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Effect of elevated embryonic incubation temperature on the temperature preference of juvenile lake (*Coregonus clupeaformis*) and round whitefish (*Prosopium cylindraceum*)

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26 Abstract

27 Anthropogenic impacts can lead to increased temperatures in freshwater environments through
28 thermal effluent and climate change. Thermal preference of aquatic organisms can be modulated
29 by abiotic and biotic factors including environmental temperature. Whether increased
30 temperature during embryogenesis can lead to long-term alterations in thermal preference has not
31 been explicitly tested in native freshwater species. Lake (*Coregonus clupeaformis*) and round
32 (*Prosopium cylindraceum*) whitefish were incubated at natural and elevated temperatures until
33 hatching, following which, all groups were moved to common garden conditions (15°C) during
34 the post-hatching stage. Temperature preference was determined at 8 (Lake whitefish only) and
35 12-months of age (both species), using a shuttlebox system. Round whitefish preferred a cooler
36 temperature when incubated at 2°C and 6°C compared to 0.5°C. Lake whitefish had similar
37 temperature preferences regardless of age, weight, and incubation temperature. These results
38 reveal that temperature preference in freshwater fish can be programmed during early
39 development, and that round whitefish may be more sensitive to incubation temperature. This
40 study highlights the effects that small increases in temperature caused by anthropogenic impacts
41 may have on cold-adapted freshwater fish.

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53 Introduction

54 Anthropogenic impacts have created scenarios where animals may be experiencing
55 thermal stress during early critical life stages. Current predictions have proposed that the great
56 lakes are expected to rise in temperature between 4-6°C by 2100 (IPCC, 2014), at a rate of about
57 0.1°C/year (Austin and Colman, 2007). More immediate concerns arise from warmer effluent
58 discharge from industrial practices that use natural bodies of water to remove waste heat. This
59 effluent can lead to increases in local habitats by 1-3°C, particularly those along the shoreline
60 (Thome et al., 2016). Nearshore environments are critical regions for aquatic species, such as
61 fish, providing regions to forage, shelter, and breed (Hampton et al., 2011). One risk for aquatic
62 species that breed in these environments is the exposure of immobile embryos to supraoptimal
63 temperature conditions throughout embryogenesis.

64 Environmental temperature exerts considerable control over the chemical process
65 orchestrating development in fish (Stevens and Fry, 1970). Water temperature is a key
66 determinant of growth in fish (Jobling, 1981; Magnuson et al., 1979), with increased temperature
67 during embryogenesis leading to accelerated growth and developmental rates (Gillooly and
68 Dodson, 2000; Nytrø et al., 2014; Sun and Chen, 2014). However, fish embryos display many
69 plastic traits that can be influenced by their developmental environment (Jonsson and Jonsson,
70 2019). For instance, a positive relationship has been noted with incubation temperature and post-
71 hatch metabolic rate in several fish species (Barrionuevo and Burggren, 1999; Bozek et al., 1990;
72 Marty et al., 1990). Interestingly, the thermal optimum of key metabolic enzymes at the adult
73 stage increases in response to temperatures experienced during rearing (Schnurr et al., 2014).
74 Taken together, this may be indicative of an increased need to elevate body temperature to meet
75 changes in metabolic demands. As ectothermic poikilotherms, several studies have demonstrated
76 that fish aggregate to their thermal preference (T_{pref} ; Kellogg and Gift, 1983; Reynolds and
77 Casterlin, 1980; Stevens and Fry, 1970), to maintain their metabolic, growth, and/or reproductive
78 optimums (Haesemeyer, 2020; Larsson, 2005). T_{pref} has been shown to vary across life-stage
79 (Edsall, 1999), season (Mortensen et al., 2007), time of day (Macnaughton et al., 2018) and
80 metabolic state (Killen, 2014). However, the impact of elevated incubation temperature and any
81 long-term change to T_{Pref} , particularly in native cold-water fish, has not been explicitly tested.

82 Lake (*Coregonus clupeaformis*; LWF) and round (*Prosopium cylindraceum*; RWF)
83 whitefish are cold-water adapted species that have an extensive range across North America
84 (Ebener et al., 2010). These fish species serve an important ecological role in food webs and
85 support commercial fisheries and indigenous communities (Ebener et al., 2010). LWF and RWF
86 occur sympatrically, co-existing due to differential habitat and resource usage within lakes
87 (Eberts et al., 2016). Both species of whitefish broadcast spawn in shallow (<10m) cobble beds
88 in late November, and embryos remain in these shallow waters until the ice melt in spring (April-
89 May; Scott and Crossman, 1973). These long incubation periods coincide with temperatures of
90 0.5-2°C at these depths (Patrick et al., 2013; Schwab et al., 1999; Thome et al., 2016), and these
91 animals may be sensitive to increases in temperature imposed by anthropogenic impacts. Indeed,
92 laboratory studies strongly support this, with whitefish exposed to elevated temperature during
93 embryogenesis generally exhibiting perturbed morphology, precocious development, and
94 increased mortality (Brooke, 1975; Eme et al., 2015; Eme et al., 2018; Lee et al., 2016; Lim et
95 al., 2017; Lim et al., 2018; Mitz et al., 2019; Mueller et al., 2015; Mueller et al., 2017; Patrick et
96 al., 2013; Price, 1940). These effects become more prevalent at constant temperatures $\geq 5^\circ$ (Price,
97 1940; Brooke, 1975; Lim et al., 2017; Lim et al., 2018; Mitz et al., 2019; Eme et al., 2015; Eme
98 et al., 2018; Mueller et al., 2015; Mueller et al., 2017; Patrick et al., 2013), with RWF appearing
99 more sensitive, experiencing mortality rates 30-40% higher than LWF (Lim et al., 2017; Lim et
100 al., 2018). Thermal stress during embryogenesis can augment and perturb the typical
101 development of both LWF and RWF, but studies exploring impacts at post-hatch stages are
102 limited. Work at later life-stages is a necessity given that whitefish embryos could be exposed to
103 temperatures as high as 5°C now and up to 8°C within ~30 years at the current rate of warming
104 (Austin & Colman, 2007).

105 This study tested the hypothesis that elevated temperature during rearing could impact the
106 resulting thermal preference of juvenile LWF and RWF. Elevated temperature can lead to
107 lethality in embryos of these species, but sublethal effects, such as changes in length and weight
108 (Brooke, 1975; Price, 1940; Mitz et al., 2019; Lee et al., 2016), may lead to altered performance
109 and function at later life-stages. To test this, we reared LWF at their optimum and natural rearing
110 conditions (2°C) and elevated constant water temperatures of 5 and 8°C. As RWF are more
111 sensitive to elevated incubation temperature and experience nearly 100% mortality at 8°C (Lim
112 et al., 2017; Lim et al., 2018), we exposed RWF to 2°C and 6°C, and a colder temperature

113 (0.5°C), to see effects in a lower range of environmental temperatures. We assessed behavioral
114 performance at 8 and 12 months for LWF, and 12 months for RWF, determining their T_{Pref} ,
115 velocity, total distance travelled, and movement across temperature gradients. The results
116 suggest that elevated incubation temperature can alter RWF T_{Pref} , but not LWF.

117

118 Methods

119 Study Species

120 Fertilized LWF embryos were acquired from Sharbot Lake White Fish Culture Station (Sharbot
121 Lake, ON) on November 30, 2017 (reared to 12-months) or November 27, 2018 (reared to 8-
122 months). Spawning RWF were obtained from Lake Ontario (Port Darlington, GPS 43°51'50"N
123 78°44'35"W) on December 10 and 11, 2018. RWF were stripped of eggs and milt and returned to
124 the water. Artificial in-vitro fertilization occurred immediately after stripping. Embryos were
125 disinfected with Ovadine® solution and transported in lake water back to McMaster University.
126 Embryos (160-310) were plated into 200mm x 20mm sterile petri dishes containing 200mL of
127 dechlorinated city tap water, and then moved to incubators (Mitz et al., 2014). Embryos were
128 initially kept at 8°C and cooled (1°C/week) until they reached a base temperature of 2°C, 5°C or
129 8°C for LWF or 2°C or 6°C for RWF (Fig. 1). To create the treatment for the 0.5°C RWF
130 embryos, an ice slurry was maintained within an incubator set to 2°C. Incubation temperature
131 was maintained for 100 days to replicate the winter period (December to March), following
132 which, embryos were warmed (1°C/week) until reaching 8°C. To confirm temperature within
133 each incubator, TidbiT® temperature loggers placed in 200mm x 200mm petri dishes with 200
134 mL of dechlorinated water. For the base temperature (excluding warming and cooling periods),
135 LWF were exposed to $2.08 \pm 0.3^\circ\text{C}$, $4.81 \pm 0.3^\circ\text{C}$, $8.05 \pm 0.1^\circ\text{C}$, and RWF to $0.54 \pm 0.2^\circ\text{C}$, 2.58
136 $\pm 0.2^\circ\text{C}$, $6.13 \pm 0.2^\circ\text{C}$. Median hatch for LWF occurred at 50 days post fertilization (8°C), 108
137 days post fertilization (5°C), and 158 days post fertilization (2°C). Median hatch for RWF
138 occurred at 88 days post fertilization (6°C), 114 days post fertilization (2°C), and 118 days post
139 fertilization (0.5°C). Hatchlings (~10) were placed in 100mm x 20mm petri dishes with 100 mL
140 of water at 8°C until successful exogenous feeding. Water in petri dishes was changed three
141 times a week for embryos and daily for larvae. Larvae were transferred to 1-10L recirculating
142 tanks and warmed (1°C/week) to 15°C, where they remained until testing (8- or 12-months post-

143 hatch). All treatment groups were maintained in common garden conditions once they were
144 warmed to 15°C. Larval fish were initially fed *Artemia* nauplii and slowly transitioned to pellet
145 feed (Otohime B1 (200-360 µm) – C2 (920-1,410 µm) larval feed).

146

147 Behavioral Assay – Shuttle Box

148 The shuttle box system (Loligo®), first described by Neill et al. (1972), consists of two
149 cylindrical tanks connected by a small rectangular ‘shuttle’ to allow movement of animals
150 between the tanks. Each tank is designated as the increasing (INCR) or decreasing (DECR) side,
151 indicating the direction of temperature change when fish occupy that tank. To regulate
152 temperature, system water was pumped through heat-exchange coils in hot (28°C) and cold
153 (4°C) water baths (60L chest coolers) with mixing in separate buffer tanks for each side. A
154 Recirculator 1/4 HP Chiller, Magnetic Drive Centrifugal Pump (300W/600W/950W @
155 0°C/10°C/20°C; VWR) and 2x400W aquarium heaters were used to maintain the temperatures in
156 the cold and warm bath, respectively. The shuttle box temperature probe can report temperature
157 units to 0.01°C accuracy. Polystyrene insulation (1/2") and foam insulation tape (1/4") were used
158 to prevent heat loss and maintain stable temperatures in the cold-water bath. System water
159 flowed at 240 mL/min via gravitational pull through temperature probes and into the shuttle box
160 where mixing between the two sides is minimized by counter-directional currents. The
161 orientation of the INCR and DECR tanks and the side to which the fish would be introduced
162 were randomized for each individual, using an online tool (random.org), to limit any potential
163 bias introduced by visual cues or side preference. Whitefish of the appropriate treatment group
164 were randomly selected from their home tank (15°C) and transported to the shuttle box system in
165 blacked-out 1L glass beakers to prevent undue stress. A plastic divider separated the two halves
166 of the arena, which when removed, started the acclimation period. Fish were acclimated to the
167 arena in a static setting, with the two arenas maintained to 14 and 16°C with a hysteresis of
168 0.25°C. After 2h in this condition, the fish were tracked using a USB 2.0 uEye Camera tracked
169 juvenile fish under infrared light (Loligo® Infrared Light Tray), recording the position of the fish
170 in the arena. The onset of warming or cooling occurred in response to whether the fish would be
171 in the INCR or DECR tank, with the difference in temperature between these two sections being
172 maintained at 2°C and warming or cooling (hysteresis = 0.1°C) occurring at a rate of 4°C/hour.

173 A maximum temperature of 23°C and a minimum temperature of 7°C was implemented to
174 prevent exposure to extreme temperatures, which could cause stress or mortality (Edsall and
175 Rottiers, 1976). T_{Pref} was calculated by the software as the median occupied temperature;
176 additional measurements calculated were velocity (cm/s), total distance travelled (cm), time
177 spent in INCR/DECR, number of passages, and avoidance temperature (temperature at which a
178 passage between tanks occurred). Following the completion of the assay period, fish were
179 removed, and measured for total length and mass before returning to a separate home tank
180 (15°C). Prior to experimentation, whitefish were fasted for 12-20 hours to prevent fouling of the
181 water and to standardize metabolic state. To account for any potential growth over the study
182 duration, the order of sampling among treatment groups was randomized using an online tool
183 (random.org). In total, 103 (12-month-old) and 87 (8-month-old) LWF, and 83 (12-month-old)
184 RWF were tested for T_{Pref} using the Loligo® shuttle box system. Differences in treatment group
185 sizes were due to differential mortality in holding tanks during rearing and were not due to
186 experimentation. Prior to experimentation, power analyses were carried out to determine the
187 optimal sample size within an acceptable power range (0.6-0.8; Harman et al., 2021).

188

189 Statistical Analysis

190 Data is presented as mean±SD unless otherwise stated. T_{Pref} , velocity, total distance travelled,
191 time in arena, number of passages, and avoidance temperature between groups were analyzed
192 using one-way ANOVA, with Tukey's HSD post-hoc for comparisons between individual
193 groups. To determine whether length or body weight influenced recorded T_{Pref} , a general linear
194 model was performed. A comparison was performed to assess species specific differences, with
195 the 12-month T_{Pref} of RWF and LWF compared using a two-tailed T-Test. Bonferroni correction
196 was applied to correct for multiple comparisons of T_{Pref} , resulting in an α taken of 0.00625 for
197 T_{Pref} analyses with LWF (a total of 4 comparisons), and 0.025 for RWF comparisons (a total of 2
198 comparisons). We developed a relationship between time (s) and temperature change (°C) in the
199 shuttle box to determine the upper threshold of the system. This was done to remove possible
200 outliers, as certain individuals were too active for the shuttle box system to determine T_{Pref} due to
201 limitations in heating/cooling rates. Outliers were identified using the robust regression and
202 outlier removal method (ROUT; Motulsky and Brown, 2006). The residuals of this fit were

203 analyzed for potential outliers, and then subjected to ordinary least-squares regression after the
204 removal of outliers. A total of 4 outliers were identified and removed using the ROUT method (2
205 x 8-month-old LWF, 2 x 12-month-old RWF). All statistical analyses were completed in R
206 (version 4.0.0 “Arbor Day”), except for outlier identification which was completed in Graphpad
207 Prism (version 8.4.3). All data and R scripts used for analysis were uploaded to a public GitHub
208 data repository (<https://github.com/WilsonToxLab/Shuttlebox-Thermal-Preference>).

209

210 Results

211 There was not a significant effect of rearing temperature on T_{Pref} on LWF of 8 months of
212 age (Fig. 2A; $F_{[2,82]} = 3.505$; $p=0.0346$). Upper and lower avoidance temperatures were similar
213 between treatment groups (Table 1). However, we note a non-significant trend, with eight-
214 month-old LWF in the 5°C treatment group displayed the lowest activity, travelling an average
215 distance of 173 m, compared to just over 190m at 2°C and 8°C. There was no observable change
216 in the number of transitions between arenas in the shuttlebox in these fish. (Table 1). Total body
217 length was similar between all treatment groups, varying less than 1mm on average (Table 1).
218 Likewise, body weight was similar across treatment groups, with the largest difference (11%)
219 between 2°C ($1.13 \pm 0.32\text{g}$) and 5°C ($1.25 \pm 0.39\text{g}$). Linear models were fit, including body
220 weight and total length as fixed effects, to determine if there was a relationship between size and
221 T_{pref} . Model results ($p = 0.068$, $p = 0.061$) indicated there was no significant interaction between
222 T_{pref} with total length or body weight.

223 At 12 months of age, LWF from all treatment groups (2°C, 5°C, 8°C) displayed similar
224 T_{pref} (Fig. 2B; One-way ANOVA; $F_{[2,100]} = 0.0765$; $p=0.468$). Upper and lower avoidance
225 temperatures were comparable between all treatment groups, suggesting 12-month-old LWF
226 were avoiding temperatures below 14.3°C and above 17.4°C on average (Table 1). Average total
227 length and body weight were similar across all treatment groups, varying less than 1mm or 0.5g,
228 respectively. Linear models were fit, including body weight and total length as fixed effects, to
229 determine if there was a relationship between size and T_{pref} . Model results indicated that body
230 weight ($p = 0.0678$) and total length ($p = 0.0607$) did not significantly affect T_{pref} at 12-months-
231 old.

232 Temperature exposure during rearing effects the T_{Pref} of 12-month juvenile RWF (Fig.
233 2C; One-way ANOVA, $F_{[2,78]}=5.509$; $p=0.0058$). The T_{Pref} RWF juveniles incubated at 2°C
234 ($13.53 \pm 1.14^\circ\text{C}$) and 6°C ($13.39 \pm 0.99^\circ\text{C}$) as embryos displayed significantly lower T_{Pref}
235 compared to those incubated at 0.5°C ($14.27 \pm 0.95^\circ\text{C}$; $p=0.0216$ and $p=0.01$, respectively), with
236 no differences between the 2°C and 6°C groups ($p=0.8764$). This change in preference is
237 reflected in recordings of upper and lower avoidance temperatures. Fish exposed to 0.5°C (15.8
238 $\pm 0.75^\circ\text{C}$) exhibit a higher upper avoidance temperature (One-way ANOVA; $F_{[2,78]}=3.51$;
239 $p=0.0347$; Table 1), with both 2 ($15.29 \pm 0.93^\circ\text{C}$) and 6°C ($15.11 \pm 0.9^\circ\text{C}$) exposed RWF
240 significantly decreased in comparison ($p=0.0216$ and $p=0.01$, respectively). Lower avoidance
241 temperatures exhibit a similar trend (One-way ANOVA; $F_{[2,78]}=3.676$; $p=0.0298$; Table 1),
242 with 0.5°C ($13.73 \pm 0.75^\circ\text{C}$) treated RWF have lower avoidance temperatures than 2 ($13.18 \pm$
243 1.01°C) and 6°C ($13.04 \pm 0.79^\circ\text{C}$) treated fish, differing significantly when compared to 6°C fish
244 ($p=0.03$). Total distance travelled (One-way ANOVA, $F_{[2,78]}=1.885$, $p=0.159$) and number of
245 passages (One-way ANOVA, $F_{[2,78]}=0.522$, $p=0.596$) were statistically similar between all
246 treatment groups. Total length was not consistent between treatment groups (One-way ANOVA,
247 $F_{[2,78]}=15.097$, $p<0.0001$) as juveniles in the 2°C group were significantly smaller than those
248 in the 0.5°C and 6°C treatment groups ($p<0.0001$). However, total length was not significantly
249 different between 0.5°C and 6°C treatments ($p=0.643$). Body weight followed the same trend as
250 total length (One-way ANOVA, $F_{[2,78]}=11.374$, $p=.000045$, Table 1), with the 2°C group
251 significantly smaller in body weight on average than the 0.5°C and 6°C groups ($p=0.0017$ and
252 0.0001 , respectively).

253 As LWF and RWF reside in similar habitats, we sought to assess whether species specific
254 differences existed in T_{Pref} . LWF reared in a similar condition to RWF (2°C), exhibit an
255 increased preference for warmer waters at 12 months of age (Fig. 3; T-test, $p<0.0001$). To
256 investigate the effect of age on T_{Pref} we compared 8-month-old and 12-month-old LWF
257 incubated at the standard temperature of 2°C. Average T_{Pref} for 8-month-old LWF was $16.04 \pm$
258 1.14°C compared to $15.27 \pm 2.67^\circ\text{C}$ for 12-month-old LWF (Fig. 4), which were not statistically
259 different (T-test, $p=0.147$).

260

261 Discussion

262 Our results reveal that elevated temperatures during rearing lead to long-term changes to
263 the T_{Pref} of RWF, but not LWF. Early-life thermal history has been shown to modulate several
264 plastic traits, including behavior, social skills, and endocrine responses in calves (Dado-Senn et
265 al., 2022), pigs (Johnson et al., 2018) and in fish (Jonsson and Jonsson, 2019, Li et al., 2021).
266 While several studies have linked acute or continuous changes in temperature as the etiology of
267 behavioral and growth changes in fish (Bartolini et al., 2015; Gillooly and Dodson, 2000;
268 Nowicki et al., 2012; Nytrø et al., 2014; Sun and Chen, 2014), studies linking solely early-life
269 incubation temperature to long-term perturbations or functional limitations are sparse (Jonsson
270 and Jonsson, 2019; Scott and Johnston, 2012). Here, we provide evidence that the resulting
271 preference of temperature for fish at the juvenile stage can be programmed during
272 embryogenesis, but this phenotypic plasticity is species specific, and the mechanisms behind
273 these changes are abstruse.

274 Most teleost fish function as ectotherms, requiring a conserved and coordinated suite of
275 physiological and behavioral responses to navigate changes in ambient temperature
276 (Haesemeyer, 2020; Stevens and Fry, 1970). For example, adults can preferentially swim to
277 more optimal habitats when encountering thermal stress, such as effluent from power plants
278 (Neill and Magnuson, 1974). These behavioral responses are key, allowing animals to maintain
279 their metabolic optimums (Haesemeyer, 2020). At the embryonic stage, fish would be subjected
280 to environmental temperatures without a recourse to navigate to more appropriate conditions.
281 Several studies have described impacts of elevated incubation temperature during
282 embryogenesis, with clear delineations of reductions in survivability and growth, but also in
283 perturbed metabolism (Barrionuevo and Burggren, 1999; Bozek et al., 1990; Marty et al., 1990).
284 In response to direct increases in ambient temperature, there is a positive linkage to metabolism
285 (Fry and Hochachka, 1970). LWF embryos incubated at constant elevated temperatures display
286 increased oxygen consumption (Eme et al., 2015), but it is unknown whether this difference in
287 metabolism persists to the juvenile stage in this species. Other studies have reported increases in
288 post-embryonic metabolism following embryonic incubations in elevated water temperatures,
289 such as in the razorback sucker (*Xyrauchen texanus*; (Bozek et al., 1990), zebrafish (*Danio rerio*;
290 Barrionuevo & Burggren, 1999), Arctic charr (*Salvelinus alpinus*; (Huuskonen et al., 2003) and
291 Japanese medaka (*Oryzias latipes*; Marty et al., 2010). This suggests that the previously noted
292 increase in metabolism (Eme et al., 2015) may persist to later life-stages. This is an important

293 point, as a functional link between basal metabolic rate and thermal preference has been
294 established in the common minnow (*Phoxinus phoxinus*), demonstrating that fish with higher
295 metabolic rates may prefer colder temperatures as juveniles (Killen, 2014). While this could then
296 suggest that RWF reared at 0.5°C have a lowered metabolism, future studies would be required
297 to confirm whether higher metabolism is at the root of lower T_{Pref} in RWF reared at 2 and 6°C.

298 Round whitefish appear to be more sensitive to elevations in rearing temperature than
299 LWF. We originally hypothesized that increased temperature during incubation would lead to
300 alterations in T_{Pref} , based upon previous observations that elevated ambient temperature increases
301 mortality in these species (Brooke, 1975; Mitz et al., 2019; Eme et al., 2018; Mueller, 2017; Lee
302 et al., 2016; Lim et al., 2017; Lim et al., 2018). At 2°C, RWF experience nearly 30% increase in
303 mortality compared to LWF, with no embryos surviving continuous exposure to 8°C (Lim et al.,
304 2018). RWF appear to exhibit considerable sensitivity to thermal challenges, forming our
305 rationale to reduce the thermal regime RWF were exposed to in this study (6°C), and the
306 inclusion of the 0.5°C incubation group. Serendipitously, this reduction revealed that 2 and 6°C
307 appear to be capable of imparting long-term alterations to T_{Pref} behavior. This possibly presents
308 an advantage in natural settings to whitefish reared in colder water, as fish experiencing a lower
309 temperature during embryogenesis would then prefer a higher ambient temperature at the
310 juvenile stage. A higher temperature is typically found higher in the water column and may
311 present a greater food supply. Indeed, zooplankton, a major food source for larval and juvenile
312 whitefish (Freeberg et al., 1990), is commonly found in higher abundances at warmer and
313 shallower water (Berger et al., 2006). Taken together, rearing in colder water might provide
314 slight behavioral advantages for RWF, suggesting that ever-increasing ambient temperature
315 driven by anthropogenic practices may be detrimental for this species.

316 Apart from temperature stressors, RWF appear to be more sensitive to environmental
317 perturbations than LWF. Population declines have been observed in RWF in New York State
318 (Bouton and Stegemann, 1993; Conley et al., 2021), leading to these fish being labelled
319 vulnerable in this state (Bouton and Stegemann, 1993). Comparing RWF to LWF, the former has
320 historically had a smaller distribution than LWF in North America, with LWF distribution
321 extending farther south beyond the great lakes (Ebener et al., 2008). While the specific causes of
322 these declines are unknown, others have speculated this could be explained by the general

323 sensitivity of RWF to abiotic stressors. For example, acid rain has impacted the Adirondack
324 lakes of New York State, lowering pH, and increasing aluminum and mercury, which interfere
325 with reproduction and survival in these fish (Conley et al., 2021). Moreover, exposure to
326 morpholine, a chemical used to prevent corrosion and damage to water pipes and is used as an
327 additive in fossil fuels, leads to increased mortality and reduced body size in RWF, when
328 compared to LWF at supraenvironmental levels (Lim et al., 2018; Thome et al., 2016). In this
329 study, comparisons between LWF and RWF revealed that when raised at a similar temperature,
330 LWF exhibit a higher T_{Pref} than RWF (Fig. 2). As adults, LWF occupy deeper (18-90 m) limnetic
331 water, with RWF residing in shallow littoral depths (Bailey, 1964; Cucin and Regier, 1965;
332 Rawson, 1951). However, as larvae and juveniles, round and lake whitefish are found feeding
333 along shorelines in shallow water, before gradually moving to deeper waters (Faber, 1970;
334 Hogman, 1971). The difference in preferred temperature may support the observation of these
335 species overlapping, but occupying distinct niches and resources (Eberts et al., 2016).

336 We originally suspected that changes in size and age may play a role in determining T_{Pref}
337 of whitefish. Previous studies have shown a significant relationship between these variables and
338 preference of temperature in LWF, with T_{Pref} decreasing as the animal grew/age (Edsall, 1999;
339 Opuszynski, 1974). In this study, we performed assessments to investigate both factors using
340 LWF, performing a regression for T_{Pref} compared to size (weight and length), and directly
341 comparing the 8-, and 12-month-old age class exposed to a similar ambient temperature during
342 embryogenesis (Fig. 3). While we note no correlations of T_{Pref} with either size or age, we must
343 acknowledge substantial differences of our study design with previous studies investigating this
344 species. Life-stage plays a significant role in determining the preference of the animal, as their
345 natural history dictates a transition to deeper waters as the animal ages (Hogman, 1971). In the
346 work by Edsall (1999) and Opuszynski (1974), thermal preference was ascertained by using
347 considerably younger and smaller LWF. This is a key point, as their comparisons were carried
348 out using fish separated by approximately 6-7 months (Edsall, 1999; Opuszynski, 1974), a larger
349 difference than the present study with a 4-month difference in age. Inherently tied to this age
350 difference is a difference in growth, as our study generated a ~10-fold increase in weight from 8
351 to 12 months of age, and the previous studies describing a more modest increase of ~2-3 fold
352 (Opuszynski, 1974; Edsall, 1999). Changes in growth can easily be attributed to holding
353 temperature of post-hatch fish, with our study implementing a common garden temperature of

354 15°C, compared to the colder holding temperature used previously (8-11°C, Eddsall, 1999). This
355 is an important consideration, as despite a substantially larger increase in absolute size in our
356 study, we note no differences in T_{Pref} . This leads to the proposal that developmental age plays a
357 more significant role in determining temperature preferences in LWF. Plankton tow data points
358 to whitefish migrating from warmer coastal waters to cooler, and deeper waters at approximately
359 4 months of age (Loftus, 1982; Ryan et al., 2014), which may suggest that the age classes
360 investigated in this study may have surpassed windows of overt change in T_{Pref} . Another
361 important difference between these studies is the implementation of a vertical testing chamber
362 (Eddsall, 1999; Opuszynski, 1974), compared to the horizontal shuttlebox used here. In vertical
363 chambers, the coldest temperature is at the bottom of the arena, which may present a behavioral
364 factor that was not considered in our design. In novel situations, fish will exhibit more bottom
365 dwelling type behavior (Blaser and Rosemberg, 2012), which may drive a larger preference for
366 colder temperature. A comparison between these behavioral paradigms would be prudent for
367 understanding how an animal's innate response during assessment may influence thermal
368 preferences.

369 There appears to be no changes in activity levels of LWF and RWF. While the shuttlebox
370 is not purposefully built to assess levels of general swimming, movement in this assessment may
371 be considered a gauge of the animal's exploratory behavior to seek an optimal environment. In
372 search of a preferred temperature, LWF and RWF of all age classes move to equivalent levels
373 and exert a similar number of chamber transitions across all temperature treatments. Increases in
374 temperature lead to increases in locomotion, and anxiety-like behaviours (Angiulli et al., 2020;
375 Biro et al., 2010), with evidence suggesting that the imprinting of temperature in early-life can
376 lead to long-term changes in behavioral responses (Li et al., 2021). While we did not explicitly
377 assess general swimming in LWF and RWF, the results generated here suggests that early-life
378 rearing temperature does not effectively alter behavior during thermal testing.

379 In conclusion, this study demonstrates a persistent effect of increased embryonic
380 incubation temperature on the thermal preference of juvenile RWF. Benthic water temperatures
381 of 2°C represent a winter of low ice cover but is sufficient to alter preferences in these fish.
382 Given that temperatures are expected to increase (0.1°C/year; Austin & Colman, 2007), and
383 thermal effluents impact coastal water temperature, these results raise concern for a fish species

384 that has been considered in decline (Bouton and Stegemann, 1993; Conley et al., 2021), and are
385 rarely seen in abundant amounts ecologically (Mraz, 1964). Coastal embayments provide a
386 thermal refuge during the spring warming (Ryan and Crawford, 2014) and ice-free conditions
387 facilitate a spring bloom of primary productivity which is important for survival of larval
388 whitefish (Faber, 1970). Round whitefish seeking cooler water temperatures may avoid prime
389 nursery grounds, which would put them at a disadvantage compared to other conspecifics. Cold-
390 adapted freshwater fish are among the taxa most vulnerable to climate change but receive a
391 fraction of the research and conservation efforts of terrestrial species (Pacifici et al., 2015). This
392 study highlights the importance of examining sub-lethal thermal effects and thermal plasticity of
393 cold-adapted species. Future studies seeking to understand the role of metabolism on thermal
394 preference are prudent, as this technique provides a non-invasive assessment of environmental
395 performance that may be used to determine at risk-populations environmentally.

396

397 List of Symbols/Abbreviations

398 LWF – Lake Whitefish (*Coregonus clupeaformis*)

399 RWF – Round Whitefish (*Prosopium cylindraceum*)

400 T_{pref} – Temperature Preference

401 °C – Degrees in Celsius

402 mL – Milliliter

403 min – Minute

404 mm – Millimeter

405 g – gram

406

407

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414

415 Competing Interests

416 D.R. Boreham received funding from Bruce Power and held a position of Bruce Power Chair in
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418

419 Author Contributions

420 Conceptualization: A.A.H., J.Y.W., R.G.M., C.S.M.; Methodology: A.A.H., J.Y.W.; Resources:
421 A.A.H., M.L.M.F., L.L.; Formal Analysis: A.A.H., W.A.T.; Investigation: A.A.H., H.M., B.A.,
422 M.M.L.F., L.L.; Data Curation: A.A.H., H.M., W.A.T.; Writing – original draft: A.A.H.,
423 W.A.T.; Writing – review and editing: W.A.T., A.A.H., J.Y.W., M.L.M.F., D.R.B., C.M.S.,
424 R.G.M.; Supervision: J.Y.W.; Project administration: A.A.H., J.Y.W., L.L.; Funding
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426

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432

433 Data availability

434 Data are available from GitHub: [https://github.com/WilsonToxLab/Shuttlebox- Thermal-](https://github.com/WilsonToxLab/Shuttlebox- Thermal-Preference)
435 Preference

436 Tables

437 **Table 1. Behavioral output and whitefish characteristics of thermal preference experiment.**

438 Fish were reared at different temperatures during embryogenesis, and then held in common
 439 garden conditions post-hatch. Lake whitefish and round whitefish embryos were brought in at
 440 8°C and cooled at 1°C/week to the base temperature (incubation treatment); after 100 days
 441 incubation, temperature was warmed at 1°C/ week until they reached 8°C. This simulated natural
 442 conditions with different base incubation temperatures. After successful exogenous feeding, fish
 443 were warmed at 1°C/week to 15°C and were held at that temperature until experimentation in a
 444 shuttlebox. Total sample size (n), avoidance temperatures (temperature when a passage between
 445 chambers occurs), distance travelled, total number of passages, and length of weight of each
 446 experiment are shown. All values are mean±SD, with different letters denoting significant
 447 differences between groups.

448

Species (age)	Incubation Treatment	n	Upper Avoidance (°C)	Lower Avoidance (°C)	Distance (m)	Number of Passages	Length (mm)	Weight (g)
Lake whitefish (8 months)	2°C	31	17.54 (± 0.73)	15.41 (± 0.81)	193 (± 47)	183 (± 113)	55 (± 5)	1.13 (± 0.32)
	5°C	29	17.23 (± 1.09)	14.93 (± 1.38)	173 (± 67)	146 (±121)	56 (± 6)	1.25 (± 0.39)
	8°C	25	17.74 (± 0.52)	15.63 (± 0.57)	191 (± 60)	170 (± 124)	55 (± 9)	1.18 (± 0.51)
Lake whitefish (12 months)	2°C	31	17.14 (± 2.45)	14.01 (± 2.62)	247 (± 112)	51 (± 95)	114 (± 12)	10.59 (± 3.04)
	5°C	40	17.72 (± 2.26)	14.68 (± 1.94)	196 (± 71)	51 (± 65)	114 (± 11)	10.76 (± 3.59)
	8°C	32	17.41 (± 1.85)	14.24 (± 2.16)	209 (± 97)	31 (± 55)	114 (± 8)	10.28 (± 2.43)
Round whitefish (12 months)	0.5°C	27	15.80 (± 0.75) ^a	13.73 (± 0.75) ^a	217 (± 44)	212 (± 81)	62 (± 5) ^a	1.60 (± 0.45) ^a
	2°C	31	15.29 (± 0.93) ^b	13.18 (± 1.01) ^{ab}	189 (± 65)	188 (± 105)	55 (± 6) ^b	*1.19 (± 0.35) ^b
	6°C	23	15.11 (± 0.90) ^b	13.04 (± 0.79) ^b	198 (± 41)	204 (±79)	62 (± 5) ^a	1.71 (± 0.52) ^a

449

450

451

452 **Table 2. Temperature preference (T_{pref}) of juvenile lake whitefish of different ages.** Age is
453 provided in years (yrs) and months. Age in months was estimated for previously published
454 studies by assuming median hatch occurs within March – April, as suggested by testing dates for
455 1-year old fish. T_{pref} from the present study is reported from 2°C treatment groups only. Edsall
456 (1999) used simulated lake water temperature during embryonic incubation. All other T_{Pref} data
457 provided by Edsall (1999). Holding temperature refers to water temperature in home tanks from
458 hatch until testing.

Age (yrs)	Age (months)	Size (g)	T_{pref} (°C)	Holding Temperature (°C)	Source
0	4-5 ^a	2.8	15.9	8-11	Edsall (1999)
0	5-6	1.1-1.7	17-18 ^b	- ^c	Opuszynski (1974)
0	5-6	1.9	16.8	8-11	Edsall (1999)
0	8	1.13	16.04	15	Present Study
1	12	10.59	15.27	15	Present Study
1	12-13 ^a	3.9	15.6	8-11	Edsall (1999)
1	12-13	5.7	10	- ^c	Opuszynski (1974)

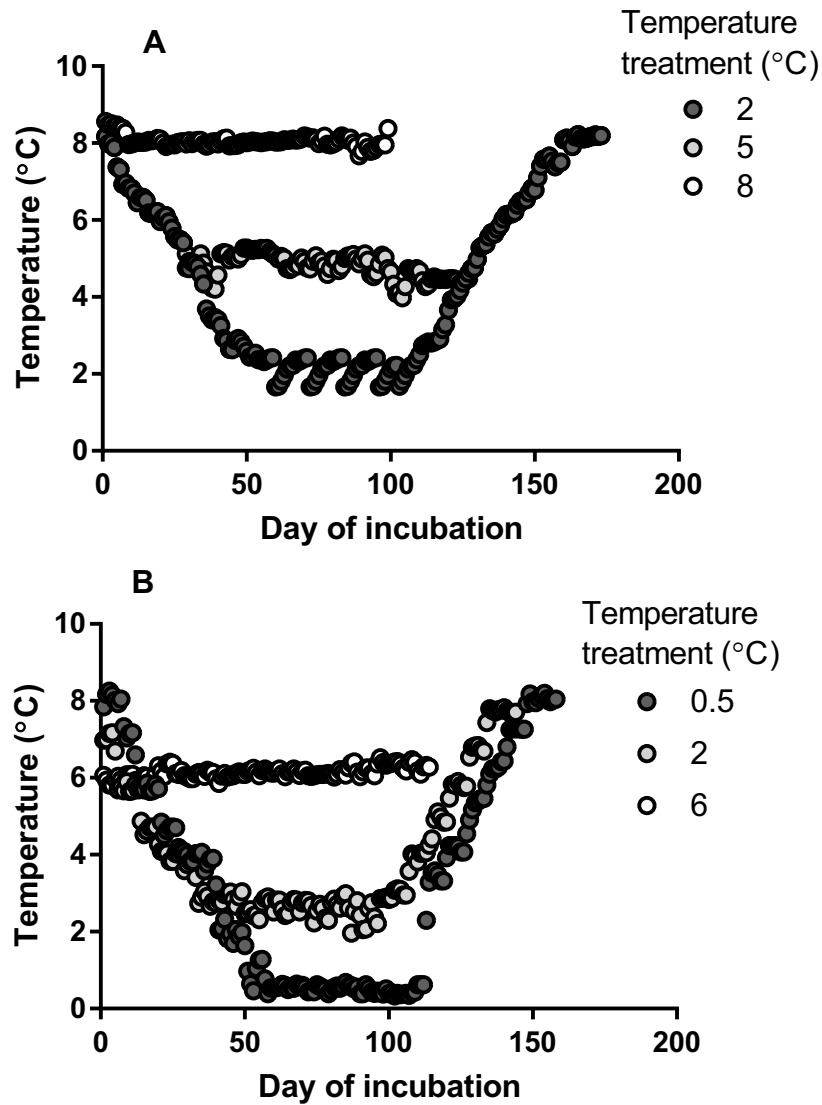
459 ^a Repeated measure on same cohort of fish.

460 ^b Temperature preference estimated via inspection by Opuszynski (1974).

461 ^c Information not available.

462

463 Figures

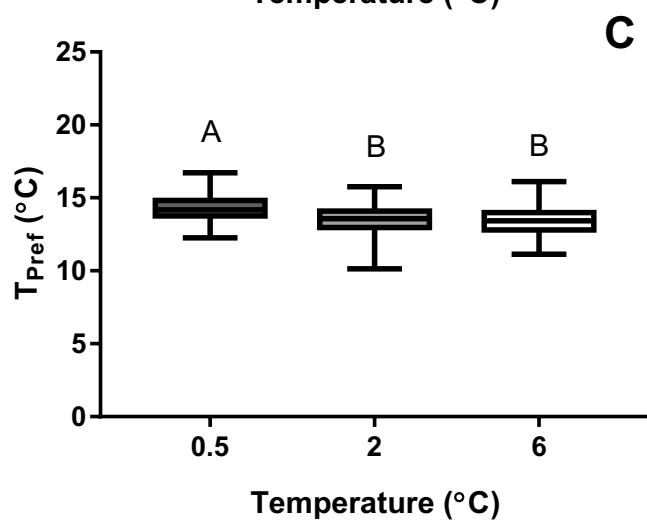
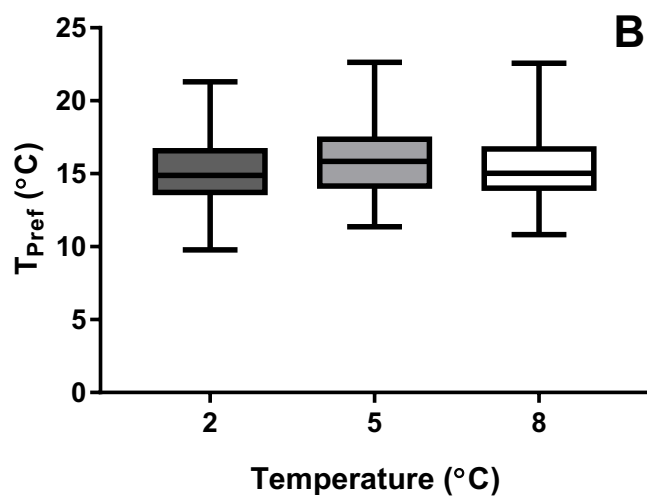
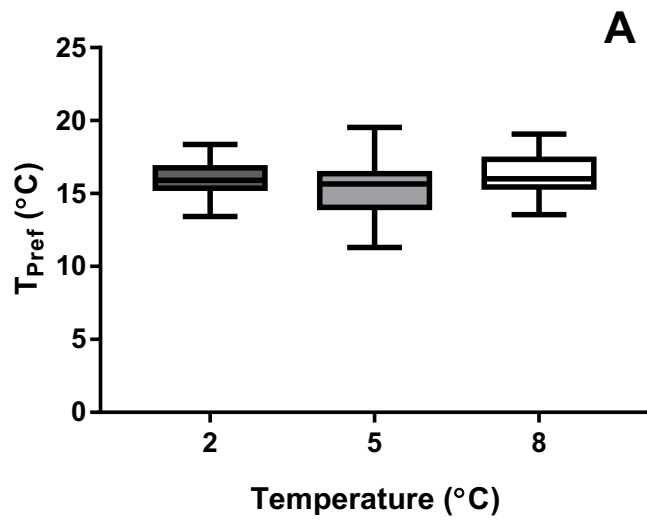


464

465 **Figure 1. Daily temperature readings of incubation of lake whitefish (LWF; A) and round whitefish**

466 **(RWF; B) until complete hatch of population. Data points represent averages of daily readings taken in**

467 15 min intervals, with error bars excluded for visibility.

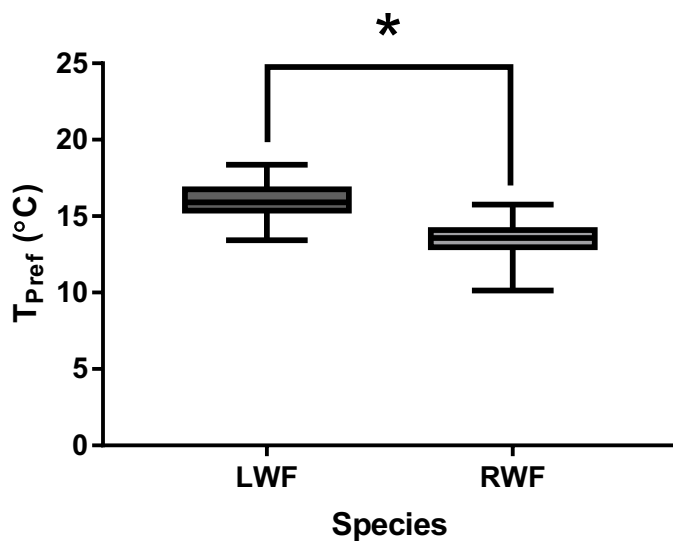


468

469 **Figure 2. Ambient temperature during rearing can alter the thermal preference of fish in a**
470 **species-specific manner.** Boxplots comparing thermal preference (T_{Pref}) of (A) 8-month-old lake
471 whitefish, (B) 12-month-old lake whitefish, and (C) 12-month-old round whitefish, after
472 exposure to different ambient temperatures during embryogenesis. Lower and upper box
473 boundaries are 25th and 75th percentiles of the data, with the line inside the box representing the
474 median, and error lines encompassing the entirety of the spread of data. Different letters above
475 the boxplots denote significant differences between treatment groups.

476

477



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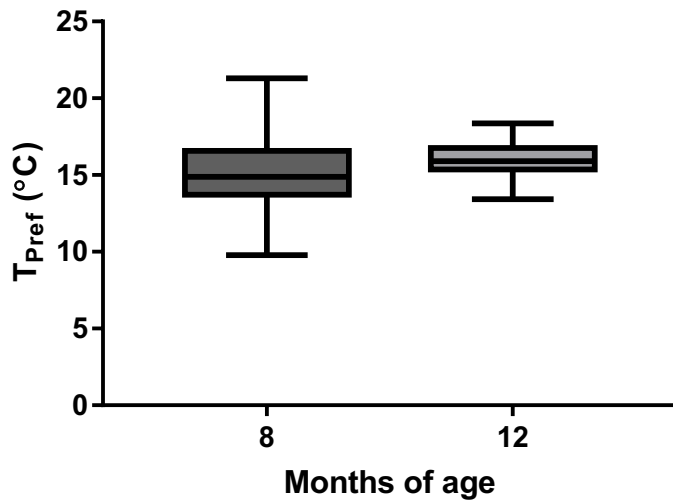
479 **Figure 3. Species exhibit unique thermal preferences following rearing at a common**
480 **temperature.** Boxplots comparing thermal preference (T_{Pref}) of 12-month-old lake whitefish
481 (LWF) and 12-month-old round whitefish (RWF), after experiencing 2°C during embryogenesis.
482 Lower and upper box boundaries are 25th and 75th percentiles of the data, with the line inside the
483 box representing the median, and error lines encompassing the entirety of the spread of data. The
484 symbol * is used to denote a significant difference between groups.

485

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489

490 **Figure 4. Thermal preference does not change during the juvenile stage of lake whitefish.**
491 Boxplots comparing thermal preference (T_{Pref}) of 8-month-old and 12-month-old lake whitefish
492 (LWF), after experiencing 2°C during embryogenesis. Lower and upper box boundaries are 25th
493 and 75th percentiles of the data, with the line inside the box representing the median, and error
494 lines encompassing the entirety of the spread of data.

495

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497

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