Thermal tolerance of the common coqui frog (*Eleutherodactylus coqui*) in East Hawaii along an elevation gradient

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ABSTRACT

Natural variation in the environment can be tolerated by animals via behavioral modification, adaptation, or physiological or developmental plasticity. Phenotypic plasticity, or acclimation, allows individuals to adjust physiological parameters to best suit the needs of their environment. Acclimation to temperature is a feature of successful invasive species and may be a contributing factor to the expansion of the coqui frog (*Eleutherodactylus coqui*) in its introduced range in Hawaii. Coqui frogs have been recorded on Hawaii Island since the late 1980s and pose a noise nuisance to humans. If the frogs expand into higher elevation, montane habitats, they may disrupt the ecosystems with noise and food web disturbances. The minimum tolerance of cold temperatures of coqui frogs along an elevational gradient in East Hawaii was measured and compared. Coqui frogs were found to have a flexible range of cold temperature tolerance, with differences in cold tolerance between populations, evidence of acclimation to lower temperatures, sex-specific thermal tolerance, and a lower thermal tolerance level in Hawaii than in their native Puerto Rico. However, a higher tolerance for cold did not correlate directly with elevation and there were no appreciable differences in hematocrit, plasma osmolality, or heart mass between acclimation treatments or populations. The overall range of shift in thermal tolerance after acclimation was 2-3°C, implying the thermal flexibility of coqui frogs may enable them to further expand their habitat in Hawaii to cooler, high elevation areas. Particularly sensitive areas should be monitored and managed to minimize ecosystem threats from coqui frogs.

TABLE OF CONTENTS

Acknowledgements……i
Abstract……..……ii
Table of Contents……..…iii
List of Tables……iv
List of Figures……iv

Introduction……1
Materials and Methods……6
Results……9
Discussion……11
Literature Cited……27

LIST OF TABLES

Table 1. Proportions of male and female *E. coqui* frogs collected from six populations in East Hawaii and proportions of frogs with lipid-rich organs (egg masses and fat bodies) removed during dissection……..17

Table 2. CTmin values associated with acclimation of *E. coqui* frogs from six populations in East Hawaii…….17

Table 3. Physiological parameters associated with acclimation of six populations of *E. coqui* in East Hawaii……..18

Table 4. Effects of sex on CTmin pre-acclimation, post-acclimation, and changes due to acclimation…..18

LIST OF FIGURES

Figure 1. Locations and elevations of *E. coqui* populations sampled in East Hawaii…….19

Figure 2.Pre-acclimation mean and SE CTmin values by population and elevation of population sites….20

Figure 3. Predicted probability of survival in the cold acclimation treatment by population, based on a binary logistic regression model ……..……..……21

Figure 4. Mean and SE CTmin of *E. coqui* frogs acclimated for thirty days……....22

Figure 5. Mean and SE difference in pre- and post-acclimation CTmin by population and by treatment...23

Figure 6. Mean and SE plasma osmolality from acclimated frogs…….24

Figure 7. Mean and SE CTmin of both frog sexes prior to acclimation and difference in CTmin post-acclimation…….25

Figure 8. Mean and SE CTmin of frogs with and without lipid-rich organs prior to acclimation and difference in CTmin post-acclimation…….26

INTRODUCTION

The coqui frog as an introduced species in Hawaii

Since the common coqui frog (*Eleutherodactylus coqui*) was introduced to the Hawaiian Islands in the 1980s, its loud, distinctive call and high population densities have made it a noticeable feature of the nighttime landscape. The frog is currently a noise nuisance to humans, but coqui frogs have the potential to affect the sensitive native biota of the Hawaiian Islands.

Invasive species are a major concern for declines in biodiversity, especially in island systems with high proportions of endemic flora and fauna (Pimm et al. 1995). Introduced species may not directly threaten native island species with extinction (Gurevitch & Padilla 2004) but invasive species can alter ecosystem processes and relationships between native species, disrupting the established ecological community of an island (Davis 2003). Extinctions of native fauna in island systems are affected by characteristics of both the ecosystem and the introduced species (Davis 2003). Under current global conditions of large-scale habitat transformation and climate change, island ecosystems may confer competitive advantages for invasive species.

The common coqui frog, *E. coqui,* has been a particularly noticeable invasive species in Hawaii. Coqui frogs are considered undesirable because their loud calls create a noise nuisance and they pose a potential threat to the tropical horticulture industry. Where frogs are present, the volume of the calls has been reported to deter tourism and lower property values by 0.16% (Kaiser & Burnett 2006). Additionally, the association of coqui frogs with Hawaii's nursery horticultural trade may have negative economic impacts on the industry. Because *E. coqui* is classified as a pest species, plants from nurseries with coqui frog infestations may be quarantined in order to reduce the risk of spread, causing economic and reputational damage to Hawaii's floriculture industry (Kraus & Campbell 2002; Rödder 2009).

Coqui frogs were first noticed in the Hawaiian archipelago by Hawaii Island landowners in 1988 (Kraus & Campbell 2002). The frogs, indigenous to Puerto Rico, are thought to have arrived in Hawaii as stowaways in nursery plants (Kraus et al. 1999; Kraus & Campbell 2002). The coqui frogs spread to several sites on Maui and Hawaii Island, where the species is now considered impossible to remove (Beard et al. 2009). Coqui frogs' success as invasive species is due in part to their life history, which features prolific reproductive ability and short generation times. As direct developers without a larval "tadpole" stage, the frogs are liberated from requiring permanent bodies of water for reproduction. In their native habitat of Puerto Rico, these frogs typically lay 4-6 clutches annually with a mean 28 eggs per clutch and have a generation time of eight months (Townsend et al. 1984). Based on genetic analysis of populations in Hawaii, it is thought the invasions on Maui and Hawaii Island were the result of two separate singular introductions (Peacock et al. 2009).

In Hawaii, the population density of *E. coqui* is dramatically different from populations in Puerto Rico. Puerto Rican populations were found to have 3265 adult frogs/hectare (Stewart & Woolbright 1996), while a study of long-term population density in Hawaiian populations found 11,800 adult frogs/ha, three

times the average Puerto Rican density (Woolbright et al. 2006). The differences in densities have been attributed to the lack of predators in Hawaii as well as an abundance of suitable retreats created by the textured terrain of lava flows (Woolbright et al. 2006).

Invasive species raise concern because of their propensity to cause drastic changes to the ecosystems they invade. The introduction of coqui frog to Hawaii could facilitate establishment of non-native flora in montane forests. Hawaiian lowland forests are dominated by non-native and invasive flora, which have higher levels of nitrogen in their tissues than native flora (Ostertag et al. 2009). Nitrogen and phosphorus are often limiting to forest productivity in new volcanic soils, such as those in Hawaii (Raich et al.1996). Montane, native-dominated forests may have an advantage over invasive flora by having evolved in nitrogen-limited soils. In Puerto Rico, exclusion of *E. coqui* decreased leaf-litter levels of nitrogen, phosphorus, carbon, and other minerals (Beard et al. 2002) while presence of the frogs increased nutrient levels, increased leaf production, and increased the leaf litter decomposition rate (Sin et al. 2008). Establishment of dense populations of *E. coqui* in montane native-dominated forests in Hawaii may alter nutrient levels and facilitate the migration of non-native flora into these habitats.

E. coqui may have direct interactions with Hawaiian fauna as well. Currently, coqui frog populations are predominantly located in areas of low elevation (0-500m above sea level) dominated by non-native vegetation (Beard et al. 2009). At present, the low-elevation coqui frogs consume predominantly non-native ants and amphipods (Beard 2007). Areas of overlap exist where coqui frogs may utilize native Hawaiian spiders and other endemic invertebrates as prey (Sin et al. 2008). Researchers have proposed a potential for coqui frogs to compete with insectivorous native birds for prey (Kraus et al. 1999). Native birds are restricted to areas of high elevation (>500m above sea level) dominated by native vegetation (van Riper et al. 1986). Under current population distributions of birds and frogs, coqui frogs are unlikely to directly compete with native birds for invertebrate prey.

However, the current neutral impact of coqui frogs is not predicted to last as the frogs continue to expand their range. In their native Puerto Rico, *E. coqui* is found at all elevations of the island, from sea level to 1200m, (Beuchat et al. 1984), though the populations with the greatest densities are found at or below 500m (Stewart & Woolbright 1996). Similarly, in Hawaii, coqui frogs are most abundant at low elevations although there are some populations at 1170m, near the maximum elevation of populations at the 1338m peak of Puerto Rico (Kraus & Campbell 2002). Because Hawaii has montane forest ecosystems at greater elevation than Puerto Rico, invasive populations of coqui frogs in Hawaii have the potential to expand into higher-elevation habitat, where native ecosystems may be disturbed (Kraus & Campbell 2002). Not all of Hawaii's high-elevation habitat is suitable for frogs (especially alpine and sub-alpine regions) but the potential for frogs to move higher is unknown. Under current climate conditions as well as under scenarios of projected anthropogenic climate change, coqui frogs are likely to find suitable habitat at higher elevations throughout the Hawaiian Islands (Rödder, 2009).

Temperature tolerance and acclimation

Temperature tolerance may be a key factor to predicting the eventual elevation distribution and potential effects of *E. coqui* on Hawaiian ecosystems. For every 1000m in elevation gained, average air temperature decreases 6.5°C (Briggs et al.1997). As amphibians are ectotherms and do not generate their own body heat, montane tropical frog body temperatures are closely linked with elevation (Navas et al. 2013). Coqui frogs are currently living at low elevations but as the frogs expand in elevation, they may be limited by physiological tolerance to cold. The mechanism of coqui frog expansion into new habitats with high degrees of environmental variation remains to be determined. Hawaiian populations are not likely to have a high degree of genetic variation, considering the introduction of frogs to the island of Hawaii is thought to have stemmed from one introduction event (Velo-Antón et al. 2007). With similar genetic make-up, different populations exposed to varying levels of thermal stress must somehow cope with the low temperatures at higher elevations in order to survive. *E. coqui* frogs in Hawaii may acclimate to new environmental conditions by adjusting physiological thermal tolerance capacity, or there may be local adaptation in response to thermal selection in these new, higher-elevation populations.

When environmental temperatures are sub-optimal, amphibians and other ectotherms can individually adjust body temperature through behavioral thermoregulation or reliance on physiological mechanisms to extend temperature tolerance (Brattstrom & Lawrence, 1962). Environmental conditions can influence an organism's phenotypic characteristic outside the constraint of the organism's genetics. Such phenotypic plasticity, whether behavioral, morphological, or physiological, confers an advantage to species in heterogeneous environments (Via & Lande 1985; Price et al. 2003). The changes occurring in an organism are reversible over the course of the individual's lifetime and are not inherited by progeny. The physiological limits of an amphibian's temperature tolerance are typically measured in the laboratory as the critical thermal maximum and minimum (CTmax and CTmin, respectively). These are the high and low temperatures, respectively, at which the animal fails to right itself when turned on its back (Lowe & Vance, 1955). This represents a functionally lethal temperature at which the animal cannot remove itself from a thermally stressful situation. In the case of coqui frogs in Hawaii, the critical thermal minimum is of more pressing interest than the thermal maximum because of the potential increases in the frogs' elevational range and subsequent effects on native ecosystems. Depending on the acclimation capacity of coqui frogs, new, as-yet uninhabited high-elevation habitats may be rapidly usable. Amphibians inhabiting tropical montane biomes that are adjacent but different in elevation are subject to different thermal regimes and therefore may be exposed to different selective pressures, even for geographically near habitats (Navas et al. 2013). Significant differences in critical thermal minima have been found for high-elevation toad populations compared to low-elevation populations of the same species (Brattstrom, 1967). Montane populations of the lowland-originated former congeneric *Craugastor* (*Eleutherodactylus) fleischmanni* have narrow thermal tolerances, suggesting a shift in thermal tolerance specific to a narrow habitable zone (Brattstrom, 1967). The ecosystem differences along elevational gradients may be

exacerbated in Hawaii, where lowland, invasive plant-dominated forest borders upland, native plant-dominated forest.

It is possible to measure phenotypic plasticity by directly measuring acclimation capacity of a species. Thermal acclimation capacity has not yet been studied for Hawaiian *E.coqui.* However, thermal acclimation capacity may be one feature of highly successful invasive species. For example, cane toads (*Rhinella marina*), another Neotropical anuran species, have been found to acclimatize to low temperatures after a mere 12 hours of exposure (McCann et al. 2014). If the acclimatization ability of coqui frogs is similar to that of cane toads, overcoming the thermal barriers to range expansion in Hawaii may be a small matter.

Acclimation to lower temperatures is likely to result in physiological changes in an animal, especially with respect to metabolism, water balance, and cardiovascular functions in ectotherms. Under cold conditions, amphibians and other ectotherms enter into dormant states, where normal resting metabolic rate can be reduced by up to 80-90% (Boutilier et al. 1997; Trzcionka et al. 2008). Suppression of basal metabolic rate is also time-dependent, with prolonged exposure to cold resulting in lower metabolic rates than short periods of cold exposure (Boutilier et al. 1997). Bullfrogs (*Lithobates catesbeianus*) acclimated at 5°C and 10°C had a reduction in plasma osmolality compared to frogs acclimated at 20°C and 30°C (Stinner and Hartzler 2000; Palenske and Saunders 2003). Reduction in plasma osmolality in both studies was explained by dilution due to increased water uptake and decreased osmoregulatory capacity at low temperatures. Bullfrogs maintained at 5°C had a decrease in hematocrit compared to frogs maintained at room temperature (Palenske & Saunders, 2003), characterized by an initial increase in hematocrit at first exposure to cold, followed by no further changes in hematocrit levels past the initial increase. This was interpreted as maintenance of body function in response to cold (Palenske & Saunders, 2003). In other studies of cold exposure, hematocrit decreased in response to cold, likely because of hemodilution due to increased water uptake (Sinsch, 1991). Cold acclimation also affects cardiovascular and muscular systems in other vertebrate taxa, though there is little study on amphibians. Cold acclimated carp (*Cyprinus carpio*) had more ventricular muscle mass, attributed to increased difficulty of pumping higher viscosity blood (Young & Egginton, 2011). In contrast, blood viscosity of bullfrogs was maintained at low temperatures over fifty days, implying an optimal plasma viscosity and hematocrit level across environmental temperatures (Palenske & Saunders, 2003). These findings led to no expectation of associated changes in cardiac muscle. The physiological changes associated with acclimation are observed primarily in temperate species with expected responses to cold.

The acclimation capacity of amphibian species may be regionally influenced, as tropical and temperate amphibians appear to have different responses to thermal acclimation. Because temperate species experience the full seasonal range of climatic variation, greater capacity for physiological adjustment might pose a selective advantage in temperate regions, known as the climatic variability hypothesis (Stevens 1989; Addo-Bediako et al. 2000). Indeed, because individuals' thermal tolerance is based on their exposure, tropical species are likely to have more narrow thermal tolerance than

temperate species (Janzen, 1967). While upper thermal limits show little variation, lower thermal limits decrease with increasing latitude, such that temperate species are more cold tolerant (Addo-Bediako et al. 2000). Some groups of tropical amphibians have this expected narrow range of physiological adjustment to temperature but other groups, specifically Ranidae and Bufonidae, have tropical species with thermal regimes shifted to a higher range than temperate species (Brattstrom, 1967). Of twenty-nine studied amphibian species, all twenty-two temperate species show metabolic acclimation while six of seven tropical species show no acclimation (Feder, 1982). Frogs exhibiting overwintering behavior, observed only in temperate regions, are more able to access stored lipids and carbohydrates in winter months (Boutilier et al., 1997). An adaptation to have greater acclimation capacity and the associated physiological adjustments is expected of temperate frogs but not necessarily of tropical frogs.

Physiological plasticity of temperature tolerance is not the sole explanation for coqui frogs inhabiting low temperature areas. Coqui frogs have a wider horizontal and vertical habitat range than other Puerto Rican *Eleutherodactylus* frogs (Stewart & Woolbright 1996), implying the ability to tolerate temperature changes better than similar species. The critical thermal maxima of *E. coqui* in Puerto Rico were not different for frogs from populations at sea level and at 700m in elevation (Christian et al. 1988). The same study found no apparent acclimation response of these frogs after acclimation for one week at 20°C (Christian et al. 1988), although this study used the same frogs to test both maxima and had a low overall sample size. In comparison to other tropical frogs from Puerto Rico, *E. coqui* did not adjust physiological parameters to changes in in temperature, implying Puerto Rican *E. coqui* have a wide thermal tolerance, in addition to potential acclimation capacity.

A wide thermal tolerance and phenotypic plasticity are two ways species can cope with the variety of existing environmental conditions in the short term. Over longer periods of time, species that easily tolerate environmental heterogeneity do so by rapidly diversifying, evolving new adaptations that are better suited to the local environment. These changes to the genetic code will be inherited by offspring, in contrast to the changes within an individual's lifetime caused by phenotypic plasticity. In some cases, phenotypic plasticity and diversification work in tandem to create local adaptations. When an environment changes rapidly, there is not sufficient time for local adaptations to arise. A species that already has a high degree of phenotypic plasticity will have progeny that are better suited to cope with the changing environment than a species with rigid habitat requirements. Over time, selective pressure will result in local adaptations mirroring the expressed phenotypes, a phenomenon called genetic accommodation (West-Eberhard 2005). Phenotypic plasticity may play a role in divergence of populations by contributing to environment-specific gene expression, where pleiotropic constraints on gene divergence are removed (Snell-Rood et al. 2011; Johansson et al. 2013). Species have the option of compensating for environmental variation with phenotypic plasticity or local adaptation but the delineation of these two strategies is not always clear.

A broad thermal tolerance may enable the coqui frog to invade higher elevation, native-plant dominated Hawaiian forests. Introduction of the frog into these sites could have negative impacts:

increased predation of native invertebrates, competition with native birds for food, and increased nutrients in leaf litter, potentially paving the way for invasive plant species to invade the montane forests. As montane tropical island ecosystems are a vulnerable biome in the contexts of global climate change, any insight into progression of invasive species will be useful in protecting native species from decline and extinction.

This study aims to describe the CTmin for *E. coqui* in Hawaii. While the thermal tolerance of *E. coqui* in their native Puerto Rico at high and low elevations was described by Christian et al. (1988), there may be differences in the introduced populations. This study also examines the effect of elevation on CTmin, looks for evidence of a thermal acclimation response, and investigates the mechanism of thermal acclimation, if present.

MATERIALS AND METHODS

Critical Thermal Minimum (CTmin)

E. coqui frogs were collected from each of six sites between September 2015 and May 2016, on the east side of Hawaii Island. The sites varied in elevation between 18.5m to 687.6m above sea level (Figure 1). Frogs were collected and tested from lower elevation sites in December 2015-March 2016 with higher frog chorus activity. Frogs from higher elevation sites were collected and tested in September 2015 and April-May 2016. Frogs were detected visually or acoustically and hand captured between the hours of 18:00 and 21:00 for all collections. Approximately twenty frogs were collected at each site, with twenty-one frogs collected from the Stainback site and eighteen frogs collected from the Glenwood site, for a total of 119 frogs from all six sites. Both male (n = 81) and female (n = 38) frogs were collected that had a snout-vent length (SVL) of over 20mm. Proportions of male and female frogs collected from each population are given in Table 1. The frogs were transported to laboratory facilities and were tested for critical thermal minimum (CTmin) within forty-eight hours of collection. Prior to CTmin testing, frogs were weighed, sexed, and measured for SVL.

To test CTmin, frogs were individually placed in mesh boxes constructed of frog-proof Teflon plastic fencing material, then placed in a glass beaker partially filled with room temperature dechlorinated tap water. A thermocouple probe was set inside the beaker to measure the water's temperature. The beaker was then placed into the water bath of a Fisher Scientific Isotemp 4100 Water Circulator filled with 8% propylene glycol and dechlorinated tap water. The starting temperature of the water bath was between 24.5°C and 25.0°C for all tests while the starting temperature of the beaker water was controlled between 23.2°C and 25.9°C for all tests. The propylene glycol and water solution was cooled from room temperature to 3°C at a rate of 0.66°C/min to allow the temperature to equilibrate through the body tissues of the frog. At five minute intervals, the frog in its mesh box was removed from the beaker and flipped onto its back. If the frog was able to right itself, it was placed back into the water bath and cooled for another five minutes. When the frog failed to right itself after 10 seconds, the temperature of the water inside the beaker at the time of frog removal was recorded as the CTmin, as a proxy for body temperature

of the frog itself. After testing, frogs were placed in a container with moss and room temperature dechlorinated tap water to recover.

Acclimation

Frogs were given one week to recover from CTmin testing at room temperature before acclimation treatments began. Frogs were randomly assigned to 'cold' or 'warm' acclimation treatment such that half of the frogs from each site would be acclimated to each treatment. Warm treatment frogs were acclimated to room temperature at sea level in Hilo, HI (25 ± 1.3 SD °C). The temperatures at the highest elevation site were used as environmentally realistic temperatures for the cold acclimation treatment. Decagon Devices Em50 Digital/Analog Data Loggers fitted with VP-3 Humidity, Air Temperature, and Vapor Pressure Sensors with radiation shields were set at the Stainback ATV park from March to June 2015. Evening and night (16:00-05:30) temperatures for March and April averaged 16.1°C (± 1.47°C) over this period. Hence, the acclimation temperature for the cold treatment was set at 16°C as an ecologically realistic temperature. Cold treatment frogs were maintained in a Thermo Scientific Precision Refrigerated Incubator set to 16°C (± 3°, according to machine specifications). Actual temperatures in the chamber ranged between 12.9 and 17.5°C. Frogs were housed in individual or paired containers (for identification) with moss substrate on a 12L:12D photoperiod. Frogs in the warm treatment were housed in the same manner, in a room with an air conditioning unit set to 25°C. Actual temperatures in the room ranged between 25.3 and 29.3°C. Frogs were maintained with dechlorinated tap water *ad libitum* and fed weekly. Frogs were acclimated for a thirty day period, after which the CTmin was retested using the procedure above.

Physiological parameters associated with acclimation

After post-acclimation testing of CTmin, frogs were maintained in their acclimation treatment temperatures. Within two days of post-acclimation testing, frogs were dissected for analysis of anatomica and physiological parameters related to acclimation capacity. Frogs were anaesthetized with 1% solution of tricaine methanesulfonate (MS-222) in a sodium bicarbonate buffer applied topically to the frog's back. MS-222 is generally used as an anesthetic for reptiles and amphibians (Cakir & Strauch, 2005). When stimulation of the frog's legs and feet elicited no response, the frog was blotted to remove excess MS-222 solution and the spinal cord was severed using dissecting scissors. Blood was collected from major vessels in heparinized micro-capillary tubes, which were then sealed and centrifuged for 3 minutes. Total blood and hematocrit lengths in the micro-capillary tubes were measured to calculate hematocrit percentage. Plasma osmolality was measured using a Vapor-Pressure Osmometer (Vapro 5520, Wescor). The osmometer was fully equilibrated to room temperature and calibrated with 100 and 290 mOsm standards before measurements were made. After blood collection, frog body cavities were opened. Hearts were removed from the body, cutting as close as possible to the atria to minimize the amount of aortic arches vasculature connecting to the heart. Any abdominal egg masses or fat bodies present were also removed, taking as little vasculature and other connective tissue as possible. The wet

masses of frog hearts and any fat bodies or egg masses present were measured after dissection. The frog carcasses, hearts, and other organs were dried for three days in a drying oven set to 70°C. The dry masses of the frogs and organs were measured to determine proportion of frog mass composed of heart, eggs, and fat bodies.

Seasonality

To take into account a possible effect of seasonality on the CTmin of *E. coqui*, a second set of twenty-four frogs were collected from the Stainback site in June 2016. The CTmin of these frogs was measured but no acclimation or analysis of physiological parameters was performed. Pilot data showed few changes in the frogs due to acclimation but did show differences in CTmin of frogs from different populations before acclimation treatments. The CTmin values for the frogs collected in June, experiencing the onset of summer temperatures, were compared to those of the frogs from the same site collected in the previous September, at the beginning of cooler temperatures at higher elevations.

Characterization of site temperature

Elevation was used as a proxy for environmental temperatures, with higher elevations experiencing colder temperatures. To verify the temperature gradient corresponded with the elevational gradient, the temperatures at the sites were compared according to air temperature modeled by Evapotranspiration of Hawaii (Giambelluca et al. 2014). Coqui frog chorus reaches its peak roughly an hour after sunset and tapers off throughout the night (Benevides et al. 2009), thus temperatures at each site were described between the hours of 17:00 and 00:00. The hourly average temperatures were compiled for December and July.

Data analysis

All distributions were checked for normality and equal variance. The observations met all assumptions for statistical tests except where noted. A one-way ANOVA was used to compare pre-acclimation CTmin of frogs between populations. The effect of elevation on CTmin was analyzed using a linear regression comparing pre-acclimation CTmin and elevation of collection sites. For post-acclimation CTmin, one observation with a Cook's distance value > 0.1 was removed from the data set. The effect of acclimation treatment (cold or warm) on CTmin was analyzed using a two-way ANOVA, with population as the second factor. The interaction between treatment and population was considered, as it was hypothesized frogs from high-elevation populations would respond differently to cold acclimation than frogs from low-elevation populations. In order to examine changes in CTmin, post-acclimation CTmin was subtracted from the pre-acclimation CTmin to yield one number as the change in CTmin. Using the change in CTmin minimizes the influence of body size on CTmin, as larger frogs take longer to cool than smaller frogs. The change in CTmin was also analyzed using a two-way ANOVA, with population as the second factor and interaction between treatment and population considered.

Similarly, two-way ANOVAs with acclimation treatment and population as factors were used to evaluate differences in dry heart mass as a proportion of total dry body mass, plasma osmolality, and proportion of hematocrit in total blood.

There was some mortality among laboratory acclimation groups of frogs. Mortality data based on population differences were tested using binomial logistic regressions, with separate models for each acclimation treatment. Separate models were used because the two treatments used different holding containers, a potentially confounding effect. Both models had no influential values and no overdispersion, meeting the assumptions for linear regression. The influence of population on mortality was tested by comparing the fitted model to a null model using a Chi-square test. The mean predicted probability of survival during acclimation for each population was calculated based on the model and graphed.

To test for an effect of seasonality, a t-test was used to compare initial CTmin frogs (without acclimation) from Stainback site from frogs collected in September 2015 and frogs collected in June 2016.

To test for effect of frog mass on pre-acclimation CTmin, post-acclimation CTmin, and the difference in CTmin pre- and post- acclimation, simple linear regressions were used. To test for effect of frog sex on frog mass, a Welch's test was used because of unequal variance between the sexes, while differences in CTmin pre- and post-acclimation were tested with t-tests. To test for effect of frog sex on CTmin pre- and post-acclimation, t-tests were used. A t-test was used to compare the effect of sex on pre-acclimation CTmin for frogs without lipid-rich organs. To test for differences in CTmin between frogs with egg masses and fat bodies and frogs without lipid-rich organs, t-tests were used. The CTmin values for this comparison were reciprocally transformed to obtain a normal distribution. All analyses were performed in R version 3.1.1 (R Core Team, 2014).

RESULTS

Critical Thermal Minimum (CTmin) and Acclimation

The CTmin values for 119 *E. coqui* frogs from six East Hawaii populations ranged between 3.4 and 8.6°C, with a mean value of 5.1 ± 1.17 SD °C, Table 2. A one-way ANOVA for pre-acclimation CTmin showed significant differences between the six populations ($F_{5,112}$=8.98, $p < 0.001$). Elevation did not significantly affect CTmin ($R^2 = 0.03$, $F_{1,117} = 3.25$, $p = 0.07$) but the removal of a single observation from the Stainback site with a Cook's D value > 0.1 established a weak positive relationship between elevation and CTmin of frogs measured directly after collection (Figure 2, $R^2 = 0.05$, $F_{1,116} = 5.52$, $p < 0.05$).

No frogs died as a result of the CTmin testing. A total of twenty-two frogs died during the acclimation period, from both warm and cold treatments, Table 2. Population had a significant effect on mortality in the cold acclimation treatment, as the test and null models had significantly different deviance ($\chi^2 = -14.14$, df = 58; $p < 0.05$). Population differences accounted for 21.9% of variation in mortality in the cold treatment. In the warm acclimation treatment, there was no significant difference between deviance for the test and null models (test model: residual deviance = 39.93, df = 54; null model: residual deviance

= 43.22, df = 59; p = 0.098). Predicted values for survival differed by population for the cold treatment (Figure 3). All populations had a high predicted survival probability (0.9 or 1.0) with the exception of Stainback, which was 0.64.

At the end of acclimation, the mean CTmin value of surviving cold acclimated frogs was 0.92°C lower than warm acclimated frogs, showing a significant difference in response to treatment ($F_{1, 85}$=32.64, p < 0.001, Figure 4). Cold acclimated frogs had mean CTmin values of 4.32 ± 0.74 SD °C, while warm acclimated frogs had mean CTmin values of 5.24 ± 0.90 SD °C (Table 2). However, there were no differences in CTmin among populations within treatments after acclimation (Figure 4, $F_{5, 91}$=1.37, p = 0.24).

There were statistically significant differences in change in CTmin before and after acclimation among populations ($F_{5, 85}$ = 9.41, p < 0.001) and a trend toward differences between acclimation treatments (Figure 5, $F_{1, 85}$ = 1.77, p = 0.19). For change in CTmin, the interaction between acclimation treatment and population also showed a trend towards significance ($F_{5, 85}$ = 2.00, p = 0.086). Post-hoc analysis showed differences in CTmin between populations were similar but not identical to pre-acclimation population differences (Figure 2 and Figure 5).

Organ and tissue changes associated with acclimation

No significant differences were found in dry heart mass as a proportion of total dry body mass between treatments ($F_{1, 83}$ = 1.62, p=0.21) or between populations ($F_{5, 83}$ = 0.58, p = 0.71). No significant differences were found in plasma osmolality between treatments ($F_{1, 77}$ = 1.58, p = 0.21). The plasma osmolality of frogs from the Stainback site was significantly higher than that of other populations (Figure 6, $F_{5, 77}$ = 12.07, p<0.001). The elevated plasma osmolality was present in both cold and warm acclimation groups (Table 3). Frogs in the cold acclimation treatment tended to have higher hematocrit percentages than frogs in the warm acclimation treatment but no significant differences were found in hematocrit levels between treatments ($F_{1, 78}$ = 3.22, p = 0.08) or populations ($F_{5, 78}$ = 1.73, p = 0.14).

Egg masses and fat bodies of twenty-three frogs from three of the six populations were removed and weighed during dissection. Due to low sample sizes within populations (n ranging from 3 to 12 among populations, Table 1), no analysis of the lipid-rich organs as a proportion of dry weight was considered. Frogs with egg masses or fat bodies post-acclimation had a lower initial CTmin than frogs without the lipid-rich organs. Mean of frogs without lipid-rich organs was 5.23 ± 0.11 SE °C and mean of frogs with lipid-rich organs was 4.58 ± 0.22 SE °C (t = -3.06, df = 116, p < 0.005). After acclimation, there was no difference in the CTmin of coqui frogs with egg masses or fat bodies (t = -1.01, df = 119, p = 0.31).

Seasonality, sex, and mass

A t-test showed no differences between CTm in of frogs collected from the Stainback site in September 2015 and June 2016 (t = -1.55, df = 38, p = 0.13).

Frog sex made a significant difference in pre-acclimation CTmin, with females having a mean of 4.69 ± 0.20 SE °C and males, 5.19 ± 0.11 SE °C, (t = -2.81, df = 116, p < 0.05, Figure 7). However,

excluding the frogs with lipid-rich organs resulted in no significant differences between the sexes (t = -0.94, df = 93, $p = 0.35$). Sex did not have an effect on post-acclimation CTmin, either by treatment or by population (Table 4, treatment: $F_{1, 93} = 1.31$, $p = 0.256$; population: $F_{1, 85} = 0.13$, $p = 0.715$). Sex did have significant effect on the change in CTmin due to acclimation, with females showing, on average, an increase in CTmin associated with a reduction in thermal tolerance. Females had a mean change in CTmin of -0.33 ± 0.27)°C and males, 0.50 ± 0.17 °C, (t = -2.73, df = 95, $p < 0.05$, Figure 7). A follow-up test showed this difference did not exist when the frogs with lipid-rich organs were excluded from analysis (t = -1.80, df = 72, $p = 0.07$) As female *E. coqui* tend to be larger than males, it was initially suspected that differences in thermal tolerance due to sex could be a result of body mass differences. Female frogs used in this study were more massive than males (Table 4, t= 4.92, df = 49.43, $p < 0.001$). However, frog mass had no significant relationship with CTmin,, for pre-acclimation ($R^2 = 0.02$, $F_{1, 116} = 2.07$, $p = 0.15$), post-acclimation ($R^2 = 0.01$, $F_{1, 96} = 0.70$, $p = 0.40$), or change in CTmin due to acclimation ($R^2 = 0.03$, $F_{1, 95} = 1.75$, $p = 0.12$).

Characterization of site temperature

The elevation gradient of the six sites had a corresponding temperature gradient at all hours between 17:00 and 00:00, for both July and December. Based on surface air temperature modelled by Evapotranspiration of Hawaii (Giambelluca et al. 2014), the highest modelled temperature was 26.1°C at Beach Road, in July, at 17:00 hours and the lowest modelled temperature was 16.0°C at Stainback ATV Park, in December, at 00:00 hours. The acclimation treatment temperatures (warm: 25 ± 1.3 SD and cold: 16 ± 3 SD °C) correspond closely to the endpoints of temperatures experienced by active coqui choruses in the natural environment.

DISCUSSION

Initial testing of CTmin of coqui frogs from six populations in East Hawaii revealed significant differences between populations, with frogs from higher elevation habitats having lower CTmin values than frogs from lower elevations. While the sites were situated on an elevation gradient and CTmin was significantly affected by elevation, there was not a strong relationship between elevation and CTmin (Table 2). The majority of variability was driven by population differences. The two highest CTmin values (indicating lower thermal tolerance) were from populations with intermediate elevations. Given that these frogs were tested directly after collection from the field, population differences are indicative of some thermal adjustment, whether acclimation to local environmental temperatures or local adaptation. While the Stainback population had a mean pre-acclimation CTmin similar to that of lower-elevation populations, the Glenwood population (at the second-highest elevation) had a much lower mean CTmin than the other populations. The low mean Glenwood value drove the weak relationship between elevation and CTmin. The Glenwood population was collected in an open field area whereas the frogs from other populations were collected in sites with a forest canopy. The elevation at Stainback is higher than

Glenwood, leading to colder temperatures at the higher site as modelled by Evapotranspiration of Hawaii (Giambelluca et al. 2014). However, the open environment at Glenwood may be a colder habitat than the insulated forest at Stainback and on-site temperature data should be used to reinforce measurements of thermal tolerance among populations in future studies.

Acclimation treatment resulted in changes in the CTmin of frogs over the thirty-day period. The differences in thermal tolerance between populations directly from the field combined with the results of the acclimation testing indicate *E. coqui* in Hawaii acclimate to local thermal environmental conditions. While the higher elevation populations initially had lower CTmin values than lower elevation populations, the differences between populations were removed during the acclimation period. Post-acclimation CTmin differed between cold and warm treatments, but not among populations, indicating population-level shifts in thermal regimes. Shifts over the lifetimes of individuals are regarded as acclimation (Angilletta et al. 2002) .

The shifts in CTmin were not uniform across populations and treatments. Overall, the CTmin of the cold treatment groups from lower elevation populations decreased, showing better tolerance of cold temperatures. Correspondingly, the CTmin of warm treatment groups from lower elevation populations stayed relatively constant. For the higher elevation populations of Glenwood and Stainback, the cold treatment groups, experiencing similar temperatures as those of the natural habitat, stayed more constant. The warm treatment groups of the higher elevation populations likely experienced a more drastic shift than the cold treatment group by maintenance at room temperature in Hilo. The CTmin of warm treatment groups for both of these populations increased, indicating a reduction in thermal tolerance (Table 2 and Figure 5). This pattern for the two highest and two lowest elevation populations supports the hypothesis of acclimation to ambient temperature. This pattern was not represented in the two mid-elevation populations. The increase in cold tolerance exhibited by the cold treatment groups from Lava Trees and Paukaa was expected. The increase in cold tolerance by the warm treatment groups from these populations was unexpected and conflicts with the acclimation pattern shown by the other four populations, Figure 5. As such, the change in CTmin as affected by the interaction between population and treatment showed a trend towards significance. A greater sample size might make the interaction between acclimation treatment and elevation of population more robust.

In addition, the mortality of frogs over the acclimation period supports the hypothesized pattern of acclimation. Population differences explained 22% of the variation in mortality in the cold acclimation treatment. The Lava Trees group, which had the highest mean CTmin value pre-acclimation, experienced 50% mortality in the cold treatment and 10% mortality in the warm treatment. This indicates the shift in environmental temperature was outside the tolerable range of the frogs' plasticity, especially for the cold group. Paukaa, with the second highest mean CTmin, also had high mortality (30%) in the cold acclimation group and 0% in the warm group (Table 2). By contrast, the higher elevation sites had no mortality in the cold acclimation groups. Glenwood had the greatest magnitude change in mean CTmin post-acclimation (for the Warm group, Table 2 and Figure 5) and exhibited no mortality for either group.

Conversely, the cold treatment group from the highest elevation site had 0% mortality while the warm group had roughly 40%. Reduction of feeding due to cold may be responsible for deaths in the cold treatment, as cold treatment frogs consistently left food uneaten over the one-week feeding interval. By contrast, warm treatment frogs rarely left food longer than one day. The reason for deaths in the warm groups are unknown. Ectotherms acclimated to cooler temperatures have lower metabolic activity (Patterson & Davies, 1984) and tadpoles maintained at cooler temperatures had lower feeding rates than those maintained at warmer temperatures (Warkentin, 1992). Lower feeding rates as a result of lower metabolic activity in the cold treatment may have contributed to mortality. While the CTmin was designated as the functional lethal temperature over a period of minutes to hours where an animal is incapable of locomotion, inability to maintain body functions such as eating represents an ecological lethal temperature in effect over periods of days to weeks. The mortality over the acclimation period introduced a bias into pre- and post- acclimation comparisons. Frogs with an inherently lower capacity to tolerate temperature change, whether as a result of individual or population level variation, were more likely to die over the course of the acclimation period. The surviving frogs were those with higher flexibility in temperature tolerance, which may have changed population-level characterization of temperature tolerance after acclimation.

Thermal acclimation responses are often the result of physiological changes in the individual, such as alterations to vascularization or epidermal tissues (Angilletta et al. 2002). These responses to environmental temperature can be non-reversible, especially in the case developmental plasticity, acclimation during larval stages (Troia et al. 2015). Coqui frogs have been present in the Stainback area for at least a decade (Coqui Frog Working Group 2007), exposing developing eggs to colder temperatures. It is possible the frogs in the Stainback population have undergone developmental changes that reduce their ability to survive at warmer temperatures. The mortality data and acclimation results together show acclimation responses consistent with acclimation to local environment but different acclimation capacity between populations. Other ectotherms show thermal tolerance specific to the local environment. Congeneric species of crabs in different thermal environments have thermal acclimation capacities limited by the temperature maximum of their respective habitats (Stillman, 2003). Genetically distinct sockeye salmon populations have different thermal tolerance based on physiological adaptations consistent with the conditions at each population's natal site (Eliason et al., 2011).

In concert with local acclimation capacity, physiology associated with sex appears to play a role in the thermal tolerance of coqui frogs. Adult female coqui frogs are on average larger than adult males (Townsend & Stewart, 1994). The higher body mass was expected to cool more slowly, resulting in a lower mean CTmin value for females. Female coqui frogs in the present study were more massive than males, with mean female mass 2.99g ± 0.18 SE and mean male mass 2.02g ± 0.07 SE and the pre-acclimation CTmin of females was significantly lower than that of males but frog mass did not have a significant effect on CTmin. Females had a lower initial CTmin than males and the sex-specific difference was eliminated by acclimation. Therefore, females had a more negative change in CTmin due to

acclimation, implying an overall lower tolerance of cold across treatments and across populations. The differences observed between the sexes may not be a factor of sex itself, but rather the lipid content of the frogs. The frogs that had identifiable egg masses or fat bodies which were removed during dissection had significantly lower initial CTmin than frogs without the lipid-rich organs. The difference between these groups was not present after acclimation. Comparison of the frogs without any lipid-rich organs resulted in no differences between the sexes, pre- or post-acclimation. The effect of lipid-rich egg masses on initial CTmin may explain the apparent differences between the sexes. All but one of the twenty-three frogs with lipid-rich organs identified and removed were female and the difference in CTmin between females and males and lipid-rich organs or no lipid-rich organs was very similar: 4.69 ± 0.20 SE °C for females and 5.19 ± 0.11 SE °C for males, 4.58 ± 0.22 SE °C for frogs with lipid-rich organs and 5.23 ± 0.11 SE °C for frogs without lipid-rich organs. Of the thirty-nine total female frogs used in the study, twenty three had lipid-rich organs removed. The lower CTmin observed for females is linked, if not attributable to the presence of egg masses and fat bodies. In addition, the collection of frogs from Glenwood, with the lowest mean CTmin, had a larger proportion of female frogs than other populations (Table 1) and all but one of the Glenwood females had lipid-rich organs. The higher incidence of females with egg masses likely drove the lower CTmin value for Glenwood as well as the lower temperature tolerance values for female frogs.

Higher lipid content in female coqui frogs at higher elevations comes with a greater capacity to tolerate colder temperatures, at least in the present study. The egg masses and fat bodies are not expected to have any insulative effect on the frogs, as ectotherms have no internal heat source to protect from a colder environment. The mechanism which allows female with egg masses to tolerate colder temperatures is unknown. Greater cold tolerance allows these frogs to continue reproductive activities (egg development, egg laying, and mating) at colder temperatures, thus conveying an advantage over less cold-tolerant populations. With a less robust tolerance for cold, males are less likely to be engaging in reproductive activities, as calling to attract mates and defending eggs require activity. Females without corresponding cold-tolerant males may rely on egg masses as energy reserves to reduce the need for foraging. These females may also be immediately ready to deposit eggs as soon as the temperatures are suitable for males and breeding activities.

An alternative explanation for population differences in thermal tolerance is local genetic divergence. The populations of coqui frogs at higher elevations were likely founded by singular introductions from lower elevation populations. With limited connectivity between populations, gene flow between isolated populations at higher elevations is unlikely and may drive genetic divergence. No investigation of genetic divergence was done in the present study, but in the period since frogs have been introduced to Hawaii, it is possible population-specific adaptations to tolerate colder temperatures have arisen.

Genetic differences may also be responsible for discrepancies between the CTmin of coqui frogs in Hawaii and in their native Puerto Rico. The mean value of CTmin for *E. coqui* frogs collected from sites

of varying elevations in East Hawaii was 5.1°C. A previous study reported the CTmin of *E. coqui* in Puerto Rico as 7.5°C for a high elevation site (700m a.s.l.) and 7.3°C for low elevation site (15m a.s.l.) (Christian et al., 1988). The frogs used in the Christian et al. (1988) study were acclimated at 20°C for a period of one week, while the frogs in the current study were acclimated at 16°C for thirty days, potentially leading to differences in observed critical thermal minimum. The Christian et al. (1998) study also did not report any acclimation response for the frogs. *E. coqui* in Puerto Rico have also been classified into subclades based on mtDNA lineages (Velo-Antón et al., 2007). Genetic diversity is not connected to altitudinal distribution, but *E. coqui* subclades are connected to the east-west distribution of frogs. The founder frogs of coqui frog populations on Hawaii Island are most closely related to the Puerto Rican populations in the eastern lowlands (Velo-Antón et al. 2007; Peacock et al. 2009). Because of the low genetic diversity of coqui frogs in Hawaii, it is possible the Christian et al. (1988) study and the current study used frogs of different subclades, which may have contributed to different thermal tolerances.

The differences in thermal tolerance between *E. coqui* in Hawaii and Puerto Rico, between cold and warm acclimation treatments, between frogs with and without lipid reserves, and between populations of frogs in Hawaii are telling of the flexibility of this introduced species. This flexibility in temperature tolerance and acclimation response is somewhat unique among tropical anurans (Feder, 1982). However, the maximum temperature differences measured here are on the order of 2-3°C. Changes in temperature tolerance at this scale are unlikely to be biologically significant. Furthermore, coqui frogs are sensitive to more environmental factors than temperature alone. During data collection for this study, the Island of Hawaii experienced several weeks of little rainfall and cool nighttime temperatures which resulted in minimal coqui frog chorus activity. Where full choruses were recorded at sites in the 950-970m elevation range in summer and autumn months, no activity was recorded in the winter months. It is unknown if the cool temperatures, the lack of moisture, or a combination of environmental factors prompted this inactivity. While the specific cause is unknown, the environmental conditions at higher elevations impede frogs from normal activity, at least under certain climatic patterns. In addition, a pilot study in which cold treatment frogs were acclimated at 12°C resulted in high mortality, prompting the increase to 16°C and indicating 12° is far below the thermal limit for coqui frogs. Seasonality did not have an effect on CTmin, as frogs collected from the same site in fall and late spring had no difference in pre-acclimation CTmin.

Acclimation treatment did not have an appreciable effect on the other physiological parameters tested. As a small acclimation response was observed between cold and warm treatments, some physiological adjustments in response to the cold temperature are expected (Angilletta et al. 2002). Other acclimation studies on anurans have examined activity of metabolic enzymes (Rogers et al. 2007), metabolic rate (Trzcionka et al., 2008), hydromineral balance (Sinsch, 1991; Stinner & Hartzler, 2000), or blood viscosity (Palenske & Saunders, 2003). If physiological changes occurred in acclimated *E. coqui*, testing of other physiological parameters might have revealed them. Coqui frogs pose a logistical challenge for physiological analyses as their small size limits multiple samples of blood or other bodily

fluids. The mean snout-vent length of frogs in this experiment was 3.02cm and blood was collected by severing the major vessels. The small size of the frogs also resulted in significant experimental variation in heart dissections. The mean heart mass was 4.26 mg; any dissection that increased or decreased the amount of major blood vessel tissues dissected with the heart was liable to change the measured heart mass. Plasma osmolality was significantly higher for the highest elevation population (Figure 6). Previous studies with findings of changes in plasma osmolality during or after acclimation found differences were due to the water balance of the animal and less related to the thermal tolerance (Palenske & Saunders, 2003; Sinsch, 1991). The cause of the higher plasma osmolality of frogs from Stainback is unknown but may be an attribute of the population, considering the higher osmolality was seen across acclimation treatments.

This study has shown coqui frogs in Hawaii have a thermal limit lower than those in Puerto Rico. Frogs in different populations were acclimated to local environmental conditions and showed an acclimation response after acclimation for thirty days. While there was not a direct relationship between elevation and cold tolerance, frogs from the second-highest elevation population showed higher incidence of females with egg masses and a corresponding lower CTmin. Different populations showed different capacity to acclimate and there may be a sex-specific acclimation response in this species on the basis of higher lipid content in female frogs The report of this acclimation response is new and the nature of the physiological shifts associated with acclimation are unknown. The capacity for thermal acclimation may enable the coqui frog to be a successful invasive species, along with other life history traits. While the thermal tolerance of this species is flexible and uncommon among tropical ectotherms, the magnitude of the shift in critical thermal minimum does not indicate coqui frogs are likely to be a threat to the higher-elevation montane ecosystems of Hawaii. However, climate envelope modeling of the potential for coqui frog expansion under different models of global climate change predicts coqui frogs may be able to find suitable habitat in the montane ecosystems of Hawaii (Rödder, 2009). These models are based on current coqui frog distributions. Thermal tolerance and acclimation, as demonstrated in this study, may slightly expand the climate envelope models of suitable habitat for coqui frogs to encompass greater areas of Hawaii's montane ecosystems. To prevent potential acoustic and nutrient related impacts of coqui frogs on these higher elevation, montane ecosystems, careful monitoring and control efforts should be deployed and maintained in habitats of concern.

Future studies on the thermal physiology of coqui frogs should be conducted with focus on the effect of cold temperature on diet, effect of sustained versus periodic cold exposure, sex-specific comparisons of cold tolerance from different populations, genetic divergence between populations in Hawaii, and comparison of temperature tolerance of Hawaiian coqui frogs and Puerto Rican coqui frogs. Future work on the effect of coqui frogs as an invasive species in Hawaii should look at diet composition of higher elevation frog populations, acoustic effect of daytime frog calling on native bird communication, and effect of coqui frog populations on nutrient dynamics at higher elevation sites.

Table 1. Proportions of male and female *E. coqui* frogs collected from six populations in East Hawaii and proportions of frogs with lipid-rich organs (egg masses and fat bodies) removed during dissection.

Population	Frogs Collected	Females	Males	Females with lipid-rich organs	Males with lipid-rich organs
Beach Road	20	3	17	2	2
Hilo	20	6	14	0	0
Lava Trees	20	7	13	0	0
Paukaa	20	7	13	5	3
Glenwood	18	12	6	11	0
Stainback	21	3	18	0	0

Table 2. CTmin values associated with acclimation of *E. coqui* frogs from six populations in East Hawaii. Negative numbers indicate an increase in CTmin, such that the frog's thermal tolerance weakened.

Population	Pre-Acclimation Mean CTmin ± SE (°C)	Treatment	Post-Acclimation Mean CTmin ± SE (°C)	Starting n	Mortality (%)	Change in CTmin Mean ± SE (°C)
Beach Road	5.20 ± 0.18	Cold	3.74 ± 0.35	10	30	1.34 ± 0.35
		Warm	5.42 ± 0.49	10	10	0.02 ± 0.47
Hilo	4.86 ± 0.23	Cold	4.15 ± 0.21	10	30	0.28 ± 0.21
		Warm	4.73 ± 0.32	10	10	0.07 ± 0.32
Lava Trees	5.81 ± 0.28	Cold	4.26 ± 0.44	10	50	1.04 ± 0.44
		Warm	5.67 ± 0.61	10	10	0.92 ± 0.61
Paukaa	5.63 ± 0.21	Cold	4.46 ± 0.40	10	30	0.63 ± 0.40
		Warm	4.69 ± 0.28	10	0	1.53 ± 0.28
Glenwood	3.91 ± 0.11	Cold	4.61 ± 0.31	9	0	-0.60 ± 0.31
		Warm	5.63 ± 0.37	9	0	-1.83 ± 0.37
Stainback	5.13 ± 0.24	Cold	4.51 ± 0.51	10	0	0.24 ± 0.51
		Warm	5.43 ± 0.33	11	36.3	-0.53 ± 0.33
			Total	**119**		

Table 3. Physiological parameters associated with acclimation of six populations of *E. coqui* in East Hawaii. Frogs from the Stainback site had significantly higher plasma osmolality than frogs from other sites ($F_{5, 77}$ = 12.07, p<0.001, see Figure 6). No other comparisons across treatments or across populations were statistically significant.

Population	Treatment	Mean ± SE Dry heart mass/dry body mass (%)	Mean ± SE Plasma Osmolality (mmol/kg)	Mean ± SE Hematocrit (%)	n
Beach Road	Cold	0.140 ± 0.009	195 ± 4.9	13.95 ± 1.43	7
	Warm	0.132 ± 0.012	183 ± 5.1	12.76 ± 0.74	9
Hilo	Cold	0.151 ± 0.008	195 ± 5.7	15.14 ± 1.31	7
	Warm	0.143 ± 0.014	182 ± 2.5	13.04 ± 1.08	9
Lava Trees	Cold	0.146 ± 0.057	187 ± 5.6	12.35 ± 1.16	5
	Warm	0.120 ± 0.015	179 ± 3.2	11.39 ± 0.76	9
Paukaa	Cold	0.143 ± 0.017	190 ± 2.0	16.30 ± 2.13	7
	Warm	0.110 ± 0.010	199 ± 2.5	14.45 ± 1.28	10
Glenwood	Cold	0.159 ± 0.019	190 ± 3.0	12.88 ± 1.28	9
	Warm	0.140 ± 0.007	192 ± 2.9	11.24 ± 1.68	9
Stainback	Cold	0.146 ± 0.025	**216 ± 8.7**	14.16 ± 2.02	10
	Warm	0.161 ± 0.032	**224 ± 12.1**	13.62 ± 1.26	7
				Total	**98**

Table 4. Effects of sex on CTmin pre-acclimation, post-acclimation, and changes due to acclimation. Females had significantly lower mean pre-acclimation CTmin than males (t = -2.81, df = 116, p < 0.05) and significantly lower mean change in CTmin after acclimation compared to males (t = -2.73, df = 95, p < 0.05). There was not an interaction between sex and treatment or sex and population for any parameters.

Sex	Mean ± SE Mass (g)	Mean ± SE Pre-Acclimation CTmin (°C)	n	Treatment	Mean ± SE Post-acclimation CTmin (°C)	n	Mean ± SE Change in CTmin (°C)
Females	2.99 ± 0.18	4.69 ± 0.20	38	Cold	4.29 ± 0.20	20	0.12 ± 0.30
				Warm	5.53 ± 0.23	18	-0.73 ± 0.43
Males	2.02 ± 0.07	5.19 ± 0.11	81	Cold	4.34 ± 0.14	39	0.55 ± 0.24
				Warm	5.11 ± 0.14	42	0.46 ± 0.23

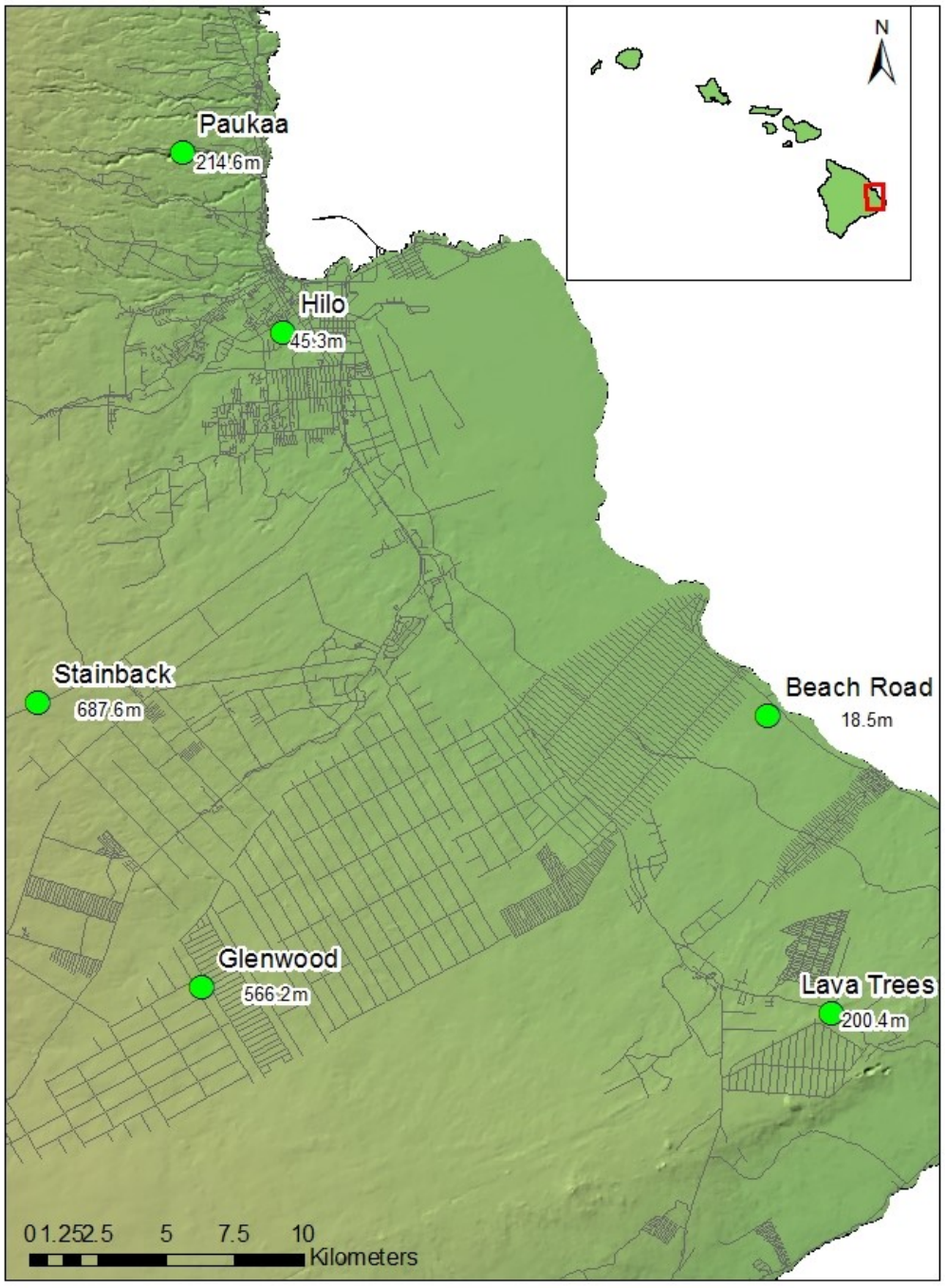

Figure 1. Locations and elevations (m above sea level) of *E. coqui* populations sampled in East Hawaii. Grey lines indicate roads.

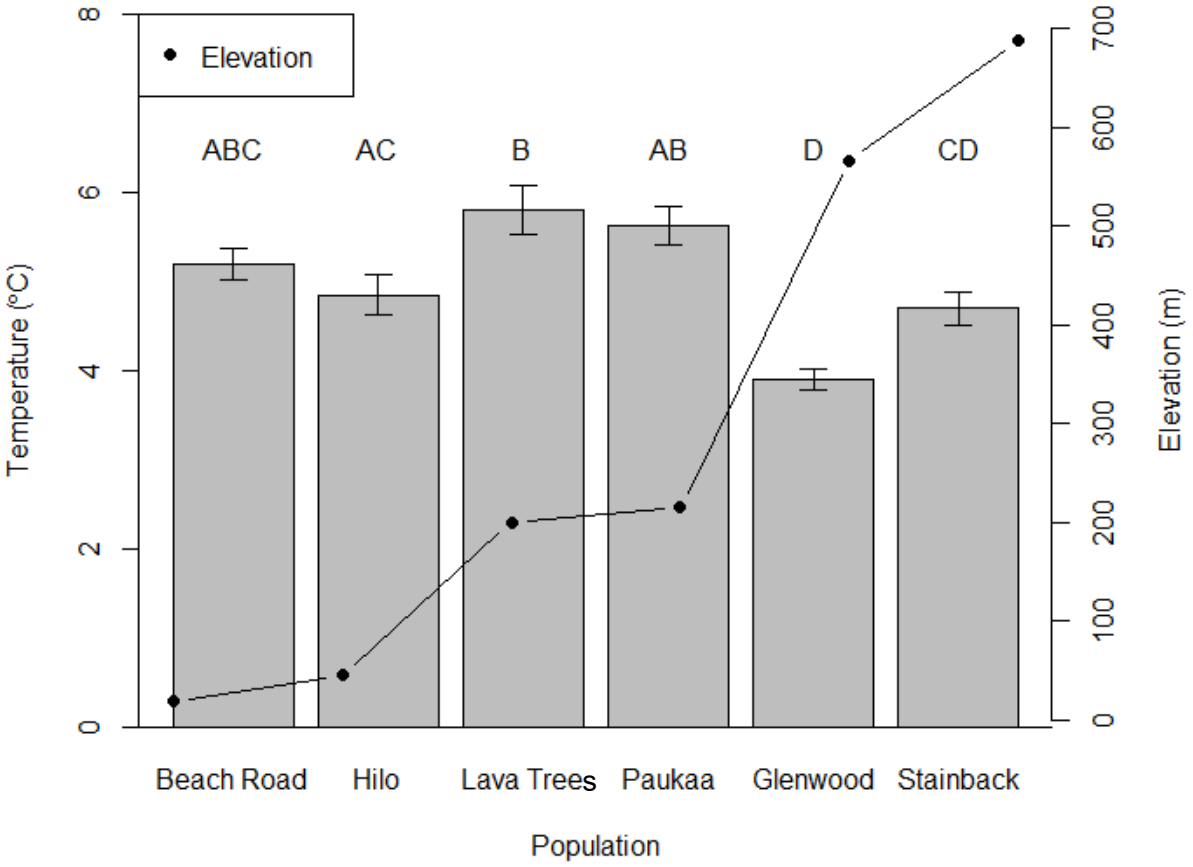

Figure 2. Pre-acclimation mean and SE CTmin values by population and elevation of population sites. Populations were significantly different ($F_{5,112}$=8.98, $p < 0.001$) and are denoted by different letter groupings. Higher CTmin values connote lower temperature tolerance, as the temperature at which the righting response of frog fails is higher. With the removal of a single observation from the Stainback group with a Cook's D value > 0.1, there was a significant relationship between elevation and CTmin ($R^2 = 0.05$, $F_{1,116} = 5.52$, $p < 0.05$).

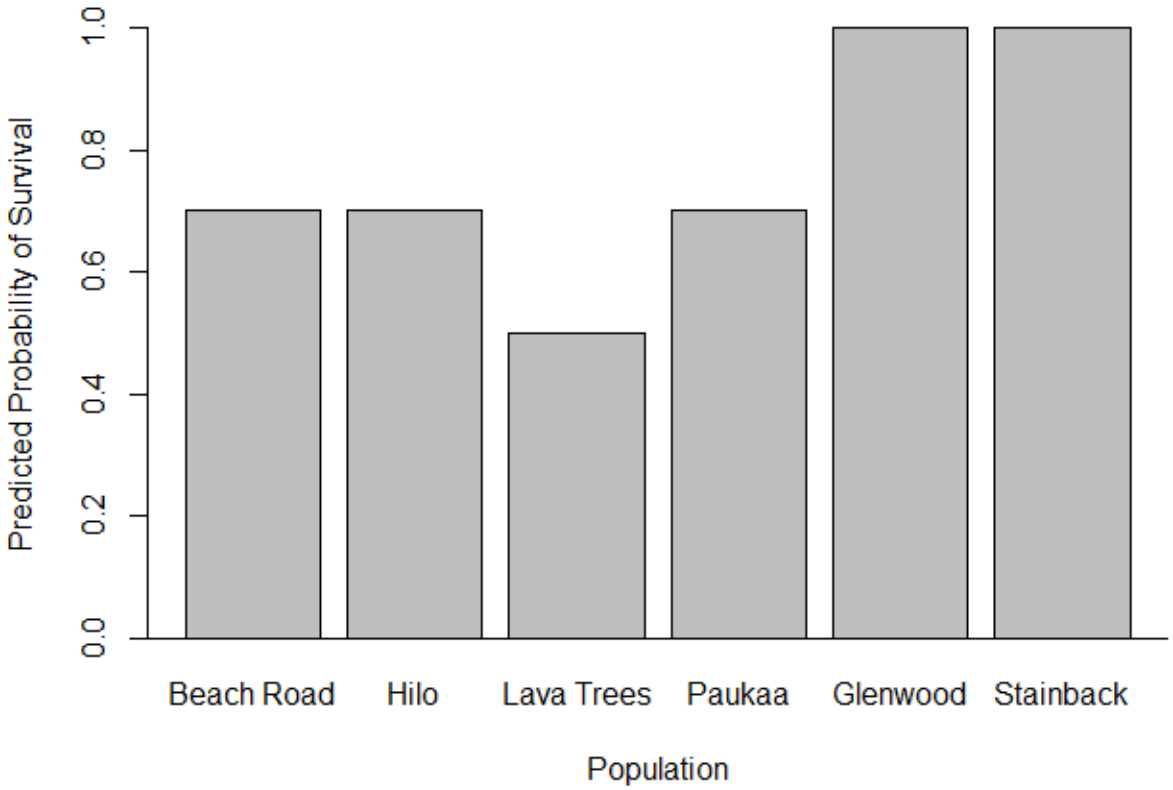

Figure 3. Predicted probability of survival in the cold acclimation treatment by population, based on a binary logistic regression model. The inclusion of population reduced variation in the null model by 21.9%. Because the model used population as the sole predictor, there was no intra-population variation and therefore no error bars.

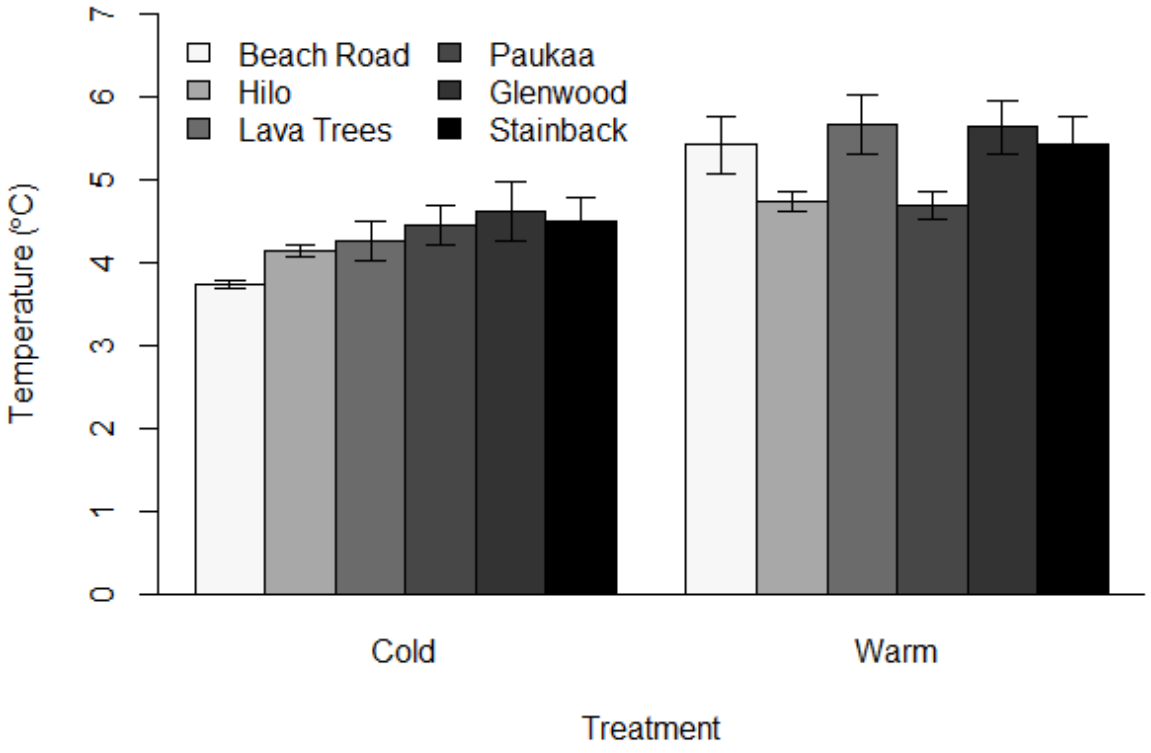

Figure 4. Mean and SE CTmin of *E. coqui* frogs after acclimation for thirty days. Acclimation treatment had a significant effect on CTmin ($F_{1,85}=32.64$, $p < 0.001$), with cold acclimated frogs having an average CTmin 0.92°C lower than warm frogs. There were no post-acclimation population differences within cold or warm treatments.

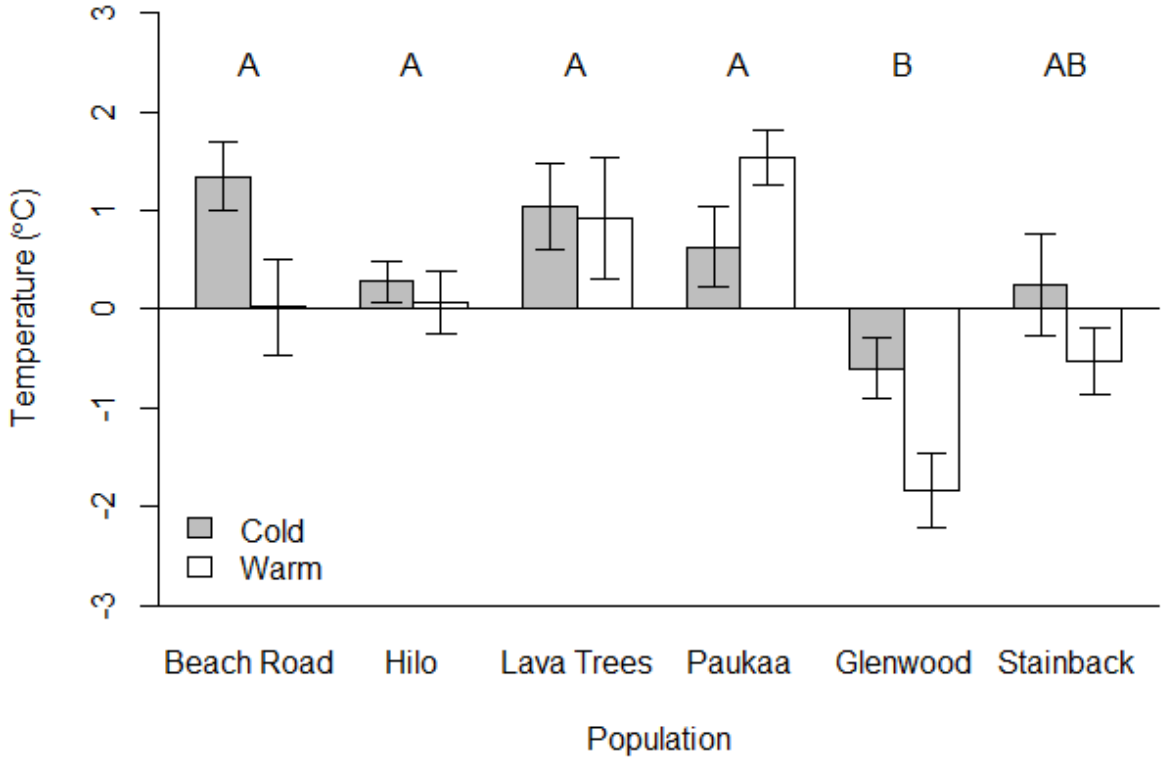

Figure 5. Mean and SE difference in pre- and post-acclimation CTmin by population and by treatment. Significant differences exist in change in CTmin between populations ($F_{5, 85}$ = 8.99, p<0.001) but not between treatments ($F_{1, 85}$ = 1.77, p = 0.19). Grey, positive bars suggest a shift towards better tolerance of cold, while white, negative bars suggest a shift towards lower tolerance of cold. Different letters denote significantly different groups.

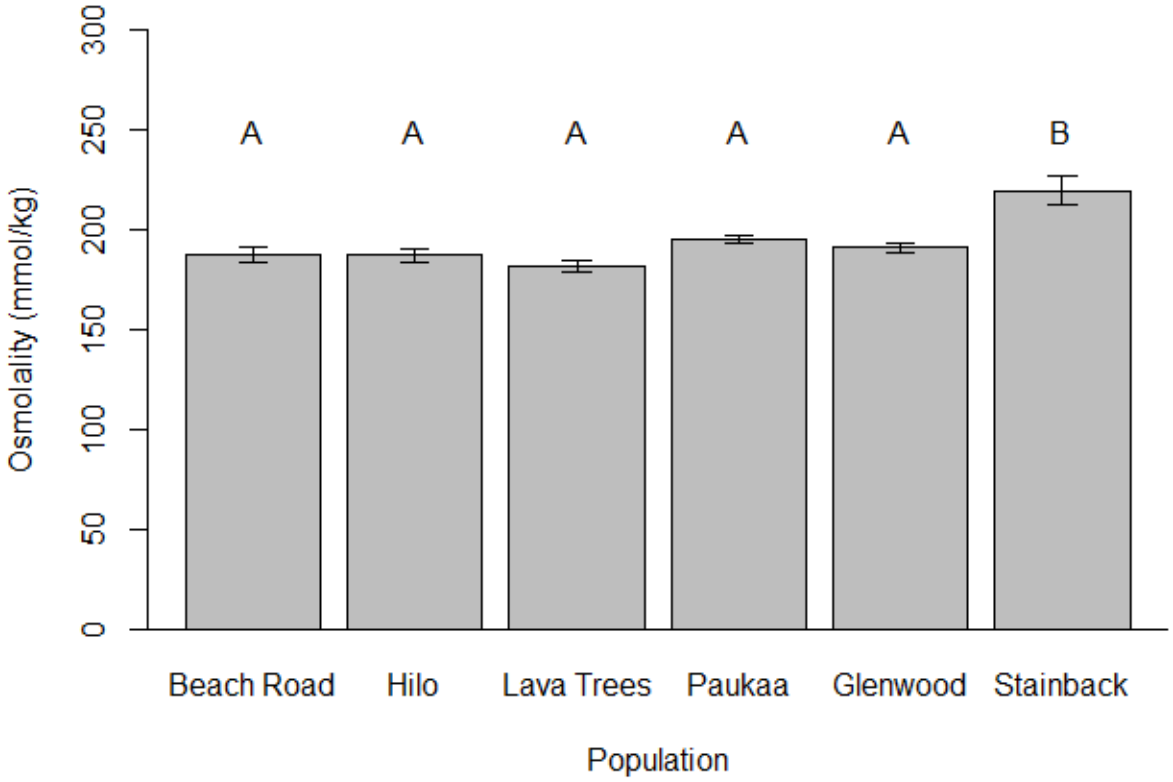

Figure 6. Mean and SE plasma osmolality from acclimated frogs. Frogs from the Stainback site had significantly higher plasma osmolality ($F_{5, 77}$ = 12.07, p<0.001) than other populations, denoted by letters. No differences existed between acclimation treatments ($F_{1, 77}$ = 1.58, p=0.21).

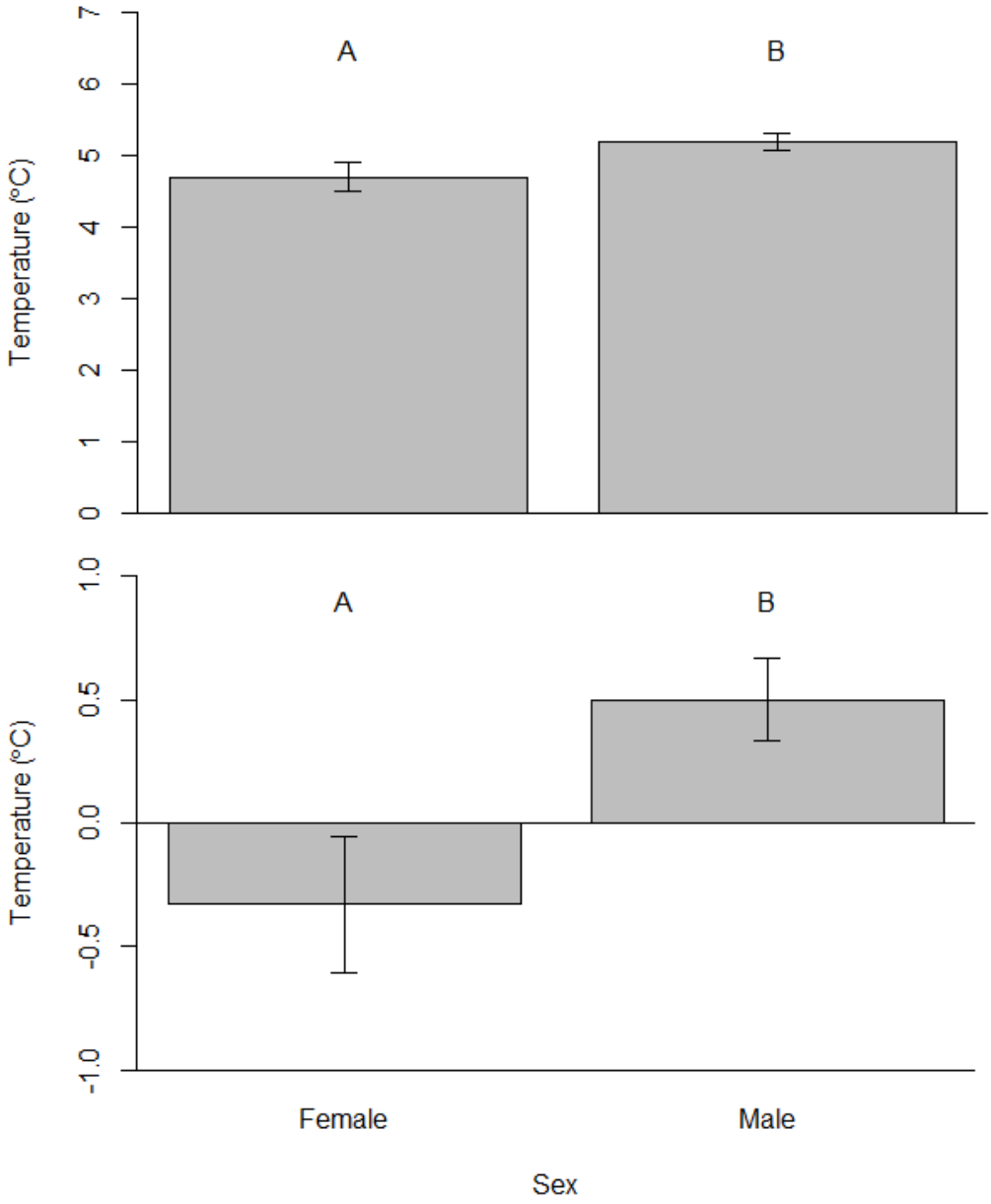

Figure 7. Top: Mean and SE CTmin of both frog sexes prior to acclimation. Females had a mean (±SE) initial CTmin of 4.96 ± 0.20 °C, which was significantly different from that of males, 5.19 ± 0.11 °C (t = -2.81, df = 116, $p < 0.05$). Bottom: Mean and SE difference in CTmin post-acclimation. Females had a significantly more negative mean difference in CTmin post-acclimation than males (t = -2.73, df = 95, $p < 0.05$). Because the post-acclimation value was subtracted from the pre-acclimation value, a negative number signifies an increase in CTmin and a lower temperature tolerance.

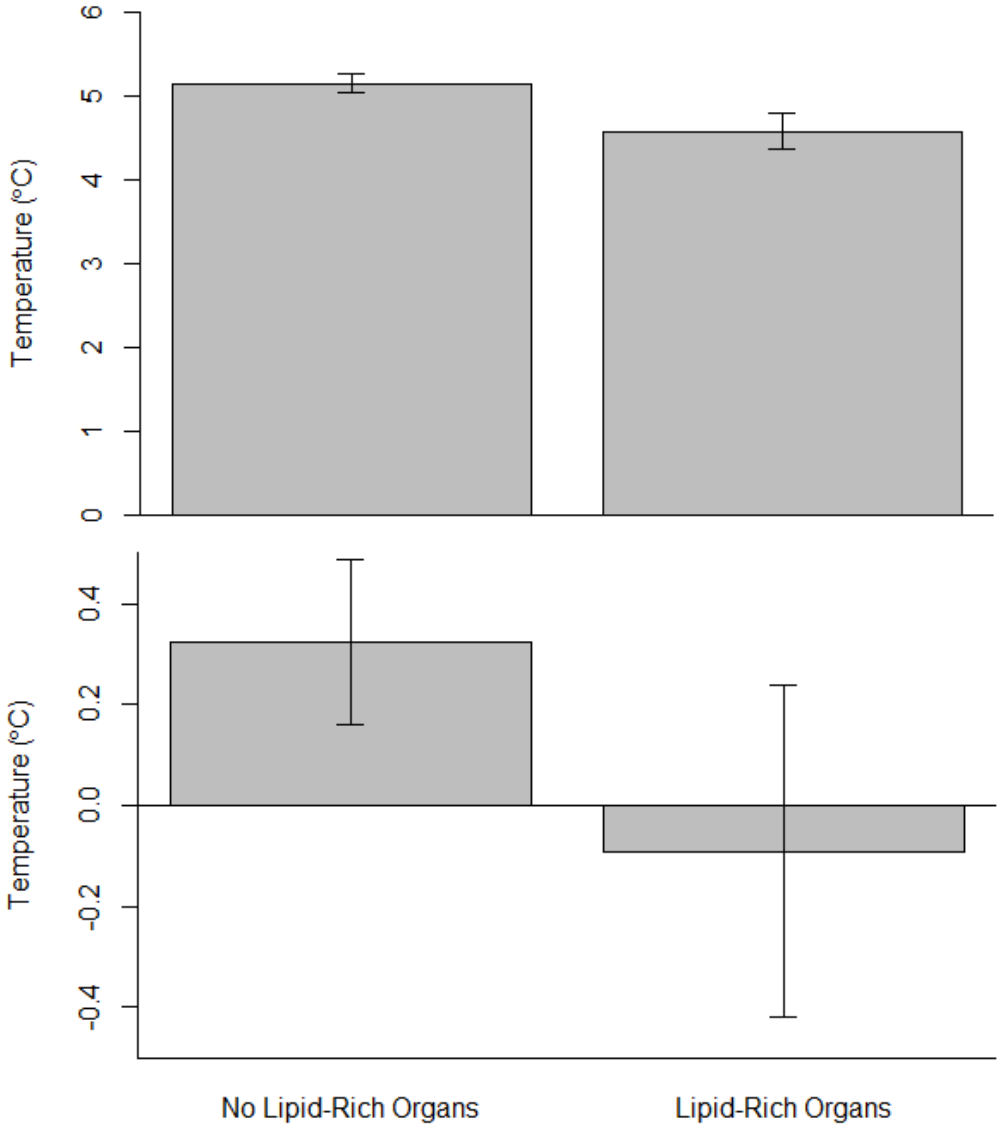

Figure 8. Top: Mean and SE difference in CTmin in frogs with and without lipid-rich organs prior to acclimation. Frogs without lipid-rich organs had a significantly lower initial CTmin (t = -2.78, df = 116, $p < 0.05$). Bottom: Mean and SE difference in difference in CTmin pre- and post-acclimation. Frogs with lipid-rich organs had a negative difference compared to frogs without lipid-rich organs but the high variation made this difference non-significant (t = 1.20, df = 95, $p = 0.23$).

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