

Projected US distributions of transgenic and wildtype zebra danios, *Danio rerio*, based on temperature tolerance data

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Abstract

A genetically modified version of the south Asian, zebra danio, *Danio rerio*, a common aquarium fish, has become the first transgenic pet sold in the USA. Mean chronic lethal maxima of wildtype (39.8 °C, $n = 16$) and transgenic (39.3 °C, $n = 10$) zebra danios initially acclimated to 30 °C were statistically (but not dramatically) different as were mean chronic lethal minima of wildtype (5.3 °C, $n = 16$) and transgenic (5.6 °C, $n = 20$) zebra danios initially acclimated to 20 °C. These temperature tolerance values were used to estimate potential geographic distributions of the two varieties in the USA. Distributions of these *D. rerio* varieties in the USA should not be limited by their upper temperature tolerances, and low-temperature tolerance data suggest that both varieties are capable of overwintering in some southern and western US waters.

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1. Introduction

Transgenic species, those containing genes extracted from other species, are becoming more common, mainly as a way to increase food production, e.g., growth hormone enhanced salmon and tilapia (Stokstad, 2002). Although transgenic fish have yet to be used in food aquaculture and are awaiting scientific evidence that demonstrate a lack of ecological harm (Gong et al., 2003; Nam et al., 2004), one transgenic fish species, the GlofishTM, is being cultured in Florida and sold in pet stores throughout the USA as of January 2004 (Yorktown Technologies, L.P., 2003).

The GlofishTM, known as the red fluorescent protein (RFP) transgenic zebra danio or commonly referred to as red glofish, was created by inserting an IndoPacific sea anemone fluorescent protein gene into zebra danios, *Danio rerio* (Hamilton, 1822) with the original purpose of

developing fish that could glow in the presence of certain chemicals (Gong et al., 2003). The gene has become incorporated into the genome of the fish so that the muscle tissue appears red instead of the silver coloration of wildtype zebra danios. These fish have yet to glow in the presence of specific chemicals, but fluoresce in the presence of blacklight. It is likely that glofish will be released into US waters since they are being sold as pets, just as the popular aquarium pet wildtype zebra danios have been found outside captivity in parts of California, Florida, Connecticut, New Mexico, and Wyoming with possible year-round survival occurring in the last two states (Courtenay, 1993; Nico and Fuller, 2003).

Little research was conducted before glofish were distributed through pet stores. The only published study conducted to estimate any kind of ecological impact was Gong et al. (2003), which found that glofish expressed no reproductive advantage over wildtype zebrafish in a one-week laboratory breeding experiment.

Since the sale of glofish, Cortemeglia and Beitinger (2005) found that the upper and lower temperature tolerances of transgenic and wildtype zebra danios were statistically different via critical thermal methodology

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(CTM), but not to an extent that would suggest the differences have biological relevance, i.e., the fluorescent gene has not dramatically altered the temperature tolerances of this species. Critical thermal trials are effective at quantifying relative temperature tolerance; however, we also wanted to determine the longer-term temperature tolerances of transgenic and wildtype zebra danios.

Temperature tolerance is one of many factors that can influence the success of non-indigenous organisms in new habitats and thus their ability to impact those ecosystems (Stauffer et al., 1988). Tropical fishes compose a large proportion of non-indigenous fish species that are being introduced to US waters. According to Lever (1996) and USGS (2005), there are 53–56 exotic fishes established in the USA with 23 (~42%) being tropical fishes. In Florida alone, 20 of 22 exotic species established have tropical origins and three of those are from south Asia. *D. rerio* has its present-day origins in the south Asian countries of Bangladesh, Burma, India, Nepal, and Pakistan (Talwar and Jhingran, 1992; Menon, 1999). This area encompasses both subtropical and tropical climates (McKnight and Hess, 2005). The range of air temperatures in Bangladesh, one of the countries in which this species is endemic, is 4.4–43.3 °C (Rahman, 1989). Other tropical fishes have been able to survive winters in the USA; hence, the establishment of zebra danios in US waters may not be prevented by winter temperatures.

The purposes of this research were to quantify the lower and upper temperature tolerances of both wildtype and RFP transgenic zebra danios (GlofishTM) via chronic lethal methodology and use these data to estimate the distribution of these two varieties in the USA. Given their origins, we hypothesize that zebra danios are more likely to be limited by winter temperatures.

Chronic lethal methodology (CLM) is a laboratory approach used to estimate temperatures that would be lethal in nature. In this approach, fishes are exposed to temperatures that are decreased or increased at slow rates of ≤ 1 °C day⁻¹ (see review by Beitinger et al., 2000). These rates of change (ΔT 's) are more than 100 times slower than CTM, and CLM trials also use death as an endpoint (Beitinger et al., 2000). The chronic lethal method has been used to estimate overwintering potential of non-indigenous tropical fishes because chronic temperature tolerances are better estimators of field temperature tolerance than critical temperature tolerances since ΔT 's are slower and exposure time is longer.

2. Materials and methods

Wildtype zebra danios were purchased from a pet store in Denton, TX while the RFP transgenic zebra danios were purchased from a research facility in Minnesota and a pet store in Carrollton, TX. Fish were divided randomly into two acclimation groups (20 °C for the chronic lethal minima (CLMin) trials and 30 °C for both the CLMin and chronic lethal maxima (CLMax) trials). All fish were

maintained under a LD 12:12 photoperiod in well-aerated glass aquaria containing dechlorinated tap water. The density (0.18–0.53 fish/L) per acclimation tank in our study was within guidelines (five growing juveniles/adults per liter of water) to prevent stress as designated in the Public Health Service and Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) approved Guide for Zebrafish users (Mattheews et al., 2002).

Fish arrived in the laboratory at temperatures between 24 and 25 °C. The 30 °C-acclimation fish were initially placed in 26.0 °C water while the 20 °C-acclimation fish were first placed in 23.0 °C water. Water temperatures were increased or decreased 1 °C d⁻¹ until the corresponding acclimation temperature was reached. Haake[®] circulating thermoregulators (Models E-52 and D-1) were used to maintain 30 °C in two 38-L aquaria insulated with polystyrene foam while the thermoregulators were used in conjunction with a 570-L submerged Living Stream[®] cooler (Frigid Units, Inc) to maintain 20 °C in two 76-L aquaria. Two CLMax trials and two CLMin trials for zebra danios and glofish were conducted. Air stones and Haake[®] circulating thermoregulators provided continuous aeration and uniform thermal mixing of the test chamber water. Fish were held at final acclimation temperatures for a minimum of 10 d and a maximum of 14 d before CLMax trials and for a minimum of 10 d and a maximum of 24 d before CLMin trials. Water quality of the acclimation tanks, measured daily, was within ranges that should have produced no adverse effects to the fish, e.g., conductivity 360–750 $\mu\text{mhos cm}^{-1}$, pH 7.0–8.2, dissolved oxygen 6.9–8.8 mg L⁻¹ (Table 10). Total ammonia (0–8.0 mg L⁻¹) and nitrite (0–2.0 mg L⁻¹) levels were above zero only during the latter parts of the acclimation period for the 20 °C acclimated wildtype and RFP zebra danios. Ammo-Lock[®] was applied to the acclimation tank water whenever measurable levels of ammonia were detected. Water chemistry during the trials, also measured daily, was within ranges that should have produced no adverse effects to the fish (conductivity 270–600 $\mu\text{mhos cm}^{-1}$, pH 7.2–8.0, dissolved oxygen 5.7–12.9 mg L⁻¹, total ammonia 0–0.25 mg L⁻¹, nitrite 0–0 mg L⁻¹). Fish were fed Tetra-min[®] tropical flake food twice daily during acclimation and during the trials until fish ceased eating. Daily water exchanges (5–25%) by siphon were performed to eliminate any uneaten food and feces.

The ΔT during the trials was 1 °C day⁻¹ and is the most commonly used rate employed in chronic temperature tolerance research (see review by Beitinger et al., 2000). Schooling, individual behavior, and feeding were noted once a day. Once fish started dying, behavior and mortality were checked each 4 h during the light part of the photoperiod, similar to Fields et al. (1987). Death was defined as no movement, including opercular movement and no response to prodding with glass rod, and was the endpoint criterion for both CLMax and CLMin trials. This definition has been used by other researchers, e.g., Stauffer

et al. (1988) and Jennings (1991); however, they found that the definition was not completely satisfactory because some apparently dead fish revived when transferred to their original acclimation temperature. Zale and Gregory (1989) considered fish dead 1 h after opercular movement ceased to account for this possibility. Once death was observed, the individual was removed from the aquarium and placed in water at its initial acclimation temperature to determine actual death, and 8% (5/65) did move. Nevertheless, the temperatures at which these fish did not move or respond to prodding during trials were used in statistical analyses. Trials continued until the endpoint was reached by all fish. Then each fish was weighed (wet weight, ± 0.5 g) and measured (total length, ± 0.1 cm), and gender was determined based on the criteria in Laale (1977) and Pyron (2003). The CLMax and CLMin for each group were calculated as the arithmetic mean temperature at which death was observed.

Two-way ANOVA was used to test for significant differences between fish varieties and gender with the CLMax data and three-way ANOVA was used to test for significant differences between fish varieties, acclimation temperatures, and gender with the CLMin data. Simple linear regression analysis determined whether or not CLMax or CLMin were related to either fish length or weight. Thermal scope (difference between CLMax and CLMin at an acclimation temperature) was calculated for fish acclimated to 30 °C. All statistical decisions were based on an α of 0.05.

3. Results

3.1. CLMax trials

During acclimation, both the wildtype and transgenic zebra danios schooled actively. When a trial was initiated and temperature was increased 1 °C day⁻¹, behavior remained the same, until the temperature reached 36 °C. At that temperature, feeding was reduced. Feeding stopped completely when the temperature reached 37 °C. The zebra danios also appeared to swim faster. At 39 °C, the fish schooled at the water surface and began to show loss of equilibrium and opercular spasms. These responses ensued for hours until death, which occurred between 39.0 °C and 40.0 °C (Table 1).

Table 1
Descriptive statistics for chronic lethal maxima trials with wildtype zebra danios and RFP transgenic zebra danios (glofish) acclimated to 30 °C

Factor	<i>n</i>	Mean $\pm$ SD (°C)	Range (°C)	<i>P</i>
Wildtype	16	39.8 $\pm$ 0.33	39.0–39.9	0.004
Glofish	10	39.3 $\pm$ 0.43	39.0–40.0	
Female	12	39.6 $\pm$ 0.49	39.0–40.0	
Male	13	39.7 $\pm$ 0.41	39.0–40.0	

Probabilities are from two-way ANOVA (interaction of variety and gender $P = 0.97$).

Two-way ANOVA with variety and gender as main effects found the mean chronic lethal maxima (Table 1) of 16 wildtype and 10 RFP transgenic zebra danios acclimated to 30 °C were significantly different ($P = 0.004$); however, neither the mean CLMax of females and males ($P = 0.96$) nor interaction between variety and gender ($P = 0.97$) were significant. The absolute difference in mean CLMax between the varieties was only 0.5 °C.

3.2. CLMin trials

Both wildtype and transgenic zebra danios schooled actively during acclimation. This behavior persisted during trials until water reached 10 °C with fish acclimated to 20, and 12 °C in the trials with fish acclimated to 30 °C. Fish acclimated to 20, and 30 °C fed similarly until temperatures reached 13, and 17 °C, respectively. Further decreases in temperature appeared to inhibit feeding. When the temperature approached 11 °C and 12–13 °C, respectively, fish did not swim to the surface to feed. Fish continued to swim until the water temperature reached 10 °C. At 9.0 °C, fish tended to huddle at the tank bottom instead of schooling in the water column. Five individuals attempted to bury themselves in the gravel at temperatures below 9.0 °C and four of these fish survived until near the end of the experiment. Loss of equilibrium was observed from 5.0 to 8.0 °C in fish acclimated to 20 °C and from 6 to 14 °C in fish acclimated to 30 °C. Opercular spasms generally preceded death, and mortality was observed from 4.0 to 7.0 °C in fish acclimated to 20 °C and from 4.0 to 14.0 °C in fish acclimated to 30 °C (Table 2).

Chronic lethal minima of zebra danios (Table 2) was significantly affected by both acclimation temperature ($P = 0.002$) and variety ($P = 0.03$); however, gender ($P = 0.74$), and the interactions between acclimation temperature and variety ($P = 0.13$), acclimation temperature and gender ($P = 0.97$), variety and gender ($P = 0.38$), and among all three factors ($P = 0.62$) did not significantly affect CLMin (three-way ANOVA).

Table 2
Descriptive statistics for chronic lethal minima trials with wildtype zebra danios and RFP transgenic zebra danios (glofish) acclimated to 20 and 30 °C

Factor	<i>T</i> _{ACCL} (°C)	<i>n</i>	Mean $\pm$ SD (°C)	Range (°C)
Wildtype	20	16	5.3 $\pm$ 0.82	4.0–7.0
Glofish	20	20	5.6 $\pm$ 0.68	5.0–7.0
Wildtype	30	20	6.8 $\pm$ 3.26	4.0–14.0
Glofish	30	20	7.6 $\pm$ 1.79	5.0–12.0
Female	20	21	5.5 $\pm$ 0.54	5.0–6.4
Male	20	15	5.4 $\pm$ 0.98	4.0–7.0
Female	30	23	7.1 $\pm$ 2.07	5.0–13.0
Male	30	14	6.4 $\pm$ 2.98	4.0–14.0

Three-way ANOVA found that CLMin were significantly different between acclimation temperatures ($P = 0.002$) and varieties ($P = 0.03$), but not between genders ($P = 0.74$). None of the factor interactions were significant $0.97 > P > 0.13$.

Absolute differences in mean CLMin between varieties were only 0.3 °C (20 °C acclimation) and 0.8 °C (30 °C acclimation).

3.3. Effects of size

Simple linear regression found no consistent relationship between length (range 2.7–4.7 cm) or weight (range 0.1–1.3 g) and temperature tolerance; therefore, the range of fish size in these experiments did not affect the results.

4. Discussion

The behavior observed at high and low temperatures for both zebra danio varieties has been reported for other species (Alcorn, 1976; Shafland and Foote, 1979; Shafland and Pestrak, 1982; Jennings, 1991; Bennett et al., 1997). The digging behavior may be a method to escape low temperatures and has been reported for other species, e.g., Jennings (1991), Bennett et al. (1997).

Results from the chronic lethal method trials are similar to those from the critical thermal method trials with these two varieties of *D. rerio* (Cortemeglia and Beitinger, 2005). The CLMax for wildtype zebra danios and RFP transgenic zebra danios are less than the critical thermal maxima (CTMax) by 1.9 and 2.0 °C, respectively. Conversely, the CLMin of wildtype and RFP transgenic zebra danios are 0.9–3.8 °C lower than the critical thermal minima (CTMin) of each variety. The chronic lethal method allows longer time at each temperature for temperature tolerance reacclimation to occur than critical thermal method trials; however, there is also more time for heat (or its lack) to exert its effects (Beitinger et al., 2000). Furthermore, death will occur at more extreme temperatures than any sublethal endpoint. The opportunity for reacclimation and a lethal endpoint were more important than the longer exposure in CLMin trials when compared to CTMin trials allowing fish to tolerate lower temperatures in the chronic trials. Conversely, longer exposure to heat at the high temperatures was more important than reacclimation and the endpoint utilized in the CLMax trials when compared to CTMax trials.

Acclimation temperature had a significant effect on chronic lethal minima temperatures with both wildtype and transgenic zebra danios similar to CTM trials with both varieties (Cortemeglia and Beitinger, 2005). This suggests that reacclimation occurring during trials could not compensate for the 10 °C difference in tolerance acclimation at the start of the trials. Thermal scope of glofish (31.7 °C) and wildtype (33.0 °C) zebra danios acclimated to 30 °C were also similar to the thermal scopes measured in the CTM trials (Cortemeglia and Beitinger, 2005).

Consistent with results from the critical thermal trials (Cortemeglia and Beitinger, 2005), gender did not influence temperature tolerances of either zebra danios or glofish in chronic lethal trials. This supports their hypothesis that

female and male zebra danios would not be thermally segregated in nature.

Although upper thermal tolerances were statistically different between wildtype and genetically modified RFP zebra danios, the similar ranges and relatively small differences in mean temperature tolerance (0.5 °C) suggest that both varieties will be found in similar geographic locations on the basis of chronic upper temperature tolerance. Summer temperatures in the USA should not be limiting to either wildtype or RFP transgenic zebra danios. For example, the mean CLMax of wildtype (39.8 °C) and glofish (39.3 °C) are approximately the same as both the ubiquitous northern largemouth bass, *Micropterus salmoides salmoides* (39.8 °C) and the Florida largemouth bass, *Micropterus salmoides floridanus* (39.4 °C) acclimated to the same temperature (Guest, 1985). Both bass subspecies can tolerate the warm summers of such states as Texas and Florida.

Although chronic lower thermal tolerances were statistically different between wildtype zebra danios and glofish, again the similar ranges and relatively small absolute differences in mean temperature tolerance at both acclimation temperatures (0.3 and 0.8 °C) indicate both varieties should be able to exist in similar geographic locations on the basis of their low temperature tolerance. Given their tropical origin, zebra danios have a surprisingly low CLMin.

Both varieties of zebra danios have relatively low CLMinima (ca. 5 °C), a characteristic indicating they may survive winters in the southern USA. For example, the minimum temperatures at which feeding was reduced (13 °C) and ceased (11 °C) and LOE began (6 °C) for wildtype and transgenic zebra danios (acclimated to 20 °C) are similar or lower than those for 17 other tropical and sub-tropical non-indigenous species, including 12 that are established in some US waters, determined by Thompson et al. (1977), Shafland and Foote (1979), Shafland and Pestrak (1982), and Zale and Gregory (1989). The CLMin of wildtype (5.3 °C) and RFP transgenic zebra danios (5.6 °C) are lower than the CLMin (8.0, 8.9, and 9.5 °C) of three non-indigenous fishes acclimated to similar temperatures that have become established in Florida (Shafland and Pestrak, 1982; USGS, 2005) and one species, the blue tilapia, *Oreochromis aureus*, that is established in 10 US states (Lever, 1996; USGS, 2005). Comparing the CLMin of wildtype and RFP transgenic zebra danios acclimated at temperatures between 20 and 30 °C (determined via interpolation) with the measured values by Thompson et al. (1977), Shafland and Foote (1979), and Shafland and Pestrak (1982), at the same acclimation temperature, the CLMin of both zebra danio varieties would be less than the CLMin of all 13 non-indigenous species, including the walking catfish, *Clarias batrachus*, which although native to Bangladesh, Borneo, Burma, India, Indonesia, Singapore, and Sri Lanka (USGS, 2005) is established in most of Florida. All of these comparisons indicate that zebra danios have the thermal tolerance capacity to become

established in areas similar to those inhabited by those 17 non-indigenous fishes, e.g., Arizona, California, Colorado, Florida, Hawaii, Idaho, Nevada, North Carolina, and Texas (USGS, 2005).

Water temperature data are more scarce and difficult to obtain than are air temperatures; therefore, it is common to use air temperature as a surrogate for water temperature in biological and water quality studies of streams. For example, Shafland and Foote (1979), Shafland and Pestrak (1982), Stauffer and Boltz (1994), and Bennett et al. (1997) compared empirically determined low temperature tolerances of non-indigenous fishes to maps of mean or maximum January air temperatures for Florida or the southern USA and cite other studies that indicate air temperatures can be used to predict water temperatures. January is usually the coldest month in the USA (Bennett et al., 1997).

The potential geographical ranges of wildtype and transgenic zebra danios are displayed in Figs. 1a and b. The different temperatures at which feeding was reduced, feeding ceased, and death occurred are designated on the maps. Fig. 1a is a liberal estimate of areas where wildtype and transgenic zebra danios could become established since it shows maximum air temperatures for January. Based on these temperatures, zebra danios are likely to become established in the 13 most southern and western states (areas where temperatures are high enough for zebra danios to feed). This possible distribution is similar to the realized distribution of blue tilapia, which has a similar CLMin to the zebra danios. This area includes three of the five states that wildtype zebra danios have been found in open waters, i.e., California, Florida, and New Mexico. Fig. 1b is a conservative estimate of the possible geographic distribution of zebra danios since it shows minimum

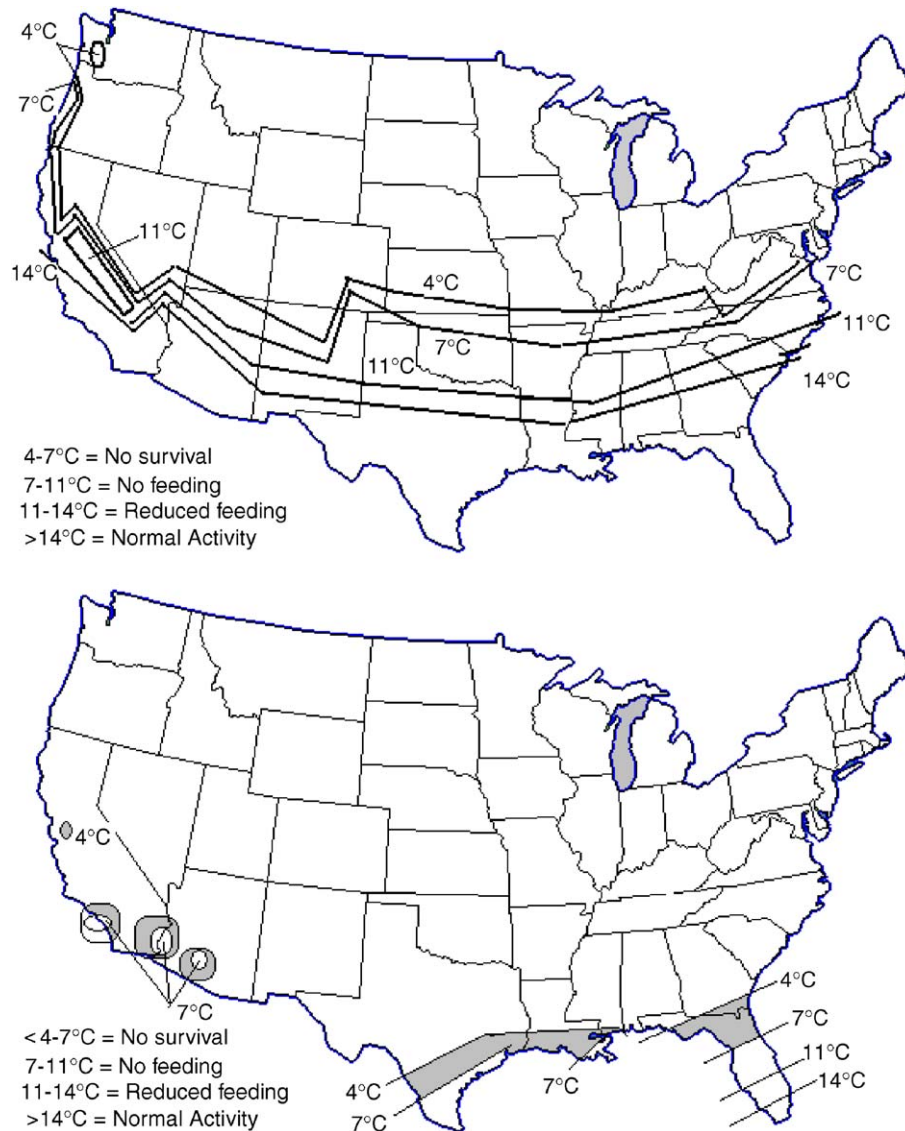


Fig. 1. Projected distributions of wildtype and transgenic zebra danios in US waters based on our CLMin results and both maximum (a) and minimum (b) January air temperatures from 1971 to 2000 (SCAS, 2004a, b).

January air temperatures. Based on this map, zebra danios are not likely to be limited by cold temperatures in nine southern and western states (anywhere where temperatures are high enough for zebra danios to strictly survive since minimum temperatures are usually short in duration).

Stefan and Preud'homme (1993) found that water temperatures of 11 US rivers were as much as 20 °C higher than the air temperatures. They analyzed weekly water and air temperatures and found that they were related: $T_{\text{water}} = 2.9 + 0.86 (T_{\text{air}})$. For temperatures relevant to our study, when air temperatures range between 4 and 14 °C, the corresponding water temperatures would be 0.94 to 2.34 °C higher. Since there is not a large difference between air and water temperatures and our maps illustrate the highest and lowest January temperatures for the past 30 years, our maps adequately describe the nationwide water temperatures of the USA for the purposes of our study. However, since winter water temperatures in the USA tend to be higher than air temperatures, the distributions of zebra danios could be even more northerly than is postulated in Figs. 1a and b.

Not only do the low CLMin values indicate that both zebra danio varieties could become established in the southern US waters, the potential exists for an even wider distribution if cold tolerance of zebra danios and glofish is extended by natural selection. More cold-tolerant zebra danios would likely be selected after winter resulting in a more cold-tolerant population of zebra danios. This possibility has been documented for other species, including threadfin shad, *Dorosoma petenense* (Rawstron, 1964 cited in St. Amant, 1967) and mosquitofish, *Gambusia affinis*, (Rawley, 1963 cited in St. Amant 1967).

In summary, results from the chronic lethal trials are similar to those from the critical thermal trials, the temperature tolerances of female and male zebra danios are not different, and wildtype and transgenic zebra danios are likely to become established in similar geographic locations. Although wildtype and transgenic zebra danios may be limited in introduced waters based on other factors, they should not be limited in the USA by their upper temperature tolerances or from the southern and western parts of the USA by their lower temperature tolerances.

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