**Low-temperature tolerances of tropical fish with potential
transgenic applications in relation to winter water
temperatures in Canada**

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Low-temperature tolerances of tropical fish with potential transgenic applications in relation to winter water temperatures in Canada

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Abstract: Application of fluorescent protein transgenes in tropical freshwater fish is used in research (e.g. in zebrafish), and for the commercial ornamental aquarium trade. To assess the overwinter potential of such fish in Canada, we examined the minimum temperature tolerance of three wild-type species (zebrafish *Danio rerio* Hamilton, 1822; black tetra *Gymnocorymbus ternetzi* Boulenger, 1895; tiger barb *Puntius tetrazona* Bleeker, 1855) used as fluorescent models in the aquarium trade, and four lines of green fluorescent protein (GFP) transgenic and wild-type zebrafish used in research. Both tetra and barb had lower temperature tolerance limits that were higher than surveyed winter temperatures in Canada, and the lower tolerance limits of zebrafish lines was only within the range of a few surveyed lakes in some years. One line of GFP zebrafish had a lower temperature tolerance limit that was significantly higher than those of other lines, suggesting some fluorescent transgene constructs may alter tolerance to extreme temperatures. When lines of zebrafish were reared at 8, 10, or 28°C for four months all lines were capable of producing viable embryos after cold rearing. These results indicate tropical transgenic fish models used in research and the aquarium trade are not expected to persist overwinter in Canadian water systems.

Keywords: *Danio rerio*; zebra danio; *Gymnocorymbus ternetzi*; white tetra; *Puntigrus tetrazona*; tiger barb; thermal minimum; temperature; fluorescent protein; transgene; risk assessment; survival; overwinter

Introduction

Transgenic tropical fish containing fluorescent protein transgenes are commonly used in research (e.g. zebrafish, Lin et al. 2016) and in the ornamental aquarium trade (e.g. GloFish®, USA). Insertion of fluorescent protein transgenes can result in directed or universal expression of exogenous fluorescent proteins in numerous organisms, including fish. Great advancements in developmental biology and gene regulation have been undertaken using tropical-origin zebrafish (*Danio rerio* Hamilton, 1822) models that have targeted fluorescent visualization of key proteins (see Udvardia and Linney 2003; Lin et al. 2016). Use of fluorescent protein transgenes as a reporter genes was first demonstrated in zebrafish in 1995 (Amsterdam et al. 1995), and has expanded to use of multiple tags in a single fish such as tagging superficial epithelial cell populations with dozens of distinguishable tags for quantitative *in vivo* imaging (Chen et al. 2016). In addition, ornamental tropical freshwater fish that have universal and/or muscle/skin-specific expression of various fluorescent transgenes to produce stunning visual colours are commercially available in some countries. For example, GloFish® (Yorktown Technologies, Austin, Texas) in the USA are available in multiple fluorescent colours three species: wild-type morph (i.e. striped) and golden morph of zebrafish (also known as golden zebra danios), the white morph of black tetras (*Gymnocorymbus ternetzi* Boulenger, 1895, also known as white tetras or white skirt tetras) and tiger barbs (*Puntigrus tetrazona* Bleeker, 1855). A transgenic red fluorescent protein (RFP) zebrafish was the first approved and marketed strain in 2003 in the US excluding California, followed by an updated RFP model and additional colour variants in following years (Hill et al. 2011; Hill et al. 2014). A GFP transgenic tetra was introduced in 2012 followed by additional colour variants, and two colour variants of tiger barbs were introduced in

2014 (Hill et al. 2014). Approval to sell GloFish® in California was granted in 2015 (California Fish and Game Commission 2015). With increasing potential application of fluorescent transgenic models in both research and the ornamental aquarium trade, information to scientifically assess the potential for such fish to establish in natural water systems is needed to inform risk assessment prior to their use to support decisions to protect Canadian environments. The purpose of our study was to determine the potential for common tropical research fish, as well as progenitor strains for commercial ornamental aquarium fish, to establish themselves in Canadian waters.

In temperate regions such as much of the high population density areas in Canada, the ability for transgenic tropical species such as zebrafish and other ornamentals to establish themselves is primarily limited by their ability to overwinter in cold water bodies. For example, Cortemeglia and colleagues (Cortemeglia and Beitinger 2005, 2006; Cortemeglia et al. 2008) examined the cold temperature tolerance of wild-type zebrafish and the original RFP zebrafish used in the ornamental aquarium trade, and contrasted it to potential survival distribution across the USA. They found that chronic lethal minimum temperature (CL_{min} , measured by gradual decreases in temperature) and critical thermal minimum temperature (CT_{min} , measured by rapid linear decline in temperature) were above 4°C for both RFP (5.6 and 7.2°C respectively) and an unrelated wild-type zebrafish (5.3 and 6.2°C respectively) when acclimated to 20°C (Cortemeglia and Beitinger 2005, 2006), and confirmed neither group of fish were able to withstand four days at 4°C in a field trial (Cortemeglia et al. 2008). Based on surface air temperature measurements, they hypothesized that projected distribution of zebrafish lines in the USA would be limited to isolated areas in the southern States (Cortemeglia and Beitinger 2006). While RFP zebrafish

displayed less cold tolerance than control fish, it should be noted that the control fish were obtained from a different source than RFP zebrafish, and whether the altered minimum thermal tolerances of RFP fish were due differences in rearing conditions or possibly background genetics is not known. As well, currently marketed transgenic zebrafish are available in both a striped and golden morph of the zebrafish, and whether these morphs alter thermotolerance in zebrafish has not been examined. In addition, whether transgenic inserts used in research may also impact thermotolerance has not been examined. Hill et al. (2014) applied the Fish Invasiveness Screening Kit (FISK) to all three species used as Glofish®, and concluded a low-risk of invasiveness within the USA based on no potential for hybridization for native species, little history of invasiveness despite high potential for release, lack of traits associated with invasiveness, small body size and predation-enhancing fluorescence. For scientifically defensible risk assessment, experiments to determine overwinter capabilities are needed to confirm this lack of potential to persist in Canadian waters, particularly in tetra and tiger barb where no published scientific data on cold tolerance exists.

In the present study, we hypothesized that fluorescent transgenic tropical freshwater fish and their wild-type progenitor strains used in research and ornamental trade would have poor capacity to overwinter in freshwater systems across Canada, as measured by survival at low temperatures and reproductive capacity after cold rearing. We compared the chronic cold tolerance in three green fluorescent protein (GFP) zebrafish lines and their wild-type progenitor line that are available for research, as well as in three wild-type tropical species/morphs (golden morph of zebrafish, white morph of black tetra, tiger barb) available as fluorescent transgenic lines in the ornamental aquarium trade in the USA, but not currently approved in Canada.

Tolerance to extreme environmental conditions can have reproductive costs in fish (e.g. Schofield et al. 2011). To address this we also examined reproductive parameters in GFP and wild-type zebrafish research lines after long-term rearing at low temperatures, to determine if GFP zebrafish would be able to reproduce after overwintering in cold systems. In addition, we surveyed government and stewardship programs collecting water temperature data to determine typical minimum water temperatures in freshwater systems across Canada. This study has relevance for the development of governmental regulations on use of such fish for commercial and research purposes in Canada and other countries with temperate climates.

Materials and Methods

Minimum temperatures in Canada

Average winter surface air temperatures for Canada were obtained from Environment and Climate Change Canada (ECCC). Surface and water column water temperatures (rivers and lakes) across Canada were obtained from various Federal, Provincial and International sources (AB Environment; Government of BC Environmental Monitoring System (EMS); Environment and Climate Change Canada; Manitoba Waterstewardship; World Lake Database; Government of Ontario Provincial (Stream) Water Quality Monitoring Network; Government du Quebec, Ministère du Développement durable, de l'Environnement et de la Lutte contre les changements climatiques; BC Lake Stewardship Society). The majority of this data was taken by government employees at designated water quality stations and available online or obtained directly from government employees. The details of how data was collected at each site are not available, and

141 frequency of monitoring ranges from hourly over several years, to once per season over a single
142 year.

143

144 *Experiment 1: Cold temperature tolerance of tropical species with potential application as*
145 *fluorescent aquarium fish*

146

147 All experiments were reviewed by institutional animal care committees and conducted under
148 institutional animal care permits meeting guidelines established by the Canadian Council for
149 Animal Care. Golden morph of zebra danios (hereafter referred to as golden zebrafish, white
150 morph of black tetras (hereafter referred to as white tetra), and tiger barbs (all wild-type
151 morphs/species for GloFish™ lines available in the US, but not available in Canada) were
152 purchased from local aquarium stores or distributors in the Greater Vancouver area, BC, Canada.
153 All three species are domesticated aquarium strains of tropical origin, i.e. zebrafish are native to
154 India, Pakistan, Nepal, Bangladesh, Sri Lanka and Myanmar (Barman 1991), and reportedly
155 tolerate a wide range of temperatures in the wild from as low as 6°C to over 38°C (see Spence et
156 al. 2008); black tetras are from the Rio Paraguay river basin in South America (see Benine et al.
157 2015); and tiger barbs are native to Sumatra (see Kottelat 1998). Fish were housed in a climate-
158 controlled room and acclimated at 24°C in 38 L tanks ($n=20$ fish per tank for zebrafish and
159 tetras, $n=15$ fish per tank for tiger barbs, 3 tanks per species) for at least one week. The
160 temperature of two tanks per species (cold-challenge tanks) were then dropped to 20°C over a
161 four-day period, and allowed to acclimate to this temperature for at least one week. One tank per
162 species remained at 24°C throughout the experiment as a control tank. Fish were fed twice daily
163 with tropical aquarium flakes during acclimation and the experiment. For the experiment,

temperature of the cold challenge tanks was dropped by approximately 1°C/day, 6 days a week, by lowering the ambient temperature in the housing room (see Supplemental Figure S1A). Control tanks were held at 24°C via submersible heaters. Water temperature was recorded every 10 min by Tidbit data loggers (Onset Computer Corporation, Bourne, MA). Fish were observed twice a day for feed intake, activity level, and loss of equilibrium until fish stopped feeding or they reached 10°C (which ever came first), at which point observations were increased to four times per day. Fish were removed once they could no longer maintain equilibrium, time and temperature recorded, and fish moved to a recovery tank kept at approximately 4°C above the cold challenge tanks. Total ammonia, nitrite and pH were measured in the tanks every 2 days, and 20% water change performed if nitrite or ammonia levels became detectable. Throughout the experiment, total ammonia nitrate levels remained well below Acute Criterion Magnitude levels as determined by USEPA (2013). Lighting was set to 10:14 light:dark to simulate winter photoperiod. CL_{min} determinations were modified from Beitinger et al. (2000) where endpoint measured was loss of equilibrium rather than death although some individuals were not rescued (e.g. those that lost equilibrium during night hours), and in these cases death was considered an equivalent endpoint to loss of equilibrium. CL_{min} was calculated as the average temperature at which each tank of individuals within a species lost equilibrium (average ± s.e.m.), and the temperature at which 50% of individuals lost equilibrium (LT₅₀) for each tank/species was calculated using the dose.p function in MASS package (Venables and Ripley 2002) in R (R Core Team. 2015) and averaged over tanks within species.

Experiment 2: Chronic cold temperature tolerance and reproductive success of laboratory transgenic and wild-type zebrafish.

187
188 Fertilized zebrafish embryos from three lines expressing enhanced GFP driven by cell/tissue
189 specific promoters (e.g. blood vessels, glial cells and epidermis-specific promoters, see Table 1)
190 as well as their wild-type progenitor line (AB line, a common research line) were purchased from
191 the Zebrafish International Resource Centre (ZIRC) at the University of Oregon, Eugene,
192 Oregon. Strains were chosen as they were the most readily available fluorescent models at ZIRC
193 (i.e. available for purchase as embryos), which may increase their potential to be used for
194 research. Fish were raised to adults (estimated as 16 weeks post-hatch) and transferred into a
195 zebrafish rack system held in an environment chamber with precise temperature control. The
196 zebrafish lines were evenly distributed among sixteen, nine-liter tanks in the rack (four
197 tanks/line, approximately 20 fish per tank, 80 fish per line total). As well, an additional 70 fish
198 per line, spread approximately evenly among the four tanks, was included for long-term
199 acclimation at different temperatures (see below). Location of each line on the zebrafish rack
200 system was assigned in a random fashion. After transfer to the zebrafish rack system, all the fish
201 were left for four weeks at 28°C to acclimate. Fish were fed daily with commercial zebrafish
202 flake diet and mortality was negligible during this period. At the onset of the experiment,
203 chamber temperature began cooling from 28°C to 12°C at a rate of approximately 1°C/day.
204 Below 12°C, the rate of temperature decrease was lowered to ~0.1°C/day to the minimum
205 temperature available in the environmental chamber (5.1°C) at which point the trial ended (see
206 Supplemental Figure S1B). Fish were monitored daily and removed once they could no longer
207 maintain equilibrium or died, and time and temperature recorded. Some fish were still alive at
208 the end of the trial, and consequently only LT₅₀ was calculated as above. Loss of equilibriums
209 from specific tanks within a species was not tracked, and consequently LT₅₀ was calculated from

all individuals from all four tanks within a species ($n=80$). Comparisons of survival curves among lines were performed with survival log-rank analysis, and comparisons of LT_{50} were performed with one-way ANOVA followed by Student-Newman-Keuls *post-hoc* test using SigmaPlot (Systat Software Inc., San Jose, CA).

During the progressive decline in temperature, 20 individuals of each line (five from each tank) were removed at 28°C, 10°C and 8°C and held at these temperatures for long-term acclimation (four months) in order to determine whether the fish could produce viable offspring after cold acclimation, once returned to 28°C. Fish were also removed and held at 6°C ($n=10$ per line), however these fish showed 80 to 100% mortality within the first week of transfer, therefore this treatment was terminated. At the end of the four-month acclimation period, which was staggered so all fish were acclimated for precisely four months, the 8 and 10°C acclimation groups were warmed back to 28°C over a one week period and, after two weeks at 28°C, a bulk breed protocol was initiated by placing a breeding tray in each tank for one week, and collecting eggs daily. Total number of individuals per genotype/acclimation temperature group was 16 to 19, and individual fish were not sexed. Domesticated zebrafish have polygenetic sex determination, i.e. sex can be influenced by environment and unidentified genetic factors (see Liew et al. 2012). All fish used had the same AB wild-type genetic background and had equal juvenile rearing conditions, and as such sex ratios are expected to be similar in each group. However, as sex was not determined this cannot be confirmed and ratio of female:male was unknown. All eggs produced were transferred to zebrafish hatchery trays and the following parameters were recorded: total egg count, total number of eggs fertilized, and total number of eggs hatched.

Results

Winter freshwater temperatures for Canadian rivers and lakes

Extensive winter water temperature was gathered from across Canada, with a total of 148 river and 107 lake locations represented. With the exception of coastal Southern BC, surface air temperatures across Canada over the winter months average below freezing (see Figure 1). Due to high mixing in rivers, temperatures are assumed to be uniform throughout the water column in these systems. Surveyed winter river water temperatures were generally $\leq 1^{\circ}\text{C}$ with maximum winter temperature recorded in rivers being $< 4^{\circ}\text{C}$ (see Figure 2, Supplemental Table S1). Unlike rivers, lakes often stratify in temperature, particularly in the winter. The majority of lakes in Canada are dimictic, which in Canada generally freeze over and have the warmest, densest water on the bottom (see Horne and Goldman 1994; BCLSS 2014). This temperature is generally around 4°C , but may be lower if sufficient wind action mixes cooler upper water with the warmer deeper water (see Horne and Goldman 1994). The exception to this is coastal southern BC lakes that are usually monomictic. These lakes mix throughout the winter, with similar temperatures throughout the water column that are generally at or above 4°C (Horne and Goldman 1994; BCLSS 2014). Surface and water column measurements in Canadian lakes confirm the majority of lakes having winter temperatures $\leq 4^{\circ}\text{C}$. A few lake locations had recorded minimum temperatures $> 4^{\circ}\text{C}$ and up to 6.8°C , and these lakes were primarily located in southwestern BC (see Figure 2, Supplemental Table S2). Only three lakes had winter temperatures greater than 5°C . The warmest lake, Lake Cowichan, had minimum temperature of 6.8°C in February 2016 measured at multiple stations across the lake on a single day (obtained from EMS database, BC Ministry of Environment), while in a different location in the same lake, monthly measurements

over one year (2012) found a minimum temperature of 5.8°C in February (BCLSS 2014). Of the other two lakes, Twin Lake minimum temperature was taken from temperature profile of the lake measure on a single day in January in 2001 (BCLSS 2004), while Spider Lake temperatures were taken on single days in February in 2010, 2013, 2016 (obtained from EMS database, BC Ministry of Environment). Considering the scarcity of data in these warmer lakes, the temperatures may not necessarily represent the coldest normal winter temperatures in the lakes. For other lakes, water column profile data for lakes are rarely taken, and warmer temperatures may be present at lower depths in dimictic lakes than those recorded, although these temperatures are not expected to be greater than 4°C.

Cold temperature tolerance of tropical species with potential transgenic applications

In the cold temperature trial using equivalent wild-type ornamental aquarium morphs and/or species as GloFish® (Experiment 1), all species lost equilibrium over a 1-2°C temperature range (6.6-4.8°C for golden zebrafish, 10.5-9.5°C for white tetras, and 13.6-12.6°C for tiger barbs, see Figure 3). Golden zebrafish had the greatest tolerance to cold water in terms of CL_{min} , followed by white tetras and tiger barbs (see Table 2). Average LT_{50} were slightly lower than average CL_{min} for all species, but this difference was less than 0.3°C (see Table 2). If CL_{min} and LT_{50} were calculated with tanks pooled per species as per Experiment 2, this did not greatly influence CL_{min} measurements, although increased LT_{50} measurements so that they were closer (within 0.1°C) to CL_{min} measurements (i.e. $5.59 \pm 0.03^\circ\text{C}$ for golden zebrafish, $9.99 \pm 0.02^\circ\text{C}$ for tetra, $13.45 \pm 0.03^\circ\text{C}$ for barb). All species decreased activity and feeding with decreasing temperature and ceased feeding as temperatures continued to drop (see Figure 3). Both zebrafish and tetras

ceased moving and rested on the bottom of the aquaria prior to loss of equilibrium, while barbs continued moderate to medium activity until loss of equilibrium. Of the fish removed at loss of equilibrium, zebrafish and tetra individuals were rescuable (i.e. returned to regular activity and feeding after warming to 24°C), but all tiger barbs died once moved to recovery tanks. There were no mortalities in control fish held at 24°C during the experiment and they had high activity and feeding level throughout the experiment.

In the cold temperature trial for research lines of zebrafish (Experiment 2), the y1 line had a more rapid decrease in survival with decreasing temperature (Figure 4, $P = 0.025$ to 0.035) as well as higher LT_{50} ($P < 0.001$, see Table 2) than the other lines, although the LT_{50} of all lines was within less than 1°C of each other (5.38 – 5.90°C). There were no other differences observed. In Experiment 1, when LT_{50} was calculated with pooled tanks as in Experiment 2, values were within 0.1°C of CT_{min} . As such, it is presumed that LT_{50} values in Experiment 2 as calculated with pooled tanks are accurate measurements of cold tolerance. Survival of control fish at 28°C was not specifically tracked, although fish reared at 28°C during this time and for an additional month (four months total, see reproductive performance below) had 85-95% survival (see Table 3).

Effect of cold-water rearing on reproductive performance of GFP transgenic and wild-type zebrafish

Survival during the four-month holding periods at 28, 10 and 8°C was not significantly affected by zebrafish line, acclimation temperature, or their interactions ($P = 0.7342$, 0.8074 , and 0.6341

respectively) and ranged from 80-95% over the four-month period (see Table 3). All fish groups from all acclimation temperatures laid eggs. There were large variations in the total number of eggs produced by the different lines of zebrafish held at different temperatures, although there was no significant factor effects of genotype or temperature on the total number of eggs laid ($P = 0.392$ and 0.551 respectively), the total number of fertilized eggs ($P = 0.394$ and 0.472 respectively), or the total number of eggs hatched ($P = 0.419$ and 0.693 respectively, see Table 3). As number of available females per group was not known, and therefore cannot be confirmed to be equal among groups, results related to total egg count as estimated of average productivity should be interpreted with caution.

Discussion

This is the first published report of cold tolerance of black tetras and tiger barbs, and the first published study examining the effect of fluorescent transgenesis on cold tolerance where background strain and rearing conditions were controlled for. Of the tropical species examined, only zebrafish (wild-type, GFP transgenic, and golden varieties) had minimum water temperature tolerances (5.38 to 5.90°C) within the range of winter water temperatures recorded for Canada. However, almost all of surveyed lakes and rivers had winter temperatures lower than tolerances of these zebrafish strains (see Figure 2). In addition, long-term rearing of wild-type and GFP transgenic zebrafish at 6°C resulted in high mortality, indicating their functional minimum temperature tolerance is higher than indicated by modified CL_{min} or LT_{50} . Long-term rearing at 8°C did not result in a significant increase in mortality, indicating the functional minimum temperature tolerance of zebrafish is between 6 and 8°C . Only one surveyed lake in

325 Southwestern BC had winter temperatures within this range, and water temperatures in the same
326 lake but different location and year were less than 6°C, indicating zebrafish lines would be
327 unlikely to persist over multiple winters even in the warmest lakes, based on temperature
328 tolerance data alone. Zebrafish released to natural systems would also be expected to be further
329 limited by varying food supply, competition and predation from native fish, and altering abiotic
330 factors such as oxygen. Fluorescent models may have even further limited persistence, as
331 currently marketed RFP Glofish® zebrafish are reported to be preyed on at a greater rate (Hill et
332 al. 2011) and have decreased spawning success and fitness (Howard et al. 2015). Of the other
333 two species examined, the higher minimum tolerated temperatures of the white tetra (9.95°C)
334 and tiger barb (13.36°C) indicate they would not be able to overwinter in surveyed freshwater
335 systems in Canada. The tiger barbs in particular displayed poor acclimation to lowering
336 temperatures as they had the highest CL_{min} , were not rescuable after loss of equilibrium, and
337 unlike zebrafish and tetras did not display modified activity as they approached CL_{min} .

339 These results provide empirical data to support limited potential for invasiveness as estimated
340 by the FISK tool in the USA (Hill et al. 2014), the tropical origins of the species in question, and
341 a lack of establishment for these species in temperate climates in North America. All three
342 species are among the most commonly sold wild-type ornamental aquarium fish (Gertzen et al.
343 2008; Strecker et al. 2011). Release of ornamental aquarium fish to natural systems in Canada
344 and elsewhere is commonplace (e.g. Marson et al. 2009 found up to 2% unwanted aquarium fish
345 in Canada were released to the wild), indicating there would be a high propagule pressure of
346 release of these fish throughout North America. Despite this, there are no reports of established
347 populations of these species in North America (Hill et al. 2014). Non-native occurrences of

zebrafish have been limited to temporary local establishments in New Mexico, USA and Columbia (Welcome 1988; Nico et al. 2013a); black tetras from the aquarium trade were transiently established in a Colorado hot spring, and isolated occurrences have been reported in Florida and Louisiana (Nico and Fuller 2005), but there are no known established populations in temperate regions; and tiger barbs have numerous isolated occurrences have been reported in Southern US, but failed to establish (Courtenay and Hensley 1979; Howells 2001; Nico et al. 2013b). In extensive surveys of waterbodies adjacent to and surrounding ornamental aquarium facilities in Florida, USA, both wild-type and Glofish™ zebrafish, as well as black tetras and tiger barbs were found adjacent to facilities, but not detected greater than 500 m away (Tuckett et al. 2017), indicating limited ability to establish and spread after escape. The authors identified potential causes for lack of establishment of escaped tropical fish, including abiotic challenges, presence of predators and competitors, and bright colouration and poor body shape for predator avoidance and competition success (Tuckett et al. 2017). It is worth noting that localized areas of warmer water, such as hot springs (Mayhood 1995; Wright 2002) and areas downstream of warm industrial effluent (Mills et al. 1993; Starling et al. 1995; Peterson et al. 2005), can result in pockets of local establishment of exotic fish species otherwise unable to withstand temperate water temperatures and able to persist and reproduce within a small spatial range, although any potential impact of this would be localized. Consequently, local conditions and potential scale of impacts should be considered when determining the establishment potential of various exotic transgenic fish species in Canada.

This is the first report of low-temperature tolerance of white tetras or tiger barbs, and their high minimum temperature tolerance is expected given their tropical origin. Minimum temperature

tolerance of golden zebrafish, as well as wild-type and GFP transgenic research lines of zebrafish, was similar to that reported for wild-type ($5.3 \pm 0.2^{\circ}\text{C}$) and red fluorescent protein (RFP) zebrafish derived from the wild-type strain ($5.6 \pm 0.2^{\circ}\text{C}$) under similar experimental conditions (Cortemeglia and Beitinger 2006). The $\text{CL}_{\min}$ of the golden zebrafish in the current study was lower than that reported in striped wild-type, suggesting the currently marketed GloFish zebrafish in a golden morph background may have less ability to withstand cold temperatures than those in a striped background (Cortemeglia and Beitinger 2006), although as the different fish morphs and strains were not examined in parallel, direct comparisons cannot be made. Of the research lines of GFP transgenic zebrafish, only the y1 line had significantly higher $\text{CL}_{\min}$ than the other GFP and wild-type lines, indicating some transgenes may alter temperature tolerance of fish. In general, GFP and other fluorescent proteins are considered neutral – i.e. their presence does not significantly affect cell function (see Stewart 2001, 2006). However, mouse (*Mus musculus* L., 1758) models with very high levels of universal GFP expression have been reported to have poor early developmental competence (Devgan et al. 2004) and renal defects and high adult mortality (Guo et al. 2007), and high cardiac-specific GFP expression increased cardiomyopathy resulting in high mortality (Huang et al. 2000), while models with lower levels of GFP expression did not show significant pathology. As well, Tao et al. (2007) found RFP (DsRed) expressing haemopoietic stem cells had poor growth, while GFP-expressing cells did not, demonstrating different fluorescent transgene constructs may have different effects. The three transgenic research lines in the current study used similar GFP gene coding regions but different promoters, resulting in different localized expression of GFP. The y1 line with inferior minimum temperature tolerance expressed GFP in blood vessels. Functional failure of circulation is thought to be a component limiting cold tolerance (see Pörtner 2001), and it is possible that

GFP expression in the blood vessels had a negative impact on circulation and hence tolerance of the line to low temperatures. Liu and Liu (2012) also found the y1 line had unspecific vascular expression and altered embryonic expression profiles of over 230 genes including immune response and embryonic development genes, suggesting this line may have overall altered response to normal or pathological conditions. Expression of GFP in glial cells (line mi2001) and developing epidermis (line zp4) did not appear to impact minimum temperature tolerance in zebrafish. Whether commercial strains with potentially ubiquitous expression (e.g. currently marketed Glofish®) would have greater potential for altered cold tolerance than laboratory strains with more directed transgene expression has not been assessed.

The differences in laboratory strains confirms other studies demonstrating the importance of considering each unique line separately when conducting tolerance or other risk assessment-related studies (e.g. Tuckett et al. 2016; Leggatt et al. 2017; see Devlin et al. 2015). Intraspecific variation, including differences among different lines of transgenics, should also be considered in risk assessment. Intraspecific variation in cold tolerance has been demonstrated in another ornamental fish, green swordtails *Xiphophorus hellerii* Heckel, 1848, where cold tolerance of escaped populations was greater than populations obtained from commercial suppliers (Tuckett et al. 2016). In salmonids, different lines of growth-enhanced transgenic salmon and trout, as well as domestication and domestication x transgenesis interactions, can influence the ability to survive in semi-natural stream environments (Crossin et al. 2015; Leggatt et al. 2016; Leggatt et al. 2017). In the ornamental aquarium trade, domestication level and life history experience may differ greatly among intraspecific populations, while in the laboratory zebrafish long-term colony maintenance may result in genetic shifts in different populations. The potential for intraspecific

variation to influence thermal tolerance or uncertainty in risk assessments should be addressed, particularly in species where thermal tolerance is closely aligned with the environments being assessed.

The reproduction trial demonstrates that wild-type and GFP transgenic zebrafish lines examined are capable of producing viable embryos after long-term rearing at temperatures as low as 8°C. Therefore, fluorescent zebrafish have the potential to reproduce in warmer water refuge areas should they survive the winter. Previous studies examining the effects of fluorescent transgenes on reproductive success or preferences in zebrafish depend on the variable examined and transgenic group considered. RFP and wild-type zebrafish had similar age at maturity for females as well as male and female fecundity (Howard et al. 2015). In a population containing equal numbers of GFP and wild-type zebrafish allowed to breed for one week, GFP zebrafish had no apparent reproductive advantage or disadvantage over wild-type (Gong et al. 2003), while in a serial breeding experiment Howard et al. (2015) found the frequency of the RFP transgene declined rapidly and was eliminated in most populations after 15 generations indicating a strong bias against the RFP transgene in reproduction. Other studies found both wild-type and RFP zebrafish females preferred to associate with RFP rather than wild-type males, but did not differ in number of mating bouts (Owen et al. 2012), lower mating success and aggression levels in RFP male zebrafish (Howard et al. 2015), greater aggression in RFP zebrafish (Jha 2010), and no effect of RFP transgene on social partner preferences in zebrafish (Snekser et al. 2006). The inconsistent effect of RFP and GFP transgenes on zebrafish reproductive effects indicate fluorescent transgenes do not impart an innate disadvantage to zebrafish, rather the effects of the transgenes are influenced by other factors, potentially including transgene type or strain utilized,

rearing history, experimental conditions, and genotype x environment interactions. However, any potential reproduction of tropical fluorescent transgenic fish is expected to be negated by poor cold tolerance, resulting in lack of establishment in Canadian waters.

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Figure Legends

Figure 1: Surface air temperatures in Canada from December through February, averaged from 1981-2010. Copy of *Temperature Climatology – Map – Average – Dec-Jan-Feb (Winter)* copied from the Government of Canada, Environment and Climate Change Canada (https://weather.gc.ca/saisons/image_e.html?format=clim_stn&season=djf&type=temp).

Figure 2: Minimum winter water temperature distribution of surveyed lakes and rivers across Canada. $n=148$ for river and 107 for lakes.

Figure 3: Survival during gradual cold exposure for three freshwater ornamental aquarium species used in production of commercial fluorescent ornamental lines. Water temperature was dropped by 1°C/day, 6 days/week starting at 20°C (40 zebrafish *Danio rerio* Hamilton, 1822; 40 tetras *Gymnocorymbus ternetzi* Boulenger, 1895; 30 tiger barbs *Puntius tetrazona* Bleeker, 1855, divided into 2 tanks per species). Arrows indicate times at which golden zebrafish (solid line/arrows), white tetras (dotted line/arrows), and tiger barbs (dashed line/arrow) altered behaviour with decreasing temperature.

Figure 4: Survival during gradual cold exposure for three lines of GFP-transgenic zebrafish (y1, mi2001, zp4, *Danio rerio* Hamilton, 1822) and their wild-type progenitor line (AB). Temperature was dropped from 28°C to 12°C at a rate of approximately 1°C per day, then from 12°C to 5.1°C at a rate of approximately 0.1°C per day (80 fish per line, divided into 4 tanks).

Table 1: Zebrafish (*Danio rerio* Hamilton, 1822) research lines used to examine the influence of enhanced green fluorescent protein (eGFP) transgenesis on chronic low-temperature tolerance and effects of cold acclimation on reproduction.

Fish line	Fish genotype	Transgene construct	eGFP localization	Reference
fli1 (y1)	Tg(Fli1a:EGFP) ^{y1}	eGFP driven by Fli-1 protein promoter	developing and adult blood vessels	Lawson and Weinstein 2002
gfap (mi2001)	Tg(gfap:GFP) ^{mi2001}	eGFP driven by glial fibrillary acidic protein promoter	developing and adult glial cells	Bernardos and Raymond 2006, Barbosa et al. 2016
msxb (zp4)	TG(-6.5msx1b:EGFP) ^{zp4}	eGFP driven by muscle segment homeobox 1b promoter	ventral epidermis during development	Pyati et al. 2006
AB (wild-type)	wild-type progenitor line	none	none	https://zfin.org/ZDB-GENO-960809-7

Note: All transgenic lines were produced in the AB wild-type line and purchased from the Zebrafish International Resource Centre at the University of Oregon, Eugene, Oregon.

Table 2: Chronic lethal minimums (CL_{min}) and temperature at which 50% of fish lost equilibrium (LT_{50}) in wild-type and transgenic tropical freshwater fish with transgenic applications in research and commercial ornamental trade.

species/line	CL_{min} ($^{\circ}C$)	LT_{50} ($^{\circ}C$)
Experiment 1		
golden zebrafish (<i>Danio rerio</i> Hamilton, 1822)	5.53 ± 0.08	5.24 ± 0.03
white tetra (<i>Gymnocorymbus ternetzi</i> Boulenger, 1895)	9.95 ± 0.03	9.81 ± 0.00
tiger barb (<i>Puntius tetrazona</i> Bleeker, 1855)	13.36 ± 0.02	13.20 ± 0.03
Experiment 2 zebrafish lines		
wild-type	n/a	5.41 ± 0.06^a
y1	n/a	5.90 ± 0.05^b
mi2001	n/a	5.45 ± 0.06^a
zp4	n/a	5.38 ± 0.07^a

Note: Data are given as mean $\pm$ s.e.m, and letters (a,b) indicate significant difference among fish lines in Experiment 2 ($P < 0.05$). Experiment 1: $n=2$ tanks with 20 (golden zebrafish and white tetra) or 15 (tiger barb) fish per tank. Experiment 2: $n=80$ fish per genotype.

Table 3: Survival of GFP transgenic and wild-type zebrafish (*Danio rerio* Hamilton, 1822) lines over a four-month period held at different temperatures ($n=20$ fish per line and temperature), and total number of eggs laid, fertilized and hatched over 7 days breeding after returning to 28°C.

	28°C	10°C	8°C
<i>Survival (%) during acclimation</i>			
y1	90	90	90
mi2001	95	90	80
zp4	90	90	85
wild-type	85	80	80
<i>Total number of eggs laid</i>			
y1	65	125	32
mi-2001	57	100	2
zp4	15	5	5
wild-type	2	102	184
<i>Total number of fertilized eggs</i>			
y1	63	38	21
mi-2001	50	91	1
zp4	13	2	1
wild-type	2	87	72
<i>Total number of hatched eggs</i>			
y1	33	34	9
mi-2001	27	39	0
zp4	4	2	1
wild-type	1	32	54

Note: Fish were also held at 6°C but experienced 80-100% mortality within the first week so this group was discontinued.

Figure 1: Surface air temperatures in Canada from December through February

71x52mm (300 x 300 DPI)

Figure 2: Distribution of minimum winter water temperatures in Canada

69x45mm (300 x 300 DPI)

Figure 3: CLmin of aquarium fish

170x79mm (300 x 300 DPI)

Figure 4: LT50 of zebrafish GFP lines

68x48mm (300 x 300 DPI)