45.1	6.0	290.0

Fish with otoliths examined are those fish with both otoliths extracted and where the images were of good quality. For each age, the number of fish with the presence of a checkmark or daily growth rings in at least one otolith are shown and the mean otolith diameter, mean estimated increment count (C), and the mean average percent error (APE) were calculated. The cumulative growing degree days above 5°C (GDD_c) are shown for each age.

Permount Mounting Medium (Fisher Chemical) and secured with a cover slip while for larger otoliths, CrystalBond (SPI Supplies) was used to cement the otoliths in place and no cover slip was used. The otoliths were mounted concave side down when the otoliths were large enough to distinguish which side was concave. Both the sagittal and lapillus otoliths were removed but only the sagittal otoliths were used for measuring and aging. The sagittal otoliths were identified based on their larger size. Otoliths were immediately inspected for quality and to see if there was a visible checkmark or daily growth rings under the compound microscope. Checkmarks are distinct and prominent bands that can form at times of stress or perturbation including hatching, yolk sac absorption, or metamorphosis [30]. A prominent checkmark observed in coregonines appears related to absorption of the yolk sac, after which time daily increments are formed [31]. In our study populations, this prominent check was observed (and was the only consistent check observed) and is herein referred to as "the check" unless otherwise indicated. Occasionally, especially for the smaller otoliths, otoliths were lost during the extraction process or crushed from the slide cover making measurements and increment counting impossible. Those samples with both sagittal otoliths present and easily identifiable and where the overall quality of the mount was amenable for accurate measurements were retained for further examination.

Once otoliths were mounted and the cementing agent was dry, the microstructure of the sagittal otoliths was captured using the 2.8 megapixel Leica ICC50W microscope camera at the 100x, 400x, and 1000x magnification. The images were then examined blindly and in a random order in ImageJ. Within the software, images were first edited to increase microstructure visibility by increasing the contrast of the images and then the straight measuring tool was used to measure the maximum and minimum diameter of the whole otolith, the maximum and minimum check diameter, and the otolith radius from the check to the posterior edge of the otolith (Figure 2). The number of increments was counted on each otolith and a confidence ranking was assigned to each otolith between 1 (low confidence) and 3 (high level of confidence). A single increment count (C) was estimated for each fish by calculating the weighted average of the two otoliths using equation (1) while the average percent error (APE) for each fish was estimated using equation (2) [39, 48]:

$$C = \frac{(X_1 \times W_1) + (X_2 \times W_2)}{(W_1 + W_2)}, \quad (1)$$

$$\text{APE} = \frac{|X_1 - C|/C + |X_2 - C|/C}{2} \times 100, \quad (2)$$

where X is the number of rings and W is the perceived confidence (between 1 and 3) in the count on sagittal otoliths 1 and 2. Subdaily rings, identified as light, nonconsistent marks that merged with another mark were not counted as increments [49].

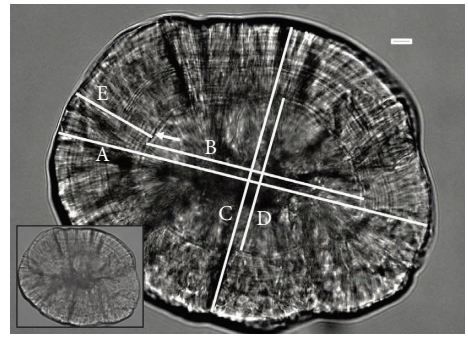

Figure 2: Measurements taken on the sagittal otoliths of larval lake whitefish. The contrast was enhanced to better see the microstructure of the otoliths. The unedited copy of the image is shown in the box at the bottom left. A is the maximum diameter of the otolith, B is the maximum check diameter, C is the minimum otolith diameter, D is the minimum check diameter, and E is the otolith radius. C and D are measured along the plane perpendicular to A and B. E is measured along the counting plane. The arrow points to the checkmark and where the counting of daily growth rings started and the white rectangle outside the otoliths is a 10 μm scale bar.

2.2. Wild-Sampled Fish. We compared the otolith microstructure and growth patterns between the hatchery population and a wild-sampled larval population from the main basin of Lake Huron. The purpose of this comparison was to determine if looking at daily increments from a hatchery population had relevance to interpreting age and growth in a wild population. Wild larval fish were collected 3–5 days per week from April 23rd–May 29th, 2018, at a combination of fixed and randomly selected sites at the Fishing Islands spawning shoal region in the main basin of Lake Huron (Figure 1; Table 2). Sampling encompassed the hatching period for lake whitefish, occurring just after ice out and continuing until the catches of larval fish dropped to 0. Larvae were sampled using a larval fish net (a 500 μm plankton plastic screen with a 50 cm diameter opening and length of 150 cm from Dynamic Aqua Supply) towed just below the surface for about 10 minutes. Trawl duration and the GPS coordinates at the beginning and end of the trawl were recorded. Larvae were placed in vials of ethanol and transported back to the laboratory for later analysis. Standard lengths were measured as mentioned above for the hatchery fish. Larval species identity was determined using DNA barcoding at the MNRF aquatic genetics laboratory at Trent University. Further details can be found in Cunningham and Dunlop [17].

Age 0 (postlarval stage) lake whitefish were also sampled in 2018 with beach seines conducted shortly after the larval hatching period at beaches in the vicinity of the larval trawling (Table 2). Following hatching over spawning shoals, lake whitefish move into nearshore beach areas that serve as productive nursery grounds [15, 42]. Age 0 whitefish inhabiting beach areas were sampled using a seine net (a 45.7 m by 1.8 m beach seine with a 1.8 m by 1.8 m by 1.8 m pocket from Memphis Net and Twine with 3.18 mm mesh) between June 11th and 26th, 2018. Fish were measured and

Table 2: Summary of the wild lake whitefish from Lake Huron sampled for otolith examination.

Sample week	Sampling method	Mean fish length (mm)	Fish with otoliths extracted	Fish with otoliths examined	Fish with yolk sac	Fish with checkmark	Fish with postcheck increments	Mean otolith diameter (μm)	Mean C	Mean APE	Min. cum. GDD5	Max. GDD_c
17	Trawl	14.0	37	28	15	6	2	85.0	7.0	11.1	0.8	7.8
18	Trawl	14.4	43	39	3	10	4	91.0	10.2	7.9	9.7	25.4
19	Trawl	14.9	63	54	2	43	32	101.3	10.5	8.5	28.7	53.0
20	Trawl	15.8	78	54	0	53	49	121.5	14.2	5.4	57.8	90.6
21	Trawl	16.6	55	45	0	45	44	138.8	21.1	4.2	96.8	138.3
22	Trawl	17.7	9	10	0	9	9	160.1	31.3	5.6	146.0	196.1
24	Seine	30.0	16	14	0	14	14	499.1	58.9	4.3	274.4	341.7
25	Seine	33.0	4	2	0	2	2	567.2	67.4	2.6	353.6	429.6
26	Seine	34.0	3	1	0	1	1	615.4	61.4	3.3	443.0	512.9

Fish with otoliths examined are those fish with both otoliths extracted and where the images were of good quality. For each sample week, the sampling method, the number of fish with the presence of a checkmark or daily growth rings in at least one otolith are shown, and the mean otolith diameter, mean estimated increment count (C), and the mean average percent error (APE) were calculated. The estimated minimum and maximum cumulative growing degree days above 5°C (GDD_c) are shown for each age.

identified to species genetically as mentioned above for the larval fish. The otoliths of these fish were extracted and analyzed in order to determine how growth during this postlarval stage compared with growth of larval fish in the wild and from the hatchery population.

2.3. Analysis. To understand biases associated with age and growth interpretation from larval otoliths, our analysis addressed two key questions [30], (1) was growth of the otolith proportional to length growth of the body? and (2) was the frequency of increment formation on the otolith constant with age? The relationship between fish length and otolith diameter provides the foundation on which larval growth history can be interpreted from an assessment of otolith increment width. The second question centers around whether daily growth rings are formed on the otolith. If increment formation is proportional to age, there is increased confidence in estimating the age of larval fish by counting the number of increments on their otoliths.

We used linear least squares regression analysis to examine the relationship between standard length of the fish and mean otolith diameter of the fish and between the number of increments and known age of the fish. Following Oyadomari and Auer [31], we calculated a percent error in aging estimation for each individual fish:

$$\%E = \left(\frac{\text{estimated age} - \text{known age}}{\text{known age}}\right) \times 100, \quad (3)$$

where the estimated age is the predicted age at increment formation (i.e., the intercept in the relationship between known age and number of increments) plus the number of increments counted on the otolith. Negative values of $\%E$ indicate that the fish has fewer increments than expected and positive values of $\%E$ indicate the fish has more increments than expected based on the known age of the fish. To examine whether growth rates influence aging error, we made a bivariate plot of $\%E$ versus growth rate (mm/day), delineated by 10-day age bins.

We also conducted a double-blind comparison of the weighted average of increments counted between two different aging technicians using a paired two-tailed t-test. This comparison among readers was performed on 20 larval lake whitefish otolith samples collected in 2017 from the Fishing Islands spawning shoal in Lake Huron.

For the wild-caught larval and age 0 lake whitefish, we analyzed the relationship between fish length and average otolith diameter using linear least squared regression, and compared this relationship to the hatchery-raised fish using analysis of covariance. Post hoc Tukey tests were used for paired comparisons. We also examined the relationship between the average check diameter (the mean of the minimum and maximum check diameter measured for the 2 otoliths) and the known age of hatchery fish or the estimated age of the wild fish. This was done to determine if the check was inconsistently identified by the reader as the fish aged [31].

Finally, to compare growth rates between hatchery and wild fish, we first accounted for differences in temperature between the two environments by estimating growth as a function of growing degree days [17, 50]. For each individual fish, a cumulative growing degree day (GDD_c) was calculated, representing the growing environment that the individual experienced from hatch to the date of sampling:

$$GDD_c = \sum \overline{T_d} - T_{\text{base}}, \quad (4)$$

where $\overline{T_d}$ is the mean daily water temperature and T_{base} is the base temperature [50]. For the hatchery-reared larvae, daily water temperature data were available from temperature loggers placed within the tanks and allowed a direct measure of $\overline{T_d}$. For the wild fish, water temperature data were only available as surface water temperature records taken at each larval fish trawl. Thus, for wild fish, we lacked temperature data from days when no sampling was conducted, making it impossible for a direct calculation of cumulative growing degree days. To overcome this, as in Cunningham and Dunlop [17], we estimated cumulative growing degree days for wild fish by performing a least square linear regression of

water temperature (taken at the trawl) versus day of the year. Previous research has shown that water temperatures in the nearshore of the Great Lakes increase linearly when greater than 4°C until the summer [51], which corresponds to the period of larval hatching and growth in our study. From the linear regression, a predicted mean temperature was then estimated for each day of the season and used to calculate cumulative growing degree days. For hatchery and wild fish, a base temperature of 5°C was chosen because larval hatching in the wild population most often occurred at temperatures >5°C [17] and the larval fish from the hatchery were sampled just after hatching at 5.1°C. Thus, temperatures below 5°C would not have a significant effect on larval growth.

3. Results

3.1. Visual Description of Otoliths. Within individuals, sagittal otoliths sometimes varied in shape, but the general layout of the checkmark and the spacing and intensity of increments was often similar. Generally, for young fish, sagittal otoliths were somewhat oval shaped with lobbed edges, gradually becoming less lobbed and more oval shaped as the fish grew (Figure 3). For larger fish, the sagittal otoliths were asymmetrical, and growth was primarily along a single axis (Figure S1). Checkmarks were usually more obvious or visible in wild fish than hatchery fish. Checkmarks were not evident at hatch in any of the otoliths examined in the hatchery fish (Table 1) and became visible in fish beginning as early as 2 days old. Visible checkmarks in the hatchery became common (>50% of the otoliths examined) at age 24 days at which point daily growth rings after the check were apparent. In wild fish, checkmarks were observed in some larvae on the earliest date of capture but were only common by sampling week 3 (Table 2). In the hatchery, checkmarks were not visible in any fish with a yolk sac, whereas in the wild, checkmarks were noted on some fish with yolk sacs. Increments were narrowly spaced immediately after the checkmark and increased in spacing further from the check. Some fish (both hatchery and wild) showed an earlier checkmark (in addition to the main check) followed by broad, less sharp concentric rings (Figure 3); however, this earlier check was not apparent in all fish and it is unclear if this was a check that formed at hatch. Subdaily rings were also observed on some fish and were more frequent in larger otoliths, most notably for later and more spaced-out increments.

3.2. Aging Validation. There was a strongly significant linear relationship between known (posthatch) age and the number of postcheck increments for lake whitefish larvae raised in the hatchery (Figure 4(a)). The slope of the relationship was 1.16 days per increment with 95% confidence intervals of 1.07–1.25, which was statistically different from 1 ($F_{1,224} = 28.52$, $p < 0.001$). Thus, increments formed on a near daily basis after check formation (0.9 increments per day). The intercept of the relationship between known age and the number of postcheck increments provides a population-level prediction of the number of days at which increments begin to form [31], here estimated as 20.1 days (referred to as the predicted age at increment formation). In fish where the true age is unknown (for example, fish sampled in the wild), the posthatch age can be estimated by adding the number of increments counted on the otolith to the predicted age of increment formation [52]. When we estimated age in the hatchery fish, we found a positive and significant linear relationship with known age (Figure 4(b)). Residuals for Figures 4(a) and 4(b) show a linear negative trend, indicating a similar magnitude of difference between predictions and observations whether using increment counts or estimated age (Figure 4(c)). For the majority of hatchery fish, the percent error in estimated age varied between ±20% (Figure 4(d)). Younger fish tended to have positive percent error while older fish tended to have more negative error, indicating that age was overestimated in young fish and underestimated in older fish (Figure 4(d)).

There was no significant difference between the number of increments counted between readers ($t = 1.32$, $df = 19$, $p = 0.202$), with the ratio between readers not significantly different from 1 (Figure S2).

We also examined whether the check diameter differed among larval fish of different ages, providing an indicator of whether the check was consistently identified throughout the larval stage. We found that the check average (mean of minimum and maximum check diameter) increased with age (Figure S3). We further observed that younger fish tended to have faster growth rates and positive higher percent error (Figure S4). When comparing wild and hatchery fish, the wild fish had a more prominent check that varied less with age although seined fish (i.e., older wild fish) had a significantly larger check diameter ($p = 0.005$).

3.3. Timing of Yolk Sac Absorption and Increment Formation. Yolk sacs in the hatchery-reared population were commonly observed in larvae less than 10 days posthatch, and almost always were found in fish that lacked visible checkmarks (only 5 fish with yolk sacs had checks, Table 1). The known age at which there was a 50% probability of having a yolk sac for hatchery fish was 9.67 days (standard error, SE = 1.29 days; $p < 0.001$), with the chances of having a yolk sac dropping to nearly 0 between 22 and 27 days (Figure 5A). None of the hatchery fish with yolk sacs had discernable increments. In the wild, only larvae sampled during the first three weeks had yolk sacs, with the majority occurring in the first week (Table 2). In the first week of sampling in the wild, 6/28 had visible checks and 2/28 had visible increments (Table 2). For wild fish, the estimated age at 50% yolk sac absorption was 22.01 days (SE = 3.65 days; $p = 0.007$), although there was high uncertainty in the probability curve because only 2 fish with yolk sacs had increments that could be used to predict the age (Figure 5B). There was a similar body length at 50% yolk sac absorption between hatchery (13.88 mm; SE = 0.19 mm) and wild (13.58 mm; SE = 0.14 mm) fish (Figures 5(C-D)), although they were significantly different from one another (z ratio = 2.393; $p = 0.017$). Larvae in the hatchery were first observed to have food in their digestive tract at age 16 days (April 11th).

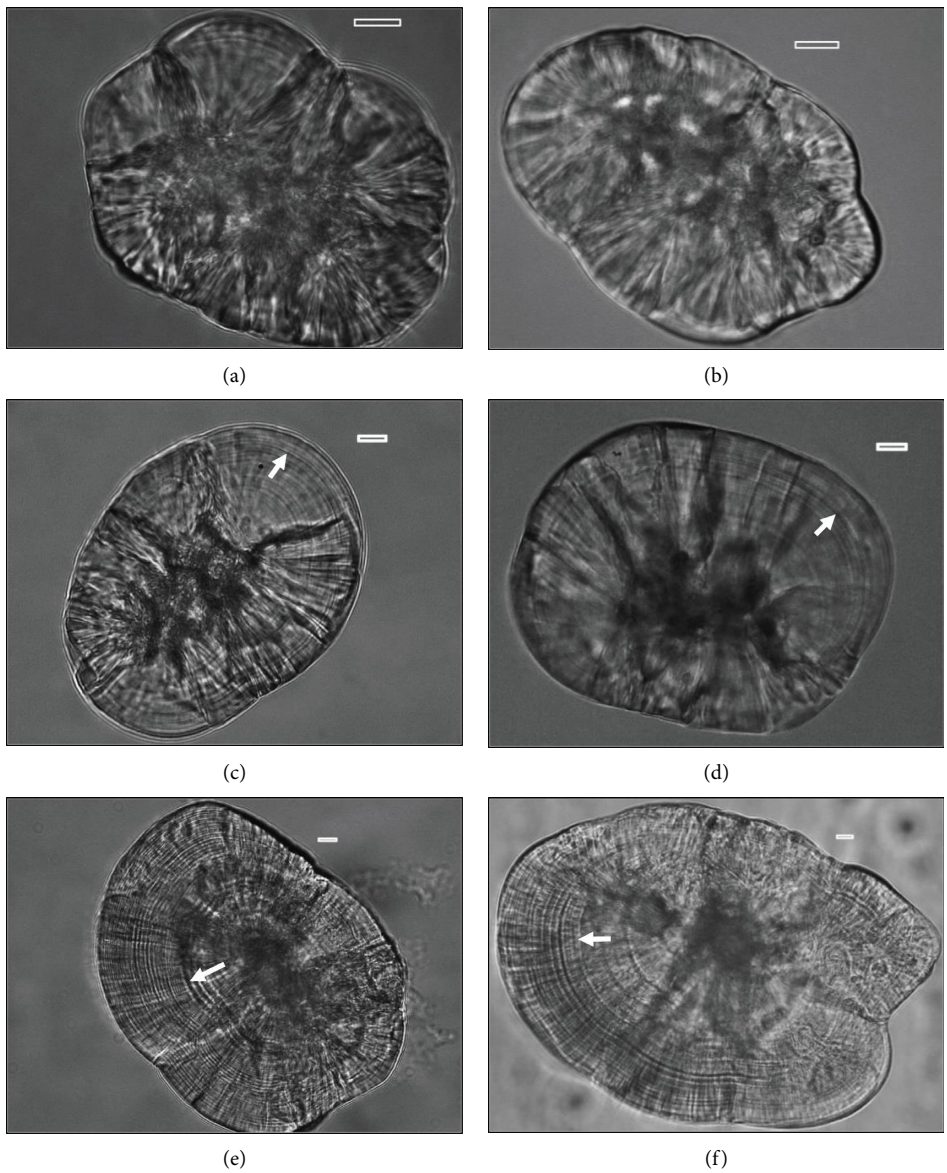

FIGURE 3: Examples of otoliths taken from wild (panels a, c, e) and hatchery (panels b, d, f) larval lake whitefish. Details on the fish include (a) zero increments or checkmark detected, standard length of 12.82 mm, and yolk sac present; (b) zero increments or checkmark detected, standard length of 12.7 6 mm, yolk sac present, and the larval fish was sampled on the hatch date; (c) the average number of increments is 6.2, standard length of 14.06 mm, and no yolk sac; (d) the average number of increments is 5.5, standard length of 14.95 mm, no yolk sac, and the larval fish was collected at 24 days posthatch; (e) the average number of increments is 25.5, standard length of 19.25 mm, and no yolk sac; (f) the average number of increments is 5.5, standard length of 20.71 mm, no yolk sac, and the larval fish was collected at 50 days posthatch. The arrows note the checkmark and where the counting of daily growth rings began. The white rectangle outside the otoliths is a 10 μm scale bar. The contrast was enhanced to better see the microstructure of the otoliths. Images (a)–(d) and (e)-(f) were taken with 400x and 1000x magnification, respectively.

3.4. Otolith and Larval Length Growth. There was a significant linear relationship between length of the larval fish and the average otolith diameter for hatchery fish, as well as for fish caught in the wild, indicating that larval fish otoliths were growing in proportion to body length (Figure 6(a)). The rate at which fish grew in relation to the otolith diameter differed between hatchery and wild fish (interaction $F_{1,386} = 47.23$, $p < 0.001$), with the rate of body growth to otolith growth being faster for hatchery fish (Figure 6(a)).

Length versus age showed a nonlinear relationship between hatch and 100 days posthatch for both wild and hatchery populations (Figure 6(b)). There was strong overlap in individual length at age values; however, on average, hatchery fish had longer lengths at age than wild fish.

Temperatures experienced during the larval growth period examined in our study showed a similar warming pattern between the wild and hatchery environments (Figure 7(a)), although with a slightly faster GDD_c increase

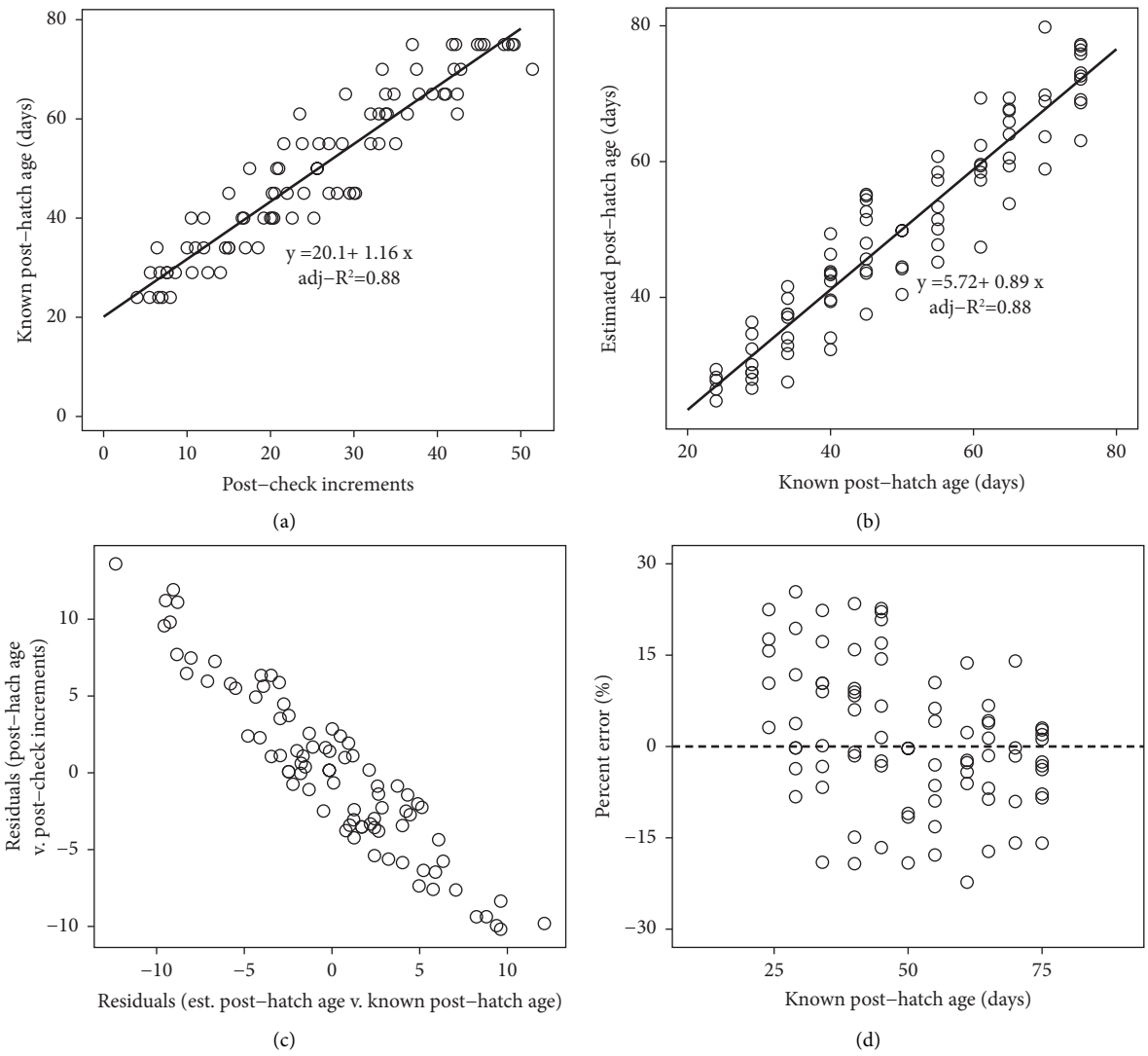

FIGURE 4: Validation of increment counts and age estimates for the hatchery population of larval lake whitefish. Panel (a) shows the relationship between posthatch age (days) and estimated postcheck increment counts which was used to estimate posthatch age (days) in panel (b). The residuals for (a) and (b) are shown in (c). Panel (d) shows the known age of the larval fish minus the mean number of daily otolith increments as a function of known posthatch age. Linear regression models and adjusted R^2 values are shown.

with date in the hatchery (Figure 7(b)). To account for these differences in warming rates, we examined trends between fish length and GDD_c and found a significant difference in the linear relationships among populations (Figure 7(c)). Growth of hatchery fish in relation to GDD_c was faster than trawled or seined wild lake whitefish (Figure 7(c), both $p < 0.001$, Tukey's method). The growth rate did not differ between wild trawled and seined fish ($t_{384} = -0.592$, $p = 0.824$).

4. Discussion

Larval lake whitefish produced from wild parents and raised in a hatchery environment formed increments at a constant rate postcheck that was proportional to the age in days of the fish. Furthermore, growth of the otolith was proportional to length growth of the body. Thus, two key aspects critical to the use of otoliths to examine age and growth of larval fish were met in our study [30]. Our results demonstrate the potential to use otoliths from larval lake whitefish to study age and growth of this early life stage, which will help with research aimed at understanding recruitment dynamics of this widely distributed fish and the recent population declines in commercially harvested lake whitefish populations in the Laurentian Great Lakes.

Lake whitefish larval otoliths in our study showed evidence of daily increment production following formation of the check. The check was estimated to form 20 days posthatch in the hatchery population, after which time increments could be readily counted and used to estimate age in days. Increments formed at a rate of 0.9 increments per day, just below (although statistically different than) 1 increment per day. Validating the rate of ring formation is useful for estimating hatch dates, survival rates, and growth

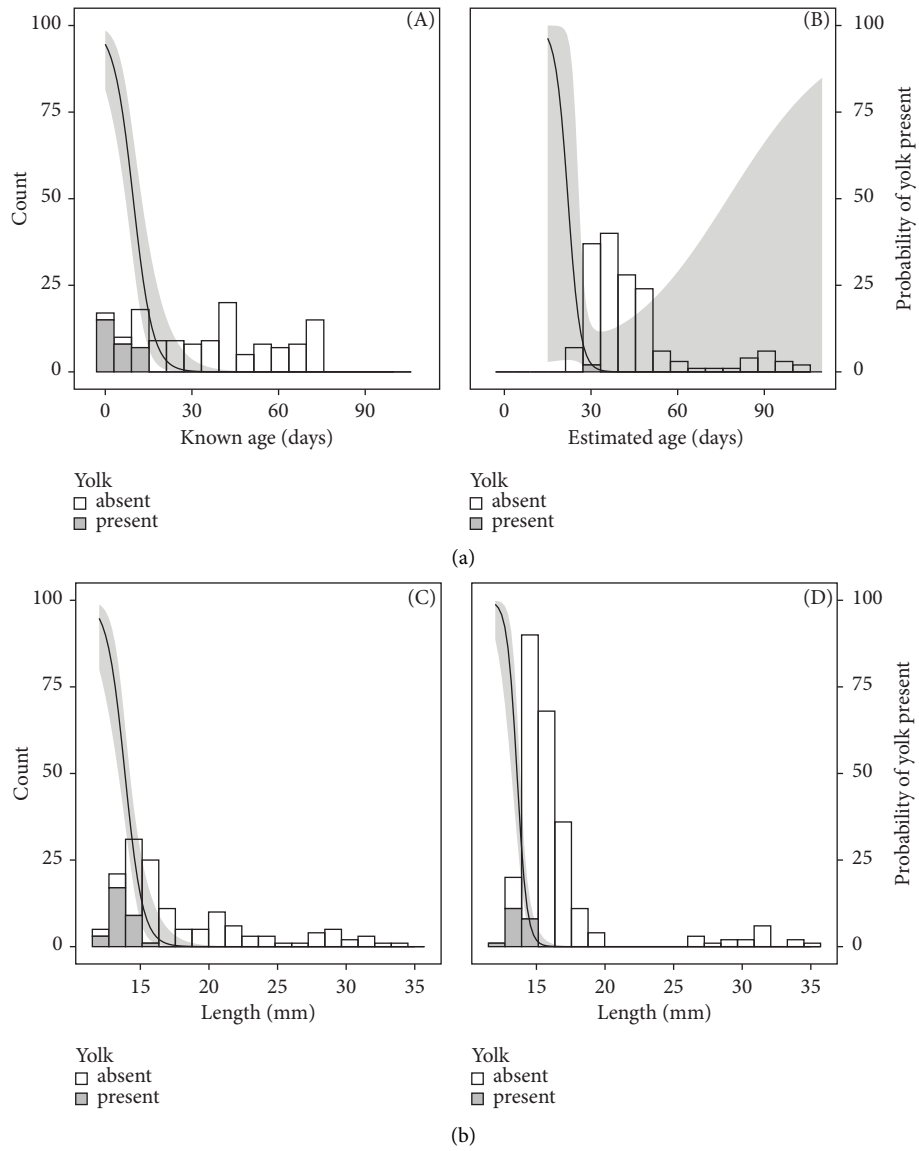

FIGURE 5: The age at 50% yolk sac absorption for the hatchery (A) and wild (B) lake whitefish (a) and the standard length at 50% yolk sac absorption for hatchery (C) and wild (D) lake whitefish (b).

rates of sampled larvae. Several other studies have demonstrated daily or near daily increment formation in coregonines [32, 33]. Likely the most comprehensive published study of larval otolith age validation in Great Lakes basin coregonines is that of Oyadomari and Auer [31] who raised larval cisco (*C. artedi*) from Lake Superior and tested otolith ring formation under various feeding and temperature conditions. Daily ring formation was confirmed in most scenarios, with the exception of a 5 day food deprivation experiment that reduced the accuracy of aging in some larval cisco [31].

Daily increment formation, while found in many populations and species, is not universal and can be influenced by environmental conditions. Several previous studies have failed to validate daily increment formation, including studies of coregonines. Vendance (*C. albula*) and European whitefish (*C. lavaretus*) collected from different wild populations showed inconsistencies in daily ring formation that disappeared when larvae were reared in a common environment [33, 53]. The discrepancy within these studies was attributed to low calcium levels in the wild, illustrating the influence of the environmental conditions on otolith age validation [33, 53]. However, in another study of larval European whitefish (*C. lavaretus*) raised from Lake Constance, daily ring formation was validated and was not affected by experimentally manipulated calcium levels [32]. Klink and Eckmann [36] raised European whitefish (*C. lavaretus*) larvae for 40 days at different temperatures and found that daily increment formation occurred at 8°C, but required the use of a scanning electron microscope for some of the larvae reared at 6 and 4°C.

Our study cannot be used to ascertain whether the rate of increment formation in larval lake whitefish or the relationship between otolith increments and larval length are

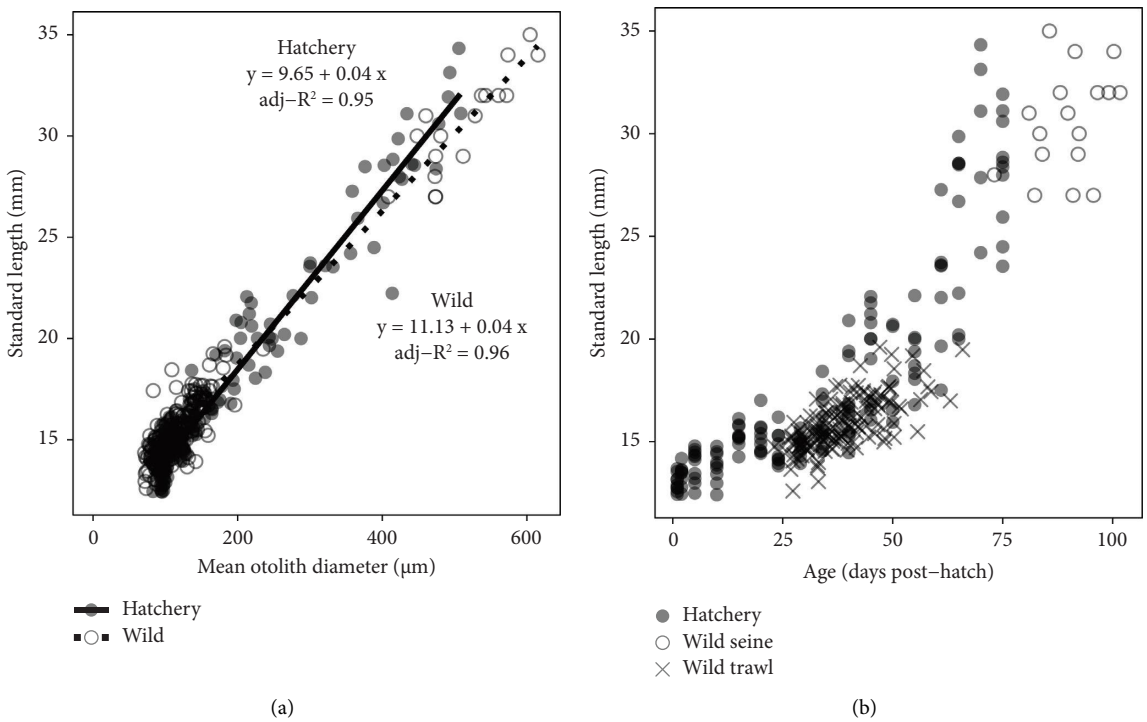

FIGURE 6: The relationship between (a) standard body length (mm) and mean otolith diameter (μm) and (b) standard body length and age in days since posthatch for hatchery raised and wild larval lake whitefish. For hatchery fish, the known age was used. For wild-sampled fish, the age was estimated using the linear relationship between the known posthatch age and posthatch increment counts derived for hatchery fish (shown in Figure 4(a)). In panel (a), the coefficients and adjusted R^2 are shown for each of the linear models.

robust across a broad range of environmental conditions. What we can confirm is that a constant and near daily ring formation occurs in lake whitefish from the Great Lakes basin experiencing relatively similar spring temperatures to that of wild population in Lake Huron. Thus, the ability to assess age of lake whitefish during the larval stage is at least expected for temperature conditions similar to those experienced by our study population and with adequate food available to support growth. We were also encouraged by the fact that while growth was slower in the wild (possibly due to differences in food availability, see paragraph below), there was nonetheless a linear relationship between length and otolith growth in the wild and overlap in larval sizes with the hatchery fish, again signalling that some patterns in otolith microstructure were preserved across these environments. While previous work on cisco (*C. artedi*) found an effect of growing conditions on larval aging error rates, it was the more extreme multiday food deprivation scenario that impacted otolith ring formation, and the authors concluded that such slow growth would be rare in the wild [31]. Additional research examining how year-to-year variation in temperatures and food affect daily ring formation in larval lake whitefish would be valuable to understanding the range of conditions under which age estimates are robust in this species.

The hatchery-reared larvae did on average grow faster than the wild Lake Huron population including when accounting for differences in the growing degree days experienced between environments, which could be attributed to more food available in the hatchery than in the wild. Hatchery larvae were fed ad libitum and were also able to consume any zooplankton naturally present in their rearing environment from White Lake, the source of water for the hatchery. Furthermore, growth of wild larvae from Lake Huron could be slower because of reductions in zooplankton prey that have occurred as dreissenid mussel populations expanded and spread across the lake [17]. Reduced food availability is a leading hypothesis for the precipitous declines of recruitment that have occurred across lakes Michigan and Huron [15, 17, 18]. A recent study of the same wild population from Lake Huron observed declines in the lengths at capture of larvae sampled between the 1970s–1980s and 2017–2021 [17], but examination of larval growth rates from otoliths would be more informative for revealing patterns of the larval growth rate in relation to food availability and invasive species. For example, individual larval growth trajectories could be estimated by measuring increments between daily rings and using them to back-calculate length at age [30]. In addition, length at capture could be related to estimated age and be used to build larval growth trajectories for the population by year or location. These growth curves (either individual or population) could be related to variation in food availability and temperature and could be used to predict year class strength, allowing a test of the hypothesis that slow growth is contributing to reduced survival and declining recruitment.

Our research was motivated by the fact that there are relatively few studies of larval otolith validation in Great Lakes

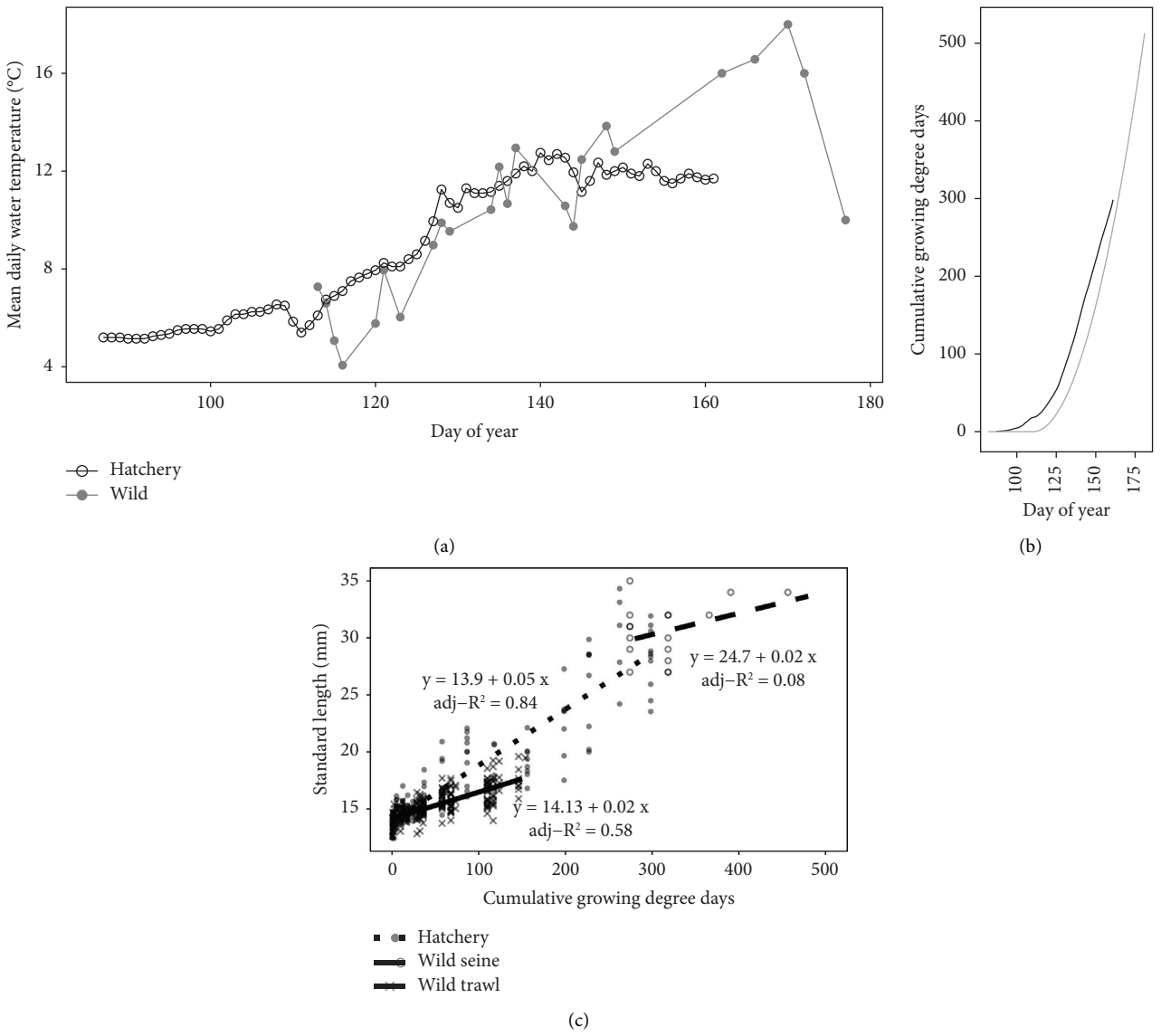

FIGURE 7: (a) Temperatures experience by larval and postlarval age 0 lake whitefish in the hatchery and wild. (b) The cumulative growing degree days (base 5°C) experienced by the hatchery (solid line) and wild (grey line) larval lake whitefish by the day of the year. (c) The relationship between length and cumulative growing degree days for the hatchery and wild lake whitefish. The coefficients and adjusted R^2 for each of the linear models are shown.

fish species, including lake whitefish (but see [31, 34, 38]). Having an estimate of the days posthatch at which the check is formed and knowing whether daily increments are consistently formed thereafter is useful considering the identified research priority aimed at understanding early life history dynamics of lake whitefish in the Great Lakes [15]. A previous study where larval otolith structure was described for early life stages of lake whitefish was by Muir et al. [38], in which age 0 lake whitefish were captured at nursery sites adjacent to several spawning shoals in Lake Michigan and one in Lake Superior. Among the lake whitefish captured from these spawning populations, two checks were noted on the otoliths, one attributed to hatching and the other (at an estimated 24 days posthatch) to the complete transition to exogenous feeding. Furthermore, in a laboratory population, Muir et al. [38] described that a prominent growth check appeared 24 to 28 days posthatch, which could be used as a benchmark for estimating days in age based on the number of postcheck increments. In our study, our linear regression of the number of postcheck rings vs. the number of days posthatch had an intercept of 20 days and the first sign of visible, complete rings occurred in larvae sampled at 24 days posthatch. In cisco from Lake Superior, a prominent check occurred at 28 days posthatch as determined by a linear regression of known age in days versus the number of postcheck increments [31]. Thus, there appears to be some similarities in the posthatch age at which increments begin to form across studies, which offer promise for estimating larval age and hatching dates from otoliths of coregonines in the Great Lakes.

In our study, the formation of a visual check and the subsequent daily formation of increments were associated with the absorption of the yolk sac. In the hatchery, the age at 50% yolk sac absorption was 9.67 days, with the probability of having a yolk sac dropping to near 0 close to the predicted age at increment formation (20 days). None of the hatchery larvae with a yolk sac had visible increments (although a few had checks). In the wild, a little over 50% (15 larvae out of 28 sampled) had yolk sacs in the first week of sampling, with the percentage dropping below 1 for the following two weeks and 0 afterwards. Out of the 15 fish sampled with yolk sacs in the first week, 6 had a check and only 2 had visible increments (6.2 and 6.4 increments, respectively, corresponding to predicted ages of ~26 days). Generally, checks were more visible and prominent in wild fish, which might explain why checks were observed in some wild fish with yolk sacs but were more rare in hatchery fish with yolk sacs. Conversely, the length at 50% yolk sac absorption was similar between the hatchery and wild population. The yolk sac provides nutrition to the larval fish after hatching; as the yolk sac begins to disappear, the fish makes a shift towards exogenous feeding [41, 54, 55]. Thus, it appears in our study that the shift towards more exogenous feeding might have occurred at similar lengths between hatchery and wild fish, but at potentially earlier ages in the hatchery. However, because we do not know the true ages of the wild fish, it is difficult to estimate the age at which yolk sacs were absorbed in our wild population. In the study by Oyadomari and Auer [31], cisco from Lake Superior raised in a laboratory at two different temperature regimes had a 50% likelihood of complete yolk sac absorption at day 28.94, which was close to the predicted age at increment formation (27.57 days). Interestingly, the length at 50% yolk sac absorption for the cisco population (13.22 mm) was very similar to our hatchery and wild lake whitefish (13.88 and 13.58 mm, respectively). The temperatures experienced by our hatchery population (Figure 7) were very similar to those experienced by the cisco in the higher temperature regime of Oyadomari and Auer [31]. Further research is needed to determine the degree to which check and increment formation vary among populations and with conditions such as temperature and food in order to determine the accuracy of predicting posthatch age and date of hatch in wild-sampled larvae. However, there is good indication that the number of increments counted on a larval coregonine otolith can be used to predict the general timing of yolk sac absorption and the switch to full exogenous feeding.

We found that the difference between known age and increment count increased with fish age in our hatchery population, as has been found in other studies [56]. This pattern of an increasing difference with age occurs because the chance of missing an increment increases with age. This explains the >1 slope in the relationship between known age and increments (Figure 4(a); slope = 1.16). The percent error showed a general pattern with known age, with more positive values at younger ages and more negative values at older ages. At younger ages, there were fewer increments counted per day while at older ages, there were more increments counted per day than expected. There was also a pattern between percent error and the growth and age of the fish. Younger fish (20–30 days old) had faster growth and more positive and larger error rates, whereas older fish (>60 days) had slower growth and negative error rates. A similar pattern was observed in cisco where faster growth was associated with positive error rates relative to slower growing larvae, although the magnitude of error increased as cisco larval growth became slower [31]. In our study, younger fish had higher growth rates and thus it could be that the growth-related effect we observed was more a function of larval age than larval growth. An aging error in a younger fish has a larger relative effect on the error rate (e.g., being off by 1 or 2 increments when young matters more than being off by the same amount in an older fish). We also found that the average check diameter increased with age, more notably in the hatchery population. Theoretically, a relationship between check diameter and age implies that interpretation of the check varies with age [31]. Wild larvae had checks that were more prominent, which might explain why the check diameter was more consistent across ages for these fish. More prominent checks in the wild might be the result of increased variability in prey density and taxa or other environmental conditions that have a stronger signal in the wild. It could be that the potential bias associated with errors in identifying the check is lessened in the wild compared to larvae raised in the hatchery, although more research is needed.

There was a strong relationship for both hatchery and wild larvae between standard body length and otolith diameter. Thus, the use of otolith growth to measure the early growth history of individuals holds promise for lake whitefish populations. Based on our results, younger fish (i.e., larvae closer to their hatch dates) may provide more reliable back-calculated estimates of daily growth than older larvae or postlarval age 0 fish if the check is more difficult to measure in older fish or because of the increasing difference in known age to increment counts as the fish ages. Furthermore, differences in slopes of the body length to otolith diameter relationship between the wild and hatchery population suggest that population (or environment)-specific relationships need to be estimated to perform back-calculations of larval length at age, rather than there being a species-specific or Great Lakes basin-specific relationship. A study by Fey and Greszkiewicz [57] on northern pike (*Esox Lucius*) found evidence that while daily increment formation was validated, the larval size-otolith size relationship varied with temperature.

The use of otoliths from early life stages of fish to examine growth histories, hatching dates, and survivorship could reveal important insights into the recruitment dynamics of coregonines and other fishes. However, this research requires validation of common assumptions about the detection of daily ring formation and the proportionality of the body growth to otolith growth relationship. Here, we found evidence that the examination of the microstructure of larval lake whitefish otoliths holds promise for future investigations into larval ecology of this species. The strong relationship we observed between larval or age 0 body length and otolith size within both a hatchery and wild population

underscores the potential of using larval otoliths to examine growth dynamics of young life stages of lake whitefish in the Great Lakes basin, and likely elsewhere. Research has also revealed that the composition of trace elements in larval otoliths of various species can be used to discriminate among different spawning populations or to infer natal origin [58–60]. Otolith morphology varies widely among species and has also been used to distinguish among fish stocks [30, 61]. Thus, further insights into stock dynamics and recruitment could be gained by collecting the otoliths of young life stages and using them for a variety of purposes, increasing the scope of information that can be provided to support management decisions.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

ESD and IH designed the study. IH and CT conducted laboratory work and analyses with guidance from ESD. ESD wrote the paper with input from IH and CT.

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Supplementary Materials

Figure S1: example otoliths taken from older (age 0) lake whitefish. Figure S2: relationship between increment counts from two different readers. Figure S3: average check diameter versus age. Figure S4: relationship between percent error in age and growth rate of the fish. (*Supplementary Materials*)

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