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The Effect of Fluctuating Temperature and Diet on the Cardiac Thermal Performance of the California Killifish (Fundulus Parvipinnis)

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SANTA BARBARA

The Effect of Fluctuating Temperature and Diet on the Cardiac Thermal Performance of the

California Killifish (*Fundulus parvipinnis*)

A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science in Ecology, Evolution and Marine Biology

By

Madison Paige Heard

Committee in charge:

Professor Erika Eliason, Chair

Professor Andrew Brooks

Professor Gretchen Hofmann

June 2023

The thesis of Madison Paige Heard is approved.

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Andrew Brooks

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Gretchen Hofmann

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Erika Eliason, Committee Chair

June 2023

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(by no means an exhaustive list, but here's an attempt)

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I would be remiss to not mention the critters and the area that made this work possible. My deepest gratitude to the California Killifish from the Carpinteria Salt Marsh. I am honored to have cared for these 362 individuals and am deeply appreciative for their service and sacrifice.

Lastly but most importantly, thank you to the Chumash on whose land this thesis was done.

ABSTRACT

The effect of fluctuating temperature and diet on the cardiac thermal performance of the

California killifish *(Fundulus parvipinnis)*

by

Madison Paige Heard

Master of Science in Ecology, Evolution and Marine Biology

University of California, Santa Barbara – 2023

Salt marshes are dynamic systems characterized by rapid and intense fluctuations in temperature. Because of this, the ectotherms residing in these environments must possess the ability to tolerate thermally variable environments. Mounting these plastic thermal responses requires energy and nutrients, which are acquired through diet. As the nutritional landscape shifts within these highly variable environments, it is critical we examine the modulating effect of diet on the capacity of ectotherms to respond to thermal variability. Using the California Killifish (*F. parvipinnis*), we tested the influence of fluctuating temperatures and diet quality on cardiac thermal performance through three specific hypotheses: (1) static acclimation temperature will yield a narrower thermal breadth when compared to fluctuating acclimation temperatures, (2) broad diets will yield higher cardiac thermal tolerance compared to a single-item diet and (3) upper thermal limits will vary when tested at the peak vs trough of a fluctuating acclimation cycle. *F. parvipinnis* were acclimated for 3 weeks at one of five ecologically relevant temperature regimes (11°C, 11-20°C, 20°C, 20-29°C, 29°C) while providing either a single-item diet or broad diet. Cardiac thermal tolerance was measured using

an Arrhenius Breakpoint Temperature test. Both maximum heart rate and cardiac upper thermal limits increased with increasing acclimation temperature. Surprisingly, diet quality had a greater influence on cardiac thermal performance at colder fluctuating acclimations when compared to warmer fluctuating acclimations. Lastly, *F. parvipinnis* were found to exhibit rapid diel plasticity by altering thermal limits throughout a diurnal phase. This work suggests that both diet and fluctuating acclimations influence species' upper thermal limits and outlines a clear need to incorporate more ecologically relevant temperature acclimations in lab-based research.

Keywords: Fluctuating temperature, diet, cardiac performance, thermal tolerance, salt marsh

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"It is advisable to look from the tide pools to the stars and then back to the tide pool again."

— John Steinbeck

While I worked in a tidepool-adjacent system and this quote is not entirely related to this thesis topic, it is nonetheless a soul-filling reminder of why I endeavored to become a master in science. To Ed Ricketts, John Steinbeck and the folks who were just as captivated with the natural world as I am; this thesis is dedicated to the giants on whose shoulders I stand, and to those that will find their footing on mine.

INTRODUCTION

Environmental variability is an inherent factor in global ecosystems. Fluctuations in conditions such as dissolved oxygen, pH, temperature, soil moisture, nutrient concentrations and salinity have been recorded at both spatial (i.e., habitat location) and temporal scales [short-term (i.e., hours, diel), long-term (i.e., seasonal), and evolutionary timescales] and the degree to which these changes occur can vary in intensity, frequency and duration (Bakun et al., 2015). Additionally, the stochasticity of these conditions is projected to increase due to anthropogenic climate change (IPCC 2014). Among these factors, temperature has been well-studied as one environmental measure that can exhibit rapid and variable fluctuations in many ecosystems and is known to be a pervasive stressor that can have profound impacts on organismal physiology and behavior (Fry, 1971; Hochachka & Somero, 2002). To survive, organisms must be able to perform throughout variable environmental conditions, which often requires the mounting of an energetically expensive acclimation response through physiological changes (Blewett et al., 2022; Seebacher et al., 2015; Somero, 2010).

An additional and well-studied factor that is known to vary within ecosystems is diet as nutrients acquired through diet provide organisms the energy needed to effectively respond to the factors present in an environment (such as mounting an energetically expensive thermal acclimation response). In the lens of ecological physiology, natural diets are being considered in the context of thermal variability to better understand how these factors influence organismal performance (Alton et al., 2020; Hardison et al., 2021; Ho et al., 2009). This thesis examines how variable temperatures and diet interact to influence ectotherm performance and persistence.

Temperature is known as a "master factor" because of the strong influence temperature has on various behavioral and physiological performance metrics (e.g., growth, swimming speed, oxygen consumption, metabolism, digestion, heart rate, etc.) (Brett, 1965; Fry, 1971; Volkoff & Rønnestad, 2020). Ectotherm performance is closely linked to temperature, where with increasing environmental temperature, organismal performance increases as well; however, performance typically reaches a plateau and subsequent declines after a certain point. From this relationship we can determine the optimum temperature at which performance is highest, and the range of temperatures where performance is effective, outside of which performance begins to decline. This relationship can be plotted as a thermal performance curve (TPC) (**Figure 1**).

Using TPCs we can characterize the effects of temperature on biological rates, and thus, performance. Thermal stress, however, can be introduced when
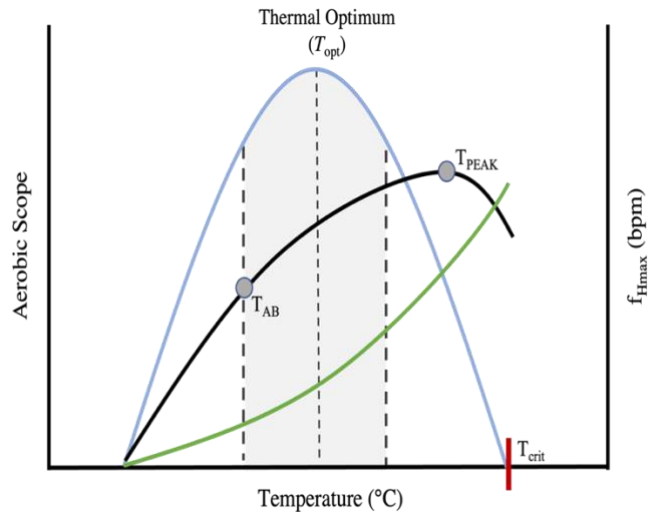
Figure 1. Theoretical performance curves comparing aerobic scope, maximum and resting heart rate (f_H). The blue line represents a theoretical performance curve showing the relationship between aerobic scope and temperature. The black line represents maximum heart rate generated from an ABT test with metrics indicating the temperature at which rate of increase begins to slow (T_{AB}) and the temperature at which maximum heart rate peaks (T_{PEAK}). The green line shows the relationship between resting heart rate and temperature. This figure demonstrates how aerobic scope varies across temperature, with the "optimum temperature" for cardiac performance falling between T_{AB} and T_{PEAK}. T_{crit} is the temperature at which aerobic scope is zero – leading to an unsustainable mismatch between supply of energy available to meet metabolic demands. Cardiac failure is imminent at this temperature.

ectotherms are exposed to acute or prolonged temperatures outside of the thermal optimum, causing a decrease in performance (Hofmann & Todgham, 2010). To maintain function under

suboptimal thermal conditions, ectotherms may mount a plastic or reversible acclimation response via a cascade of mechanisms across various levels of biological organization (e.g., change in capillarization, relative ventricular mass, cardiac stroke volume, heart rate, upregulation of genes associated with heat stress, a shift in aerobic:anaerobic metabolism, or change in hematocrit; (Eliason & Stecyk, 2021; Hrubec et al., 2000; Somero, 2010). Ectotherms must possess a capacity to meet the demands of their environment – i.e., those living in highly variable environments must have rapid reversible (i.e., plastic) responses, or display a broad thermal performance in order to cope with a wide range of thermal conditions (Kefford et al., 2022).

As ectotherms experience suboptimal conditions, the ability to mount an acclimation response is supported by energy and nutritional building blocks gained through diet (Moyes & Schulte, 2005). Emerging research suggests that diet has an important impact on the magnitude of the acclimation response in marine ectotherms (Hardison et al., 2021, 2023) and holds both ecological and physiological implications. Ecologically, diet can vary across tidal cycles, across habitats, or seasonally – presenting a challenge for ectotherms as they forage. Considering how ectotherms respond to these suboptimal conditions, it may be beneficial for ectotherms to display a generalist strategy when in search of food. The term "generalist" in regard to feeding strategy implies the consumer has no highly developed preference for a food source, and instead opts for a broad spectrum of prey (Barry et al., 1996; Gerking, 2014; Hoar & Cottle, 1952). This strategy may allow ectotherms to meet energy demands throughout periods of high and low food availability (Selleslagh & Amara, 2015). Viewing fluctuating nutrition on a larger scale, global climate change can shift diet items in their composition, or how much nutrition is available within an item (e.g., shifting ratios of C:N in *Macrocystis*

pyrifera due to warming ocean conditions) (Alton et al., 2020; Birnie-Gauvin et al., 2017; Lowman et al., 2022). Anthropogenic impacts (i.e., pollution, landscape modification, invasive species, overfishing, etc.) are causing further shifts in diet abundance, composition, and nutritional content (Birnie-Gauvin et al., 2017). Physiologically, a change in diet can impact individual performance traits (e.g., swimming speed, cardiac performance, metabolic rate, growth, etc.) and can influence the magnitude of an acclimation response across various traits that can be linked to overall evolutionary fitness (Hardison et al., 2021; McLean & Todgham, 2015). To understand the full capacity of acclimation responses, considering shifting diet along with variable environmental conditions, like temperature, are critical and relatively understudied. This study aims to combine temperature variation and diet type to better understand the thermal acclimation capacity of ectotherms.

Thermal acclimation responses vary across traits, with a complex suite of interacting mechanisms that drive overall thermal tolerance (Rohr et al., 2018). One trait that is thought to be central in declining performance is cardiac function (Eliason & Anttila, 2017); Farrell, 2002). The heart is a critical organ for supporting blood circulation which underpins organismal performance through delivering oxygen, nutrients and hormones throughout the body. Cardiac output increases with warming, which is primarily driven by an increase in heart rate (f_H) (Eliason & Anttila, 2017). Notably, cardiac function is limited and ultimately fails when exposed to high temperatures, perhaps due to an insufficient supply of oxygen to meet demand (Pörtner & Knust, 2007; Pörtner et al., 2017), mitochondrial dysfunction or lessened activation of excitable cells (Ern et al., 2023).

To measure heart function across temperature and quantify species-specific upper thermal limits of cardiac performance, one can use the Arrhenius Breakpoint Temperature

(ABT) test. During an ABT test, the fish is anaesthetized, maximum heart rate (f_{Hmax}) is pharmacologically induced via the intraperitoneal injection of atropine sulphate (to block vagal tone) and isoproterenol (to activate cardiac adrenergic β-receptors). Maximum f_H is induced so as to measure upper thermal limits of heart function as maximum f_H is thought to limit scope for cardiac performance (**Figure 2**) and thus aerobic capacity (**Figure 1**, Eliason et al., 2013; Eliason & Anttila, 2017). Temperature is acutely increased at a rate of 10°C h^{-1} until arrythmia occurs. An acute thermal performance curve is plotted so that several cardiac thermal performance metrics can be extracted (**Figure 3**). (1) The ABT test is founded on the Arrhenius equation, which when applied to the rising phase of the TPC,
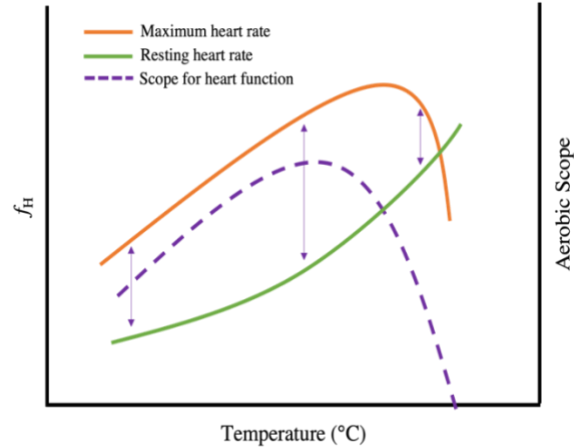
Figure 2. **Maximum vs resting f_H and corresponding scope for f_H.** *The orange line represents maximum heart rate across temperature, while the green line represents resting heart rate. The dashed purple line shows how scope for f_H (scope = maximum – resting) increases, peaks, and promptly declines as temperature increases.*
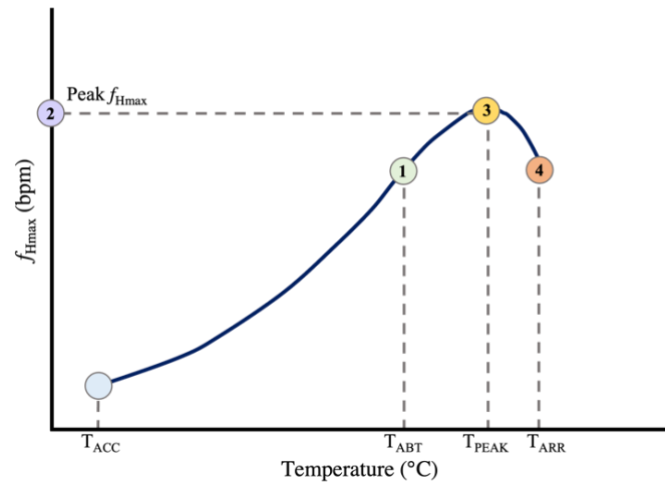
Figure 3. Theoretical diagram generated from the ABT test. Fish begin test at the acclimation temperature (for fluctuating treatments, T_{ACC} can be either the peak or trough of the temperature cycle. (1) T_{ABT} refers to the breakpoint, or the inflection point where the curve decreases from exponential growth and heads towards a plateau. (2) refers to the maximum f_{Hmax} and is linked to (3) T_{PEAK} or the temperature at which rhythmic heart performance peaks. (4) Refers to the temperature at which the heart shifts from rhythmic beating to arrhythmic beating.

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can mathematically identify the inflection point where cardiac performance begins to slow down from the previously exponential increase. This point is known as the Arrhenius Breakpoint Temperature (or T_{ABT}), and it indicates the point at which f_{Hmax} becomes constrained with increasing temperature. T_{ABT} typically corresponds to temperatures within the optimal range for aerobic scope (**Figure 3**) (Anttila et al., 2014, 2013; Casselman et al., 2012; Gilbert & Farrell, 2021; Muñoz et al., 2014; Muñoz et al., 2015). (2) The highest heart rate measured is referred to as peak f_{Hmax}, (3) with the corresponding temperature referred to as T_{PEAK}, at which point there is very little scope for heart rate remaining as resting f_H also increases with increasing temperature (**Figure 2**). Thus, T_{PEAK} occurs at a supraoptimal temperature for aerobic scope (**Figure 1**). (4) The temperature at which arrythmias occur is known as T_{ARR}. At this point, the heart has reached its upper critical temperature for performance and oxygen delivery is severely impaired. (Casselman et al., 2012; Farrell, 2002; Gilbert & Farrell, 2021).

The effects of temperature acclimation on cardiac function have been well studied in fishes (Eliason & Anttila, 2017). TPCs can change their height, position, and shape in response to thermal acclimation (Schulte et al., 2011). Moreover, thermal limits of the heart are known to be plastic and linked to thermal acclimation where increasing acclimation temperatures can increase upper cardiac thermal limits (Anttila et al., 2014; Drost et al., 2016; Safi et al., 2019; Schwieterman et al., 2022). For example, Common Killifish (*Fundulus heteroclitus*) acclimated to 5°C, 15°C or 33°C displayed considerable plasticity in the upper thermal limits of cardiac performance, with the warmest acclimation group maintaining rhythmic beating 10°C higher than the coldest acclimation group (Safi et al., 2019). Thermal breadth – or the range of temperature over which an organism can function, is another useful metric that can

be extracted from a thermal performance curve. A wider thermal breadth suggests a greater span of temperatures for cardiac function, while a narrower breadth suggests a constrained thermal range for performance (**Figure 4**). To quantify TPCs that allow for the most insight into an organism's performance across a suite of environmental conditions, it is critical that lab-based experiments testing organismal performance yield results comparable to natural settings, otherwise results draw inaccurate comparisons. Historically, studies on cardiac thermal tolerance have typically
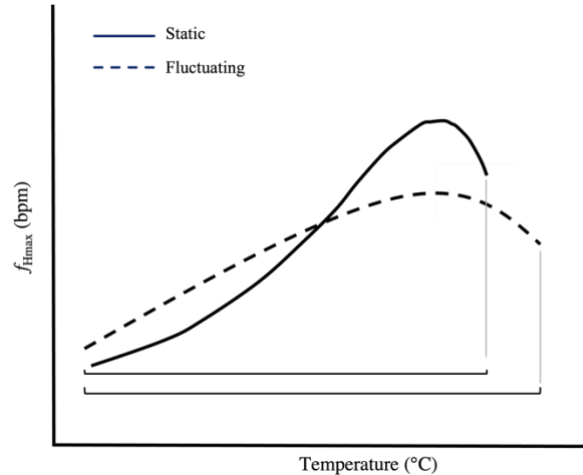
Figure 4. Static vs fluctuating acclimations and comparative thermal breadth. *The solid line represents static acclimation groups, while the dashed line represents fluctuating acclimation group. The bars below the curves correspond to the relative thermal breadth between acclimation regimes. This depiction suggests groups that experience limited thermal variability may reach greater performance across a narrower range of temperature while groups that experience high variability will have greater range over which performance can occur at the cost of decreased performance.*

relied on a static acclimation approach, with findings that are species and system specific (Anttila et al., 2014; Drost et al., 2016; Safi et al., 2019). To date, only one study has examined the effect of fluctuating temperatures on cardiac thermal tolerance in fish (Schwieterman et al., 2022). Recognizing that thermal variability is a ubiquitous phenomenon within natural systems (da Silva et al., 2019; Drake et al., 2017; Kern et al., 2015; Morash et al., 2018; Podrabsky & Somero, 2004) it is critical that *ex-situ* studies incorporate components of this natural variability to ensure findings are applicable beyond the lab.

Considering diet as a factor, emerging studies investigating diet effect on thermal performance have found a varied influence on traits across levels of biological organization

suggesting a complex relationship between diet and thermal acclimation responses (Christen et al., 2020; McKenzie, 2001; McLean & Todgham, 2015; McLeod et al., 2013). At the whole-organism level, Rodgers et al. (2019) found upper thermal limits to be influenced more so by the acclimation temperature rather than food restriction, while specific growth rate (SGR) was more affected by either food restriction or cold acclimation independently. McLean and Todgham (2015) investigated food limitation and thermal physiology in juvenile Dungeness Crab (*Metacarcinus magister*) and found evidence of physiological trade-offs as Dungeness Crabs were found to prioritize stress tolerance over growth when exposed to food limitation and elevated temperatures. At the level of the heart, an omnivorous diet stunted performance when compared to carnivorous diet under 20°C static acclimation in opaleye (*Girella nigricans*), (Hardison et al., 2021). However, few studies have simultaneously examined the impact of diet and fluctuating temperature on organismal performance. One study using *Daphnia* and diel vertical migration as a model found that dietary eicosapentaenoic acid (EPA) supplementation in combination with fluctuating acclimations supported greater population growth rates and offspring production when compared to a static acclimation (Navarro et al. 2019). Additionally, a study by Gresens (1997) looks at the interactive effects of diet and thermal regime on the midge (*Pseudochironomus richardsoni*) and found the combined effect of food quality (diatoms vs algae) and thermal environment to be significantly different than the independent effects of these factors, with specific growth rate varying across thermal regimes but remaining highest for pupae that were fed a diatom diet. Despite the key role of cardiac function determining thermal performance in ectotherms, no known studies to date have measured the effect of ecologically relevant diets and fluctuating temperatures on cardiac thermal performance.

To address this pressing research gap, I selected a tidally influenced salt marsh system as the site to investigate this question. These highly variable environments experience regular and drastic changes in temperature, dissolved oxygen, salinity and food presence/abundance across both daily and seasonal timescales (Teal & Howes, 1996). The ecosystem services salt marshes provide (i.e., serving as a nursery habitat for commercially important species, buffering the coast from erosive surf and absorbing excess nitrogen thereby minimizing harmful eutrophication events) are often overlooked (Johnson et al., 2016) and along the coast of California, these once plentiful but now sparse habitats have begun to decline due to a combination of urban development and sea level rise (Rosencranz, 2017).
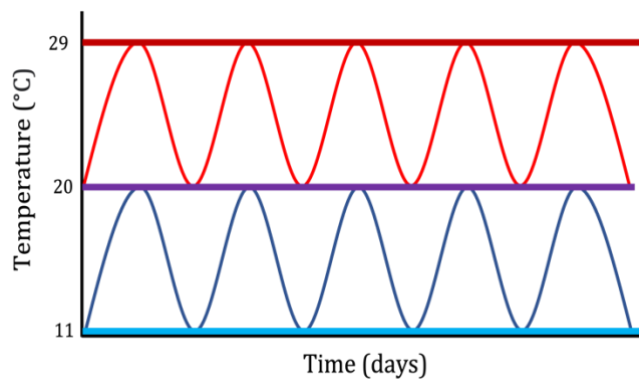
Figure 5. Graphical depiction of the 5 temperature regimes for acclimations: *11°C (light blue line), 11-20°C (dark blue line), 20°C (purple line), 20-*

My study system, the Carpinteria Salt Marsh, is one of the few remaining protected coastal salt marshes along the southern California coastline. Sea water temperatures recorded for this system show daily fluctuations of >10°C, along with a significant seasonal difference across summer temperatures

(maximum: 29-33°C; minimum: 15-20°C) and winter temperatures (maximum: 18-23°C; minimum: 5-11°C) (Kraskura, *unpublished data;* **Figure 5**). Individuals of the California Killifish (*Fundulus parvipinnis*) are year-round residents in this dynamic habitat (Geographic range: Morro Bay, CA through Baja California Sur; Love and Kalman-Passarelli, 2020). Additionally, the congeneric Atlantic Killifish (*Fundulus heteroclitus*) has been used as a model system for studying acclimation responses and thermal plasticity (Dhillon & Schulte,

2011; Fangue et al., 2006; Healy et al., 2017a; Healy et al., 2017b; Healy & Schulte, 2012). *F. parvipinnis* are schooling fish that are mostly found within estuarine systems in large densities. Small in nature, individuals of this species reach a maximum total length of 10.8 cm (Love, 2011). *F. parvipinnis* is an annual species, where breeding season occurs in late fall and peak reproductive output is late spring/early summer (Fritz, 1975; Pérez-España et al., 1998). *F. parvipinnis* utilize a generalist feeding strategy and can shift the items they consume based on the type and amounts of food available – a strategy that may be useful in periods when food is limited (i.e., during low tide), or during times when an environmental stressor is present (i.e., warming or hypoxia) (Sokolova et al., 2012).

The overall objective of this thesis was to determine how thermal variability and diet type affect the cardiac thermal performance of the California killifish. To test this, I acclimated fish to static and fluctuating conditions across temperatures that are commonly experienced in the Carpinteria Salt Marsh and provided either a restricted, single-item diet where only one food item was available or a broad diet that consisted of a diverse assemblage of food items. After acclimating fish to treatment for 3 weeks, ABT tests were performed to examine thermal tolerance and cardiac performance. Specifically, this study was designed to answer the following three questions:

(i) How does a static vs fluctuating acclimation regime affect upper thermal limits of cardiac performance? I hypothesized that a) warm acclimation would produce higher upper thermal limits (e.g., higher T_{ABT}, T_{PEAK} and T_{FAIL}) compared to cold acclimation and b) acclimation to fluctuating temperatures would produce a broad thermal performance curve compared to static thermal exposures (da Silva et al., 2019; Schwieterman et al., 2022)

(ii) How do solo vs broad diets affect upper thermal limits of cardiac performance under fluctuating acclimation? I hypothesized that a broader diet would provide more nutrition to support thermal stress responses and would result in increased upper thermal limits (i.e., higher T_{PEAK} and higher peak f_{Hmax}) compared to the simple diet treatment.

(iii) How do upper thermal limits of cardiac performance vary when tested at the trough vs peak of fluctuating temperature cycle? I anticipated starting temperature would drive the cardiac thermal performance responses, with tests starting at 11°C yielding thermal limits (T_{ABT}, T_{PEAK}, T_{FAIL}) similar to 11°C static, while thermal limits for tests starting at 20°C performing would be similar to 20°C static.

The non-linear relationship between temperature and biological processes suggests more research is needed to better understand cardiac function in natural systems. Results from this study will further our knowledge of the complex relationship between diet and temperature and how these factors interact to influence organismal function. From this, we can better understand the underlying mechanisms that influence thermal tolerance – which will be essential as ecosystems face a warming and increasingly variable future.

METHODS

Fish Collection and Husbandry

Adult *F. parvipinnis* were collected from the Carpinteria Salt Marsh, Santa Barbara County, CA (34.401177° N, 119.536432° W) between late May 2022 and early June 2022 (CDFW permit #: S-213430003-21346-001) using minnow trapping and seining. Once collected, fish were transferred to a 410 L tank at the UCSB seawater lab and maintained at ambient seawater temperature (13-20°C) for at least 48 h before being transferred to 57 L experimental tanks. Pre-experiment diets consisted of brine shrimp (brineshrimpdirect.com) provided *ad libitum*. Fish were fed twice a day at 9:30 and 15:30 local time. Tanks were siphoned daily to remove uneaten food from the tanks. All protocols and personnel for this experiment were approved by the University of California, Santa Barbara's Institutional Animal Care and Use Committee (IACUC).

Acclimation and Treatments

After completing quarantine/lab acclimation, *F. parvipinnis* were randomly assigned to one of 7 treatments (**Table 1**) with 3 replicates per treatment (57 L tanks, 13 fish per tank) for at least 3 weeks. The five experimental temperature regimes chosen include: 11°C, 11-20°C, 20°C, 20-29°C, 29°C (**Figure 5**). These temperature values were selected as they are ecologically relevant: the 11-20°C range is representative of a winter regime, while the 20-29°C group serves as a representative of a summer regime as determined by data collected within the Carpinteria Salt Marsh using HOBO TidBiT loggers (Onset Computer Corporation, Bourne, MA) deployed over a two-year period, 2019-2021) (**Figure 6**).

Individuals in the static temperature treatments were fed only one diet type (solo diet of brine shrimp, see diet treatment details below), while individuals held under one of the two

fluctuating temperature regimes were fed one of two different diets (single-item diet or broad

diet, see diet treatment details below).

Table 1. Configuration of the 7 treatments. "Treatment" is used to aide in identifying the various treatments, however these terms are not used throughout this thesis. Rather, the acclimation regime, temperature and diet are used to refer to the various treatments.

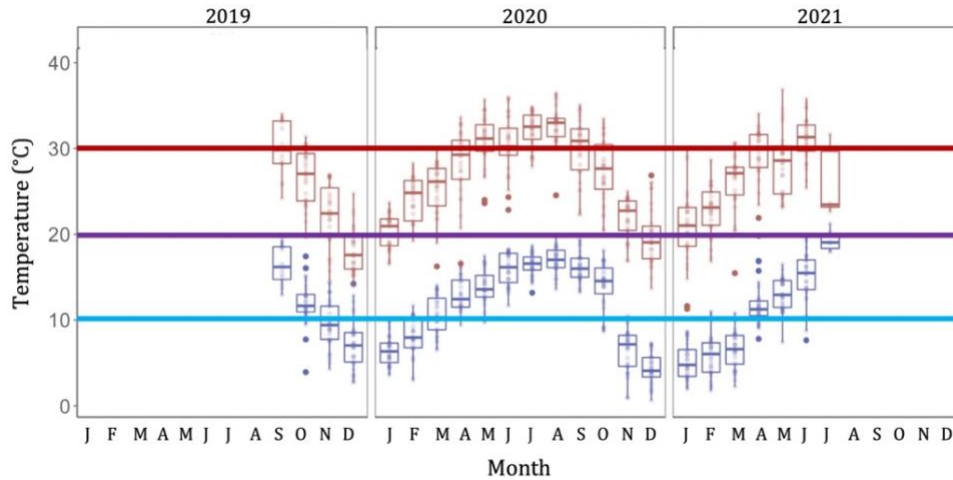| Treatment #	Acclimation regime	Temperature	Diet
Treatment 1	Static	11°C	Single item
Treatment 2	Fluctuating	11-20°C	Single item
Treatment 3	Fluctuating	11-20°C	Broad
Treatment 4	Static	20°C	Single item
Treatment 5	Fluctuating	20-29°C	Single item
Treatment 6	Fluctuating	20-29°C	Broad
Treatment 7	Static	29°C	Single item

Figure 6. Seasonal variability of maximum and minimum temperatures*. Temperature data from
a HOBO logger (Onset Computer Corporation, Bourne, MA) that was submerged in a tidal channel
within the Carpinteria Salt Marsh from September 2019 through July 2021. Red boxplots represent
average daily maximum temperatures within each month and blue boxplots represent average daily
minimum temperatures within each month. The blue line at 11°C represents the static coldest
acclimation temperature. The purple line at 20°C represents the static medium acclimation
temperature. The red line at 29°C represents the warmest static acclimation temperature. Together,
this plot provides a foundation for the range of temperatures chosen for this study.*

On a daily timescale, these data show that it is not uncommon for the amplitude of daily

temperature fluctuations to reach 9-10°C (**Figure 7**). Thus, fluctuating treatments experienced

9°C changes in temperature to represent conditions experienced in the marsh. Each

experimental tank was plumbed with

three sources of seawater: chilled

seawater (10°C), heated seawater (20°C)

and ambient seawater (temperature

varied with ambient ocean conditions).

To simulate a diurnal tidal cycle, 800W

aquarium dip heaters (Finnex, McCook,

IL, USA) were affixed to either side of
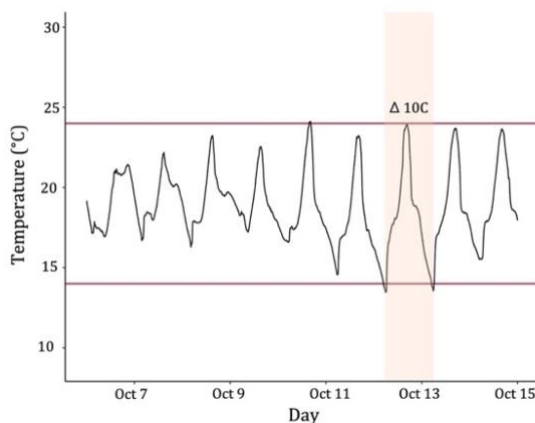
the 57 L tanks and interfaced with digital

***Figure 7. Daily temperature fluctuations within the
Carpinteria Salt Marsh.*** *These data illustrate the
range of daily fluctuations that varied by as much as
5°C and 10°C across 9 days in October 2021. The
shaded region outlines a single tidal cycle.*

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outlet timers (Nearpow, Amazon, Seattle, WA) to schedule the daily increase and decrease of temperatures within the tanks. While the Carpinteria Salt Marsh experiences a semi-diurnal tide, our experiment was limited to recreating a single high tide and low tide per day due to logistical constraints. Tank temperature was monitored 1-2 times daily using a Digi-sense Traceable Singe RTD thermometer (Cole Palmer, IL, USA) and dissolved oxygen content was measured using an OxyGuard handy Polaris 2 dissolved oxygen meter (OxyGuard International A/S,
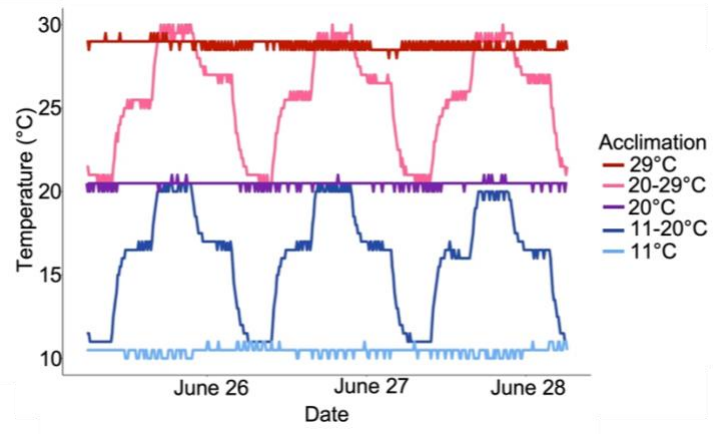
Figure 8. Temperature readings from tanks illustrating 5 acclimation temperatures over 3 days. Colors represent different acclimation temperatures. Warming phase began at 9:30 AM and ended at 3:30 PM. Cooling phase began at 3:30 PM and ended at 9:30 PM. (iButtons, Maximum Integrated, San Jose, CA)

Farum, Denmark). Real-time and retroactive temperatures were collected via Inkbird (London, England) and iButton (Maximum Integrated, San Jose, CA), respectively (**Figure 8**). Water oxygen was maintained at >80% air saturation and fish were held on a 14:10 light:dark cycle throughout the study. Visual assessments of animal health occurred at least twice, but often 3 or more times per day. As collections coincided with the peak/end of the reproductive cycle for these annual fish, signs of disease were sometimes present during acclimations. Fish that were found to be infected were immediately removed from the experiment and humanely euthanized. All fish used in testing were visually healthy and showed normal behavior.

Diet Treatments

Two diet treatments were used for this study: a single-item diet consisting of solely brine shrimp (*Artemia sp.*) and a second multi-item diet consisting of brine shrimp, bloodworms *(Chironomid sp.)* and arctic copepods (*Calanus sp.)*. Below is a breakdown of diet usage across my three hypotheses:

H1 - Static vs. fluctuating acclimation: Single-item diet.

H2 - Diet & fluctuating acclimation: Single-item and broad diets.

H3 - Peak vs. trough of fluctuating acclimation cycles: Single-item and broad diets.

Diet items were selected based on composition (protein vs fat vs fiber) and aimed to serve as an analog for common prey items consumed by *F. parvipinnis* based on food web studies conducted within the Carpinteria Salt Marsh and published life history information on the California killifish (Brooks, 1999; Love, 2011). Brine shrimp (*Artemia sp.*) were used as a proxy for shrimp-like invertebrates, bloodworms (*Chironomid sp.*) served as a proxy for polychaete-like prey items, and arctic copepods (*Calanus sp.*) served as a proxy for zooplankton while providing a source of essential marine lipids (proximate analysis shown in **Table 2)**. The broad diet treatment was composed of 60% brine shrimp: 20% blood worms and 20% Arctic copepods. Fish in the warmer treatments required more food (i.e., due to elevated metabolic rates) than fish in the colder treatments. Each tank was fed twice daily: 11°C – 15 g; 11-20°C – 50 g, 20°C – 60 g, 20-30°C – 75 g and 29°C – 90 g.

Nutrient category	Brine shrimp	Bloodworms	Arctic copepods
Moisture	92.2%	92.2%	83.3%
Protein	4.3%	4.3%	9.2%
Ash	1.5%	1.5%	NA
Fat	0.3%	0.2%	5%
Other	1.7%	1.3%	NA
Total	100.0%	99.5%	97.5%

Marine lipids such as Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) are essential omega-3 fatty acids (FAs) that cannot be synthesized by the body and can only be acquired through diet. These FAs have been shown to support membrane integrity under colder temperatures (Christen et al., 2020) and may provide a key function in cardiac thermal tolerance (McKenzie, 2001). As these essential marine FAs are not available in freshwater-reared brine shrimp, it was critical to include arctic copepods in the broad diet treatment to not only satisfy the fibrous/chitinous component for a diverse diet composition, but to provide a source of marine FAs to contrast the single-item diet treatment that would be lacking in these key FAs.

Arrhenius Breakpoint Temperature (ABT) test

All tests began at acclimation temperatures. For fluctuating temperatures, individuals were tested in both the morning (at the lowest temperature they experienced) and the evening (at the highest temperature they experienced) (**Figure 9**). Fish were acclimated at least 21 days prior to testing.

To measure cardiac thermal tolerance and performance in *F. parvipinnis*, we employed the Arrhenius Breakpoint Temperature (ABT) test as described by Casselman et al. (2012). Two

fish from each tank were randomly selected and anesthetized in 0.125 g L^{-1} MS-222 buffered with 0.25 g L^{-1} NaHCO$_3$ at the temperature that matched the assigned treatment temperature at that time. Once fish were unresponsive to tactile stimulus, they were placed ventral side up and secured to an experimental sling in a maintenance anesthetic solution that consisted of 0.1 g L^{-1} MS-222 buffered with 0.2 g L^{-1} NaHCO$_3$ in seawater at the tank temperature. Custom

Figure 9. Diagram of testing for Hypothesis 3. Testing at "Peak" occurred at 16:00 as tank temperatures reached 20°C. Testing at "Trough" occurred at 09:00 as tank temperatures reached 11°C.

made mouthpieces ensured continuous gill irrigation throughout test. Once secure and stable in the experimental sling, a small hole was created anterior to the pectoral girdle above the heart by a 32-gauge needle. This allowed for a Stainless-Steel Needle Tip Electrode (ADInstruments Inc., Colorado Springs, CO, USA) to be inserted under the skin to detect an ECG signal. The resulting ECG signal was amplified using a Dual Bio Amp and Powerlab data acquisition system (ADInstruments Inc.) and filtered (filters: 60 Hz Notch filter; mains filter; low pass: 2 kHz; high pass: 10 Hz; range: 2 mV).

After a 15-minute equilibration period, atropine sulfate was injected intraperitoneally (1.2 mg kg^{-1} in 0.9% NaCl) to block vagal tone (Safi et al., 2019) and induce $f_H$max. Isoproterenol (4 ug kg^{-1} in 0.9% NaCl) is commonly used to maximally stimulate β-adrenoreceptors, however preliminary tests with isoproterenol injections showed inconsistent results (either very little increase in f_H, a decrease in f_H or no effect) after 4-hour drug dosage trial runs and

was, therefore, removed from our ABT protocol. Any fish that did not respond to the drug injections or for which experimental error occurred (e.g., water pump failure, organ puncture upon injection, etc.) were removed from the analysis (n=65; 50% of total trials). Final n-values were 6-8 per treatment.

After a 30-minute period following the atropine sulfate injection, water temperature was increased 1°C every 6 minutes using a Polystat recirculating heater/chiller (Cole-Palmer, Vernon Hills, IL, USA) and stainless-steel coil. At each 1°C interval, f_{Hmax} and temperature were allowed to stabilize for 30 seconds to record a value for f_{Hmax}. I repeated this process until the onset of cardiac arrhythmia or a sudden bradycardia (decline of f_H) which marks the point of cardiac failure (T_{FAIL}). As *F. parvipinnis* exhibited variable markers of cardiac failure (i.e., some displayed arrhythmias after sudden bradycardia while other displayed solely arrhythmias), I developed a metric that marks the moment of cardiac failure, denoted as T_{FAIL}. I will use this metric for the remainder of this thesis to describe cardiac collapse instead of T_{ARR}. Once fish had been removed from the testing sling, the fish was euthanized, the ventricle and gonads were removed and weighed to determine relative ventricular mass [RVM = (heart mass/body mass) x 100] and gonadosomatic index [GSI = (gonad mass/body mass) x 100]. Morphology metrics can be found in **Table 3**.

Table 3. Morphology metrics for all fish tested across all 3 hypotheses. *Tests at "Peak" temperature for 20-29°C acclimation groups are excluded due to low sample sizes (means ± S.E.M. for all test metrics across treatments).*

Acclimation Temperature	Diet	Testing timepoint	n	Sex ratio	Body mass (g)	Total length (cm)	Gonadosomatic Index (GSI; %)	Relative ventricular mass (%)
11°C	Single	-	6	2F:4M	5.3 ± 0.7	7.2 ± 0.3	F: 10.8 ± 4.6; M: 2.0 ± 0.1	0.1 ± 0.006
11°C – 20°C	Single	Peak	6	3F:3M	6.6 ± 0.7	7.4 ± 0.4	F: 13.2 ± 4.5; M: 2.3 ± 0.6	0.07 ± 0.009
11°C – 20°C	Single	Trough	8	2F:6M	8.1 ± 1.0	8.1 ± 0.3	F: 24.4 ± 5.8; M: 2.0 ± 0.1	0.09 ± 0.005
11°C – 20°C	Broad	Peak	7	2F:5M	7.8 ± 1.1	8.0 ± 0.3	F: 16.7 ± 6.2; M: 1.3 ± 0.2	0.09 ± 0.004
11°C – 20°C	Broad	Trough	8	4F:4M	6.9 ± 0.8	7.6 ± 0.3	F: 12.8 ± 2.1; M: 1.2 ± 0.2	0.08 ± 0.004
20°C	Single	-	6	3F:3M	8.8 ± 1.2	8.0 ± 0.4	F: 19.9 ± 2.9; M: 2.0 ± 0.1	0.08 ± 0.005
20°C – 29°C	Single	Trough	6	4F:2M	9.4 ± 1.5	8.0 ± 0.5	F: 9.3 ± 1.0; M: 2.1 ± 0.8	0.2 ± 0.1
20°C – 29°C	Broad	Trough	8	4F:4M	8.6 ± 0.8	7.9 ± 0.2	F: 8.5 ± 2.5; M: 1.7 ± 0.1	0.08 ± 0.009
29°C	Single	-	8	4F:4M	8.2 ± 1.1	7.8 ± 0.4	F: 7.0 ± 2.0; M: 1.2 ± 0.2	0.08 ± 0.009

Data analysis for ABT tests

I calculated f_{Hmax} for each temperature increment from at least 15 continuous seconds of measurements using LabChart software (www.adinstruments.com). Peak f_{Hmax} is the highest maximum heart rate recorded across the entire test, and the corresponding temperature at which peak f_{Hmax} occurs is denoted as T_{PEAK}. As *F. parvipinnis* arrhythmias oftentimes occurred after a marked decline in cardiac performance, I used T_{FAIL} as the metric to identify the moment of drastic decline in cardiac performance (created by either a missing QRS peak, or a sudden bradycardia marking a notable decrease in effective cardiac function). The breakpoint

temperature (T_{AB}) was quantified by running Arrhenius breakpoint tests on f_{Hmax} for the rising phase of the TPC using the *segmented* package in R (version 4.1.2).

Statistical analysis

Data are presented as mean ± SEM unless otherwise stated and significance levels were set to $p < 0.05$. All statistical tests and visualizations were run using R. All data were visually and statistically assessed for normality using Shapiro-Wilk tests and q-q plots, and for heteroscedasticity using Levene's test. I used One-way ANOVA to test for differences in morphometrics across treatments (body mass, total length and RVM). Analyses of Covariance (ANCOVAs) were run on all hypotheses to assess whether body size (using length as the co-variate) played a significant role in cardiac thermal tolerance. All ANCOVAs yielded non-significant results for body size as a covariate and hypothesis testing proceeded with the use of ANOVA (Analysis of Variance). Information on statistical analyses can be separated into the three following hypotheses:

H1 – Static vs fluctuating acclimation

I used a One-way ANOVA to test for differences in acclimation temperature for the following four test metrics – T_{AB}, T_{PEAK}, T_{FAIL}, peak f_{Hmax} (**Appendix 1**). As ABT tests for the 29°C Static treatment began above the breakpoint, this group was excluded from Arrhenius breakpoint analyses. Differences in thermal breadth [i.e., range between starting test temperature (T_{ACC}) and the temperature of cardiac failure (T_{FAIL})] for H1 were statistically analyzed using a one-way ANOVA. A *post-hoc* Tukey HSD was used to identify significant pairwise interactions.

H2 – Diet and fluctuating acclimation

I used a Two-way ANOVA was used to test for differences in acclimation temperature and diet for the following four test metrics (T_{AB}, T_{PEAK}, T_{FAIL}, peak f_{Hmax}) (**Appendix 2**). A *post-hoc* Tukey HSD was used to identify significant pairwise interactions.

H3 – Peak vs. trough of fluctuating acclimation

I also used a Two-way ANOVA to test for differences in timepoint (peak vs trough of fluctuating acclimation cycle) and diet on T_{ABT}, T_{PEAK}, T_{FAIL}, peak f_{Hmax} (**Appendix 3**). Differences in thermal breadth [i.e., range between starting test temperature (T_{ACC}) and the temperature of cardiac failure (T_{FAIL})] for H3 were statistically analyzed using a Two-way ANOVA on time in fluctuation cycle and diet. A *post-hoc* Tukey HSD was used to identify significant pairwise interactions.

RESULTS

Morphology metrics

Fish mass, total length and relative ventricular mass did not differ across treatments (total length p = 0.84, fish mass p = 0.15, RVM p = 0.38; One-Way ANOVA, **Appendix 4**).

Thermal Performance Curves

TPC's displayed similar trends across treatments with f_{Hmax} increasing under acute warming until a peak in performance was reached at T_{PEAK} (**Figure 10**). Sample size for T_{FAIL} is n<6 for several treatments (**Table 4; Table 6**), however I've included a TPC using means across temperatures up to T_{FAIL} to convey the trend where performance (f_{Hmax}) reaches a peak, followed by a sharp decline (**Figure 11**).
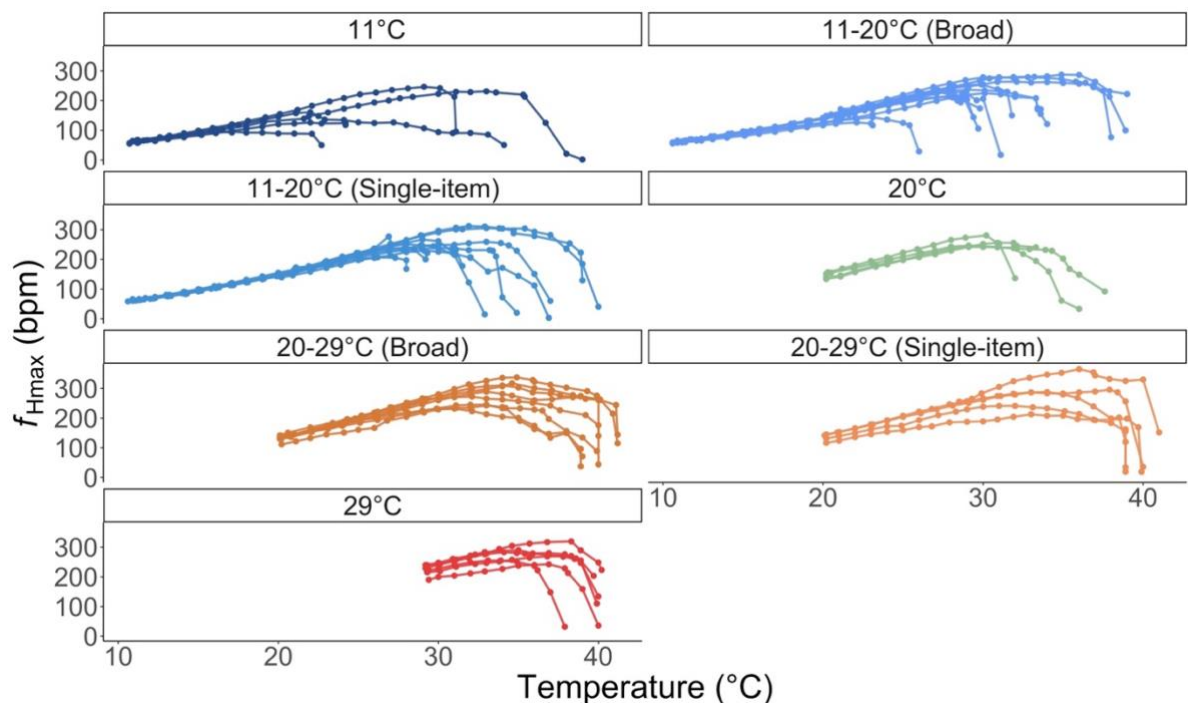

Figure 10. Thermal performance curves separated by treatment. *TPCs show f_{Hmax} for all fish from T_{ACC} to T_{FAIL}. Note, individual fish with missing endpoints were excluded from this plot. All fluctuating groups were tested at the trough of the acclimation cycle. N = 6-8 per treatment.*
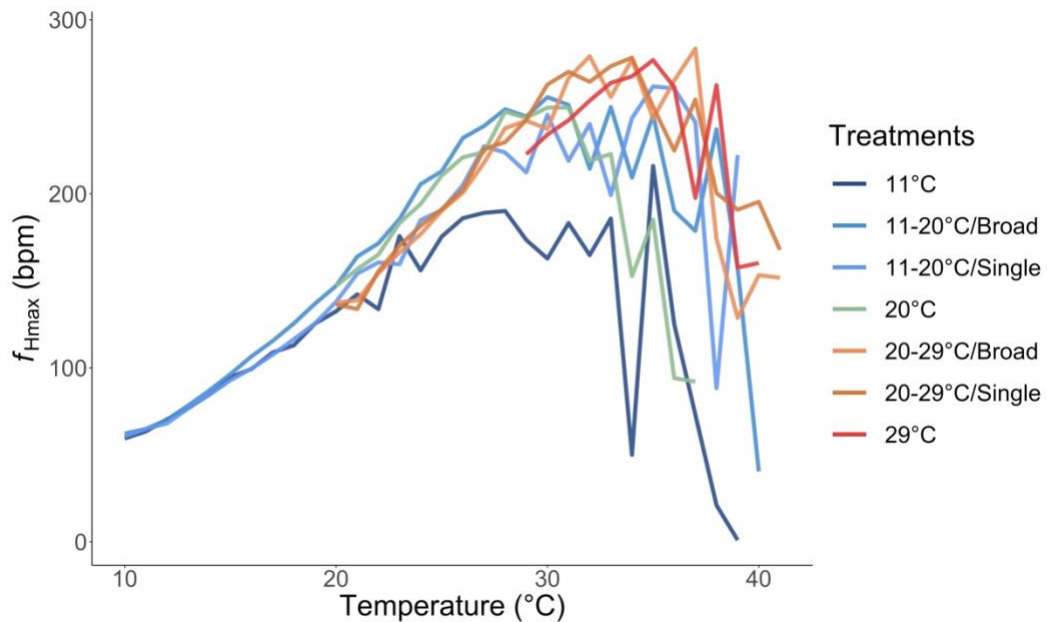
Figure 11. Combined thermal performance curves for all seven treatments. TPCs show f_{Hmax} for all fish from T_{ACC} to T_{FAIL}. All fluctuating groups were tested at the trough of the acclimation cycle. N = 6-8 per treatment.

Hypothesis 1: Static vs fluctuating temperatures

All metrics for upper thermal limits of cardiac performance (peak f_{Hmax}, T_{ABT}, T_{PEAK} and T_{FAIL}) increased steadily with increasing acclimation temperatures (**Figure 12; Table 4**). In general, across metrics, the fluctuating temperature treatments did not significantly differ from their corresponding low and high static temperature treatments [e.g., 11-20°C did not differ statistically from 11°C static (T_{ABT}: p = 0.8; T_{PEAK}: p = 0.06) or 20°C static (T_{ABT}: p = 0.3, T_{PEAK}: p = 0.9, T_{FAIL}: p = 0.9, peak f_{Hmax}: p = 0.9) and 20-29°C did not differ from 20°C static (T_{ABT}: p = 0.8, T_{PEAK}: p = 0.8, T_{FAIL}: p = 0.2, peak f_{Hmax}: p = 0.9) or 29°C static (T_{ABT}: NA, T_{PEAK}: p = 0.7), T_{FAIL}: p = 0.9, peak f_{Hmax}: p = 0.8). The notable exceptions were that the 11-20°C fluctuating treatment had significantly higher T_{FAIL} (p = 0.02) and f_{Hmax} (p = 0.006) compared to the 11°C static treatment. Interestingly, peak f_{Hmax} in the 11-20°C group was significantly higher than 11°C static and did not differ from the treatments maintained at 20°C

and above [20°C (p =0.9), 20-29°C (p = 0.9) and 29°C (p = 0.9)] **(Figure 13; Table 4)**. The 11-20°C acclimation supported an impressive 45% increase in peak f_{Hmax} compared to 11°C (p = 0.006). Comparing only static temperature treatments, the cold (11°C) treatment was significantly lower than the warm (29°C) treatment for all metrics (except T_{ABT}, which was not determined at 29°C) (T_{PEAK}: p = 0.00002; T_{FAIL}: p = 0.0003; peak f_{Hmax}: p = 0.002). However, the 20°C static treatment did not differ from the 29°C static treatment for any metrics (T_{PEAK}: p = 0.1; T_{FAIL}: p = 0.2; peak f_{Hmax}: p = 0.9). The two fluctuating treatments did not differ in any of the test metrics, with the exception of a higher T_{ABT} in the warm fluctuating group (T_{PEAK}: p = 0.4; T_{FAIL}: p = 0.3; peak f_{Hmax}: p = 0.9).
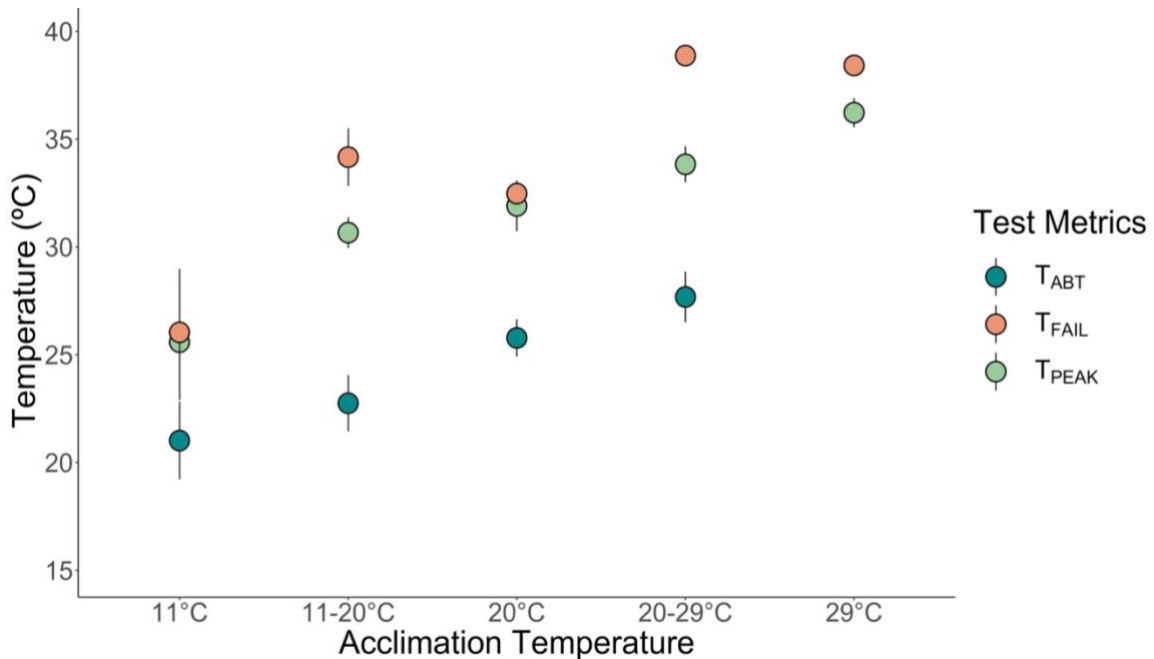
Figure 12. Upper thermal tolerance metrics for Hypothesis 1. *Arrhenius breakpoint temperature (T_{ABT}), peak temperature (T_{PEAK}) and failure temperature (T_{FAIL}) are shown. T_{ABT} is excluded from 29°C as this acclimation temperature was beyond the Arrhenius breakpoint temperature. All Arrhenius breakpoint temperature tests for fluctuating groups began at the trough temperature.*
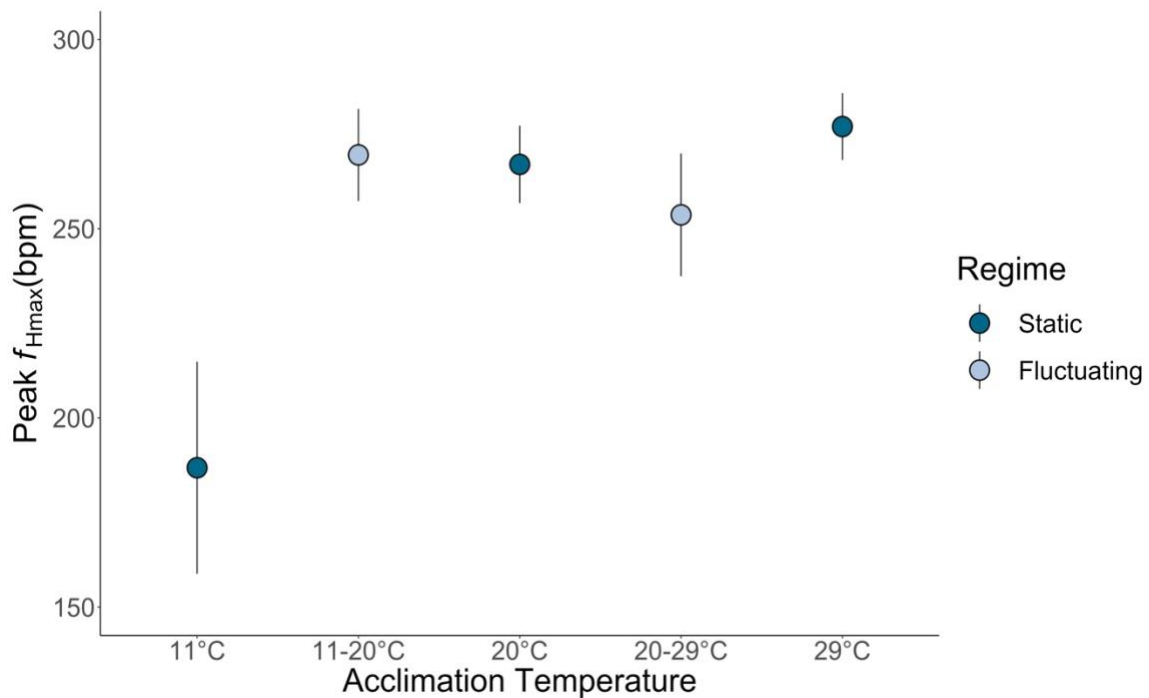
Figure 13. Peak maximum heart rate (f_{Hmax}) for Hypothesis 1. *Testing began at the trough temperature for all fluctuating treatments). The dark blue circles represent "static" while the light blue circles represent "fluctuating" treatments.*

Thermal breadth

The distance between starting test temperature (i.e., acclimation temperature) and T_{FAIL} was significantly larger in fluctuating groups compared to their corresponding static groups (**Table 4**), with the exception of the warm fluctuating group which did not differ from the 20°C static groups (p = 0.2) (**Figure 14**). The static 29°C treatment did not significantly differ from either the static 20°C (p=0.7) or the static 11°C (p= 0.2). The two fluctuating groups did not significantly differ in thermal breadth (p=0.4).

Table 4. Mean ± SEM of ABT test metrics for Hypothesis 1. *Fluctuating groups were only tested at the trough of temperature cycle (i.e., 11°C for cold fluctuating groups and 20°C for warm fluctuating groups). Test temperature refers to the temperature the ABT test begins. Differing letters indicate statistically significant differences across treatments (p<0.05).*

Acclimation temperature	11°C	11-20°C	20°C	20-29°C	29°C
Regime	Static	Fluctuating	Static	Fluctuating	Static
Test Temperature	11°C	11°C	20°C	20°C	29°C
n	6	8	6	6	8
n (T_{FAIL}; Thermal Distance)	5	6	4	4	6
T_{ABT} (°C)	21.0 ± 1.8^a	22.8 ± 1.3^a	26.1 ± 0.9^{ab}	27.8 ± 1.0^b	NA
T_{PEAK} (°C)	25.6 ± 2.7^a	30.7 ± 0.7^{ab}	31.7 ± 1.1^{bc}	33.8 ± 0.8^{bc}	36.2 ± 0.7^c
T_{FAIL} (°C)	26.0 ± 3.0^a	34.2 ± 1.4^b	32.5 ± 0.6^b	38.9 ± 0.2^b	38.4 ± 0.5^b
Peak f_{Hmax} (beats min^{-1})	186.8 ± 28.0^a	269.5 ± 12.2^b	267.0 ± 10.2^b	254.0 ± 16.0^b	277.0 ± 8.8^b
Thermal distance	15.0 ± 3.2^{ab}	23.2 ± 1.5^c	12.5 ± 0.7^{ab}	18.9 ± 0.3^{bc}	9.4 ± 0.5^a
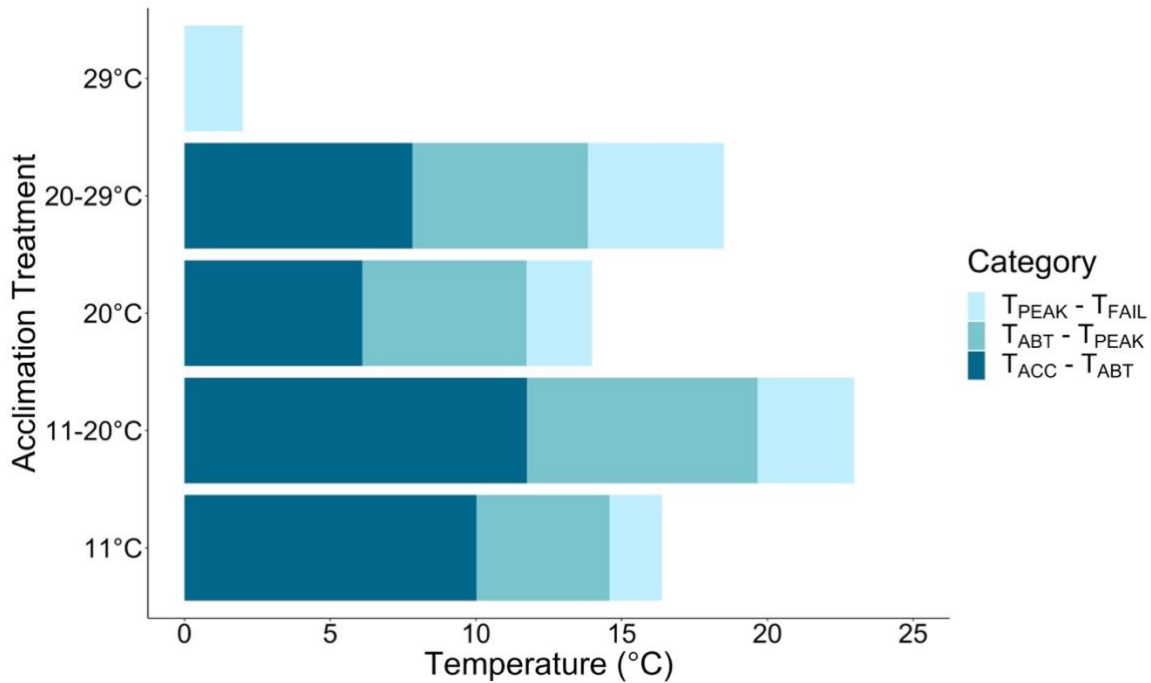
Figure 14. Thermal breadth between thermal tolerance metrics from ABT tests on F. parvipinnis acclimated to one of five acclimation conditions. Thermal distance for "$T_{ACC} - T_{ABT}$" and "$T_{ABT} - T_{PEAK}$" are absent from this plot for 29°C as fish were acclimated to temperatures above the breakpoint and T_{ABT} could not be measured.

Hypothesis 2 – Diet and fluctuating temperature

Upper thermal limits for cardiac performance increased with acclimation temperature, however diet was found to have a mixed effect based on temperature (**Figure 15; Table 5**). Surprisingly, in the cold fluctuation group (11-20°C), all metrics were significantly lower in the broad diet treatment compared to the single-item diet treatment (T_{ABT}: p = 0.0008; T_{PEAK}: p = 0.002; T_{FAIL}: p = 0.04; f_{Hmax}: p = 0.004). By contrast, no difference in cardiac thermal performance metrics was found between the broad and single-item diet treatments in the warm fluctuation (20-29°C) group (T_{ABT}: p = 0.9; T_{PEAK}: p = 0.1; T_{FAIL}: p = 0.9; f_{Hmax}: 0.6). In the 11-20°C group, peak f_{Hmax} for fish fed a broad diet was significantly lower than fish fed a single-item diet (p= 0.004), conversely, fish fed a single-item diet were able to increase peak

f_{Hmax} by 38%. Moreover, fish fed a single-item diet in the 11-20°C group had similar peak f_{Hmax} to 20-29°C fish for both the broad diet (p= 0.9) and the single-item diet (p = 0.9) (**Figure 16**).
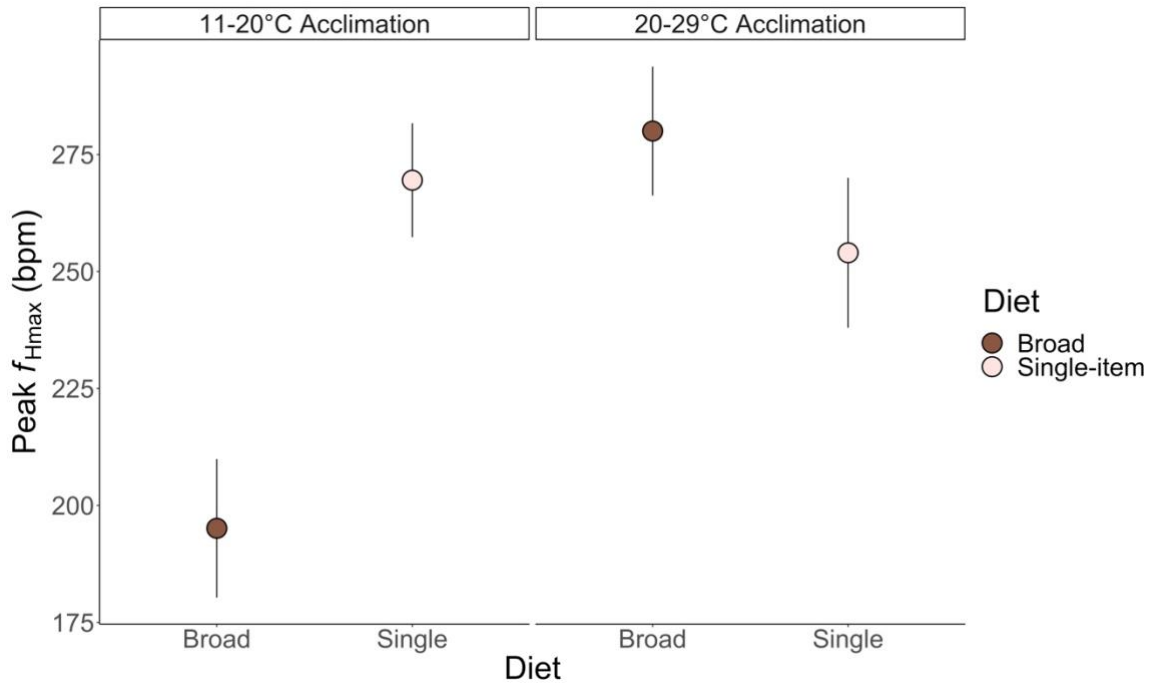
Figure 16. Peak maximum heart rate (f_{Hmax}) Hypothesis 2. Testing began at the trough temperature for all fluctuating treatments. The dark pink circles represent a "broad" diet while the light pink circles represent a "single item" diet.
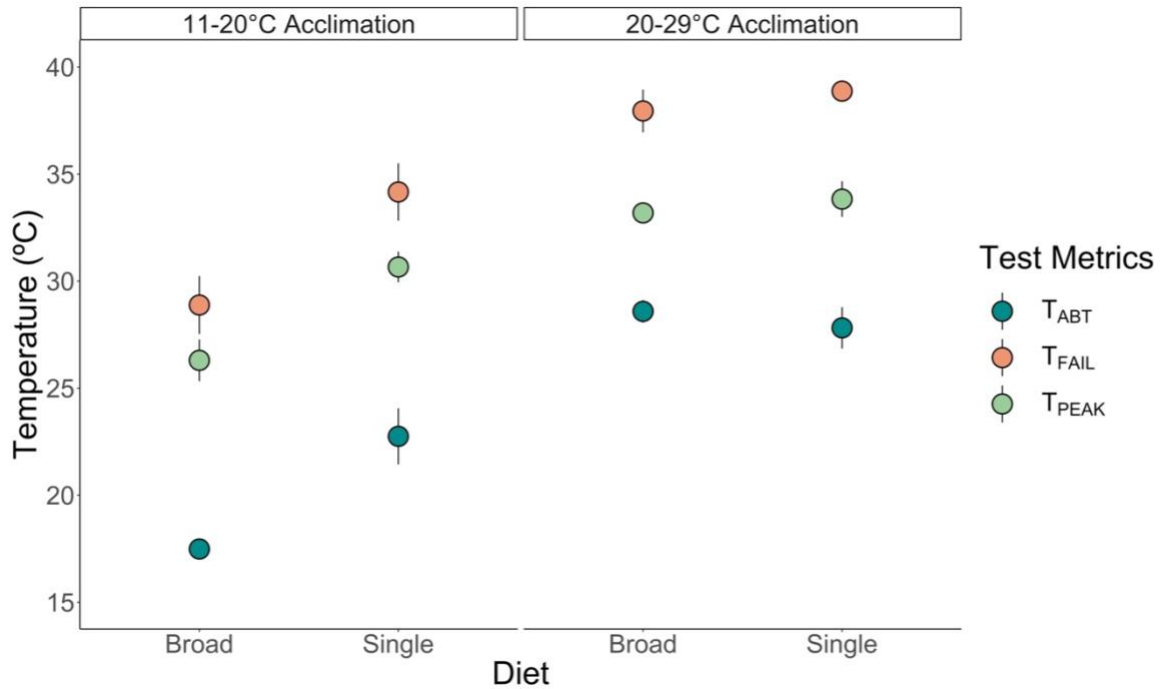
Figure 15. Thermal tolerance metrics for Hypothesis 2. *Plot is split into panels that represent winter temperatures (11-20°C) and summer temperatures (20-29°C). Within each panel, groups are split between diet treatments All ABT tests for fluctuating groups began at the trough temperature. Mean ± SEM is presented.*

Table 5. Mean ± SEM of ABT test metrics for Hypothesis 2. *Fluctuating groups were only tested at the trough of temperature cycle (i.e., 11°C for cold fluctuating groups and 20°C for warm fluctuating groups). Differing letters indicate statistically significant differences across treatments*

Acclimation temperature	11-20°C		20-29°C	
Diet	Single	Broad	Single	Broad
n	8	8	6	8
n (T_{FAIL})	6	7	4	8
T_{ABT}	22.8 ± 1.3^a	17.5 ± 0.3^b	27.8 ± 1.0^c	28.6 ± 0.5^c
T_{PEAK}	30.7 ± 0.7^b	26.3 ± 1.0^a	33.8 ± 0.8^c	33.2 ± 0.5^{bc}
T_{FAIL}	34.2 ± 1.4^b	28.9 ± 1.4^a	38.9 ± 0.2^b	38.0 ± 1.0^b
Peak f_{Hmax}	269.5 ± 12.2^b	195.1 ± 14.8^a	254.0 ± 16.0^{ab}	280.0 ± 13.8^b

Hypothesis 3 – Thermal tolerance at peak vs trough of temperature fluctuations

Hypothesis 3 was only examined using the 11-20°C acclimation group. The impact of diet on upper thermal limits of cardiac performance varied on whether the test occurred at the trough vs the peak of the fluctuating temperature range (**Figure 17**). Interestingly, while upper thermal limits were lower in the broad compared to the single-item diet treatments when tested at the trough (11°) of the temperature cycle (see results from hypothesis 2 above and Figure 14, Table 6), the upper thermal limits for fish tested at the peak of the temperature cycle were statistically indistinguishable between broad and single-item diet treatments (T_{ABT}: p = 0.9; T_{PEAK}: p = 0.5; T_{FAIL}: p = 0.6; f_{Hmax}: p = 0.9). As expected, all cardiac metrics with the exception of T_{FAIL} were significantly higher when tested at the peak vs the trough of the cycle for the broad diet treatment (T_{ABT}: p = 0.0000009, T_{PEAK}: p = 0.007, peak f_{Hmax}: p = 0.01; **Figure 17; Table 6**). In the single-item diet treatment, fish tested at the peak of the cycle had decreased thermal breadth compared to those tested at the trough (p = 0.0004) (**Figure 18; Table 6**). Moreover, thermal breadth did not differ for the broad diet treatment between fish tested at the peak vs trough (p = 0.2) of the thermal cycle. In the broad diet group, peak f_{Hmax} was 31% higher when tested at peak temperature compared to trough (p = 0.01). Peak f_{Hmax} in fish tested at peak in the broad diet group did not differ from fish fed a single-item diet at either the peak (p= 0.9) or trough (p = 0.8) temperatures (**Figure 19; Table 6**).
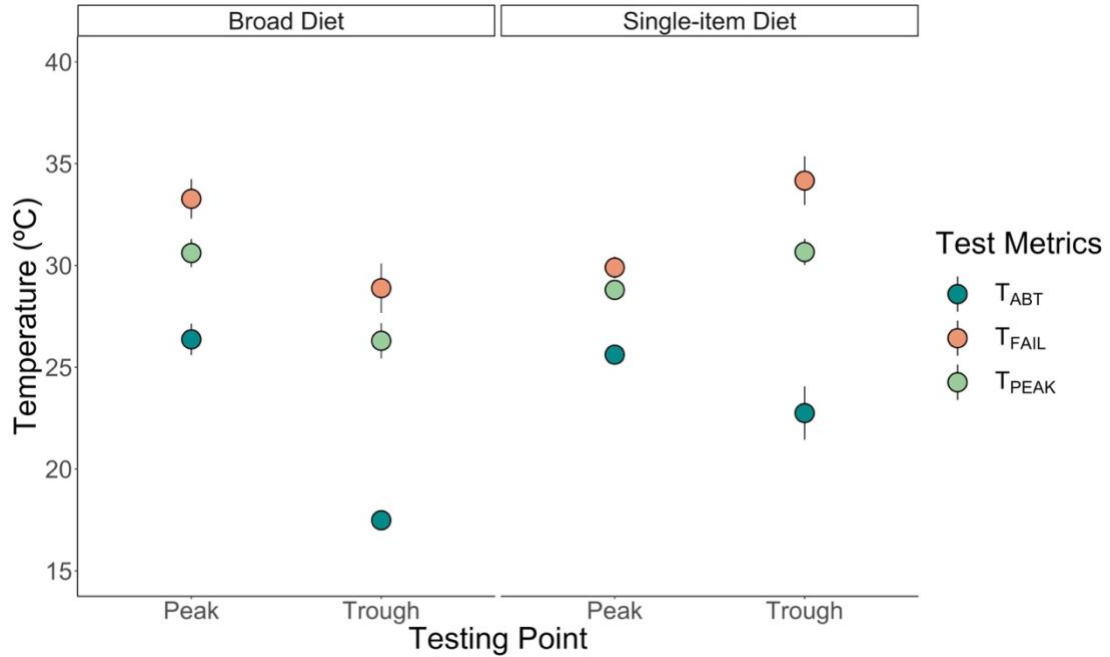
Figure 17. Thermal tolerance metrics for Hypothesis 3. *Only 11-20°C fluctuating acclimation groups were tested for Hypothesis 3. Plot is split into panels that represent diet. Within each panel, groups are split between test timepoints (i.e., peak at 20°C and trough at 11°C). Mean ± SEM is presented.*

.
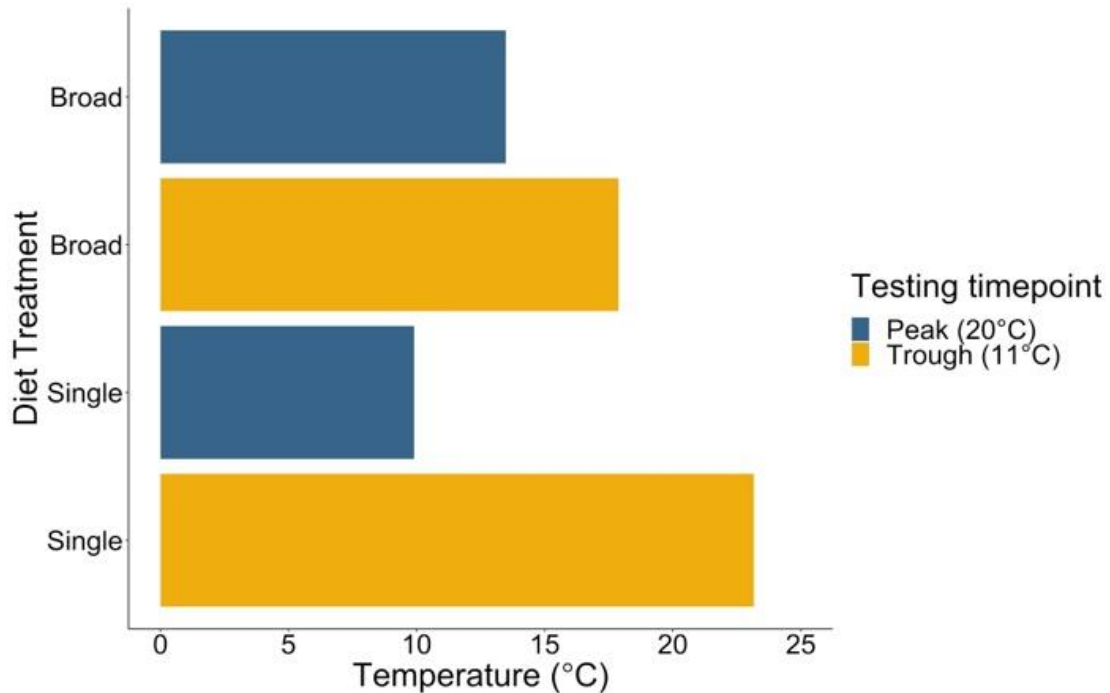
Figure 18. Bar plot of thermal distance for peak vs trough and diet type. *Only 11-20°C acclimations were tested. "Single" refers to the single-item diet, "Broad" refers to the broad diet treatment. Means are presented.*
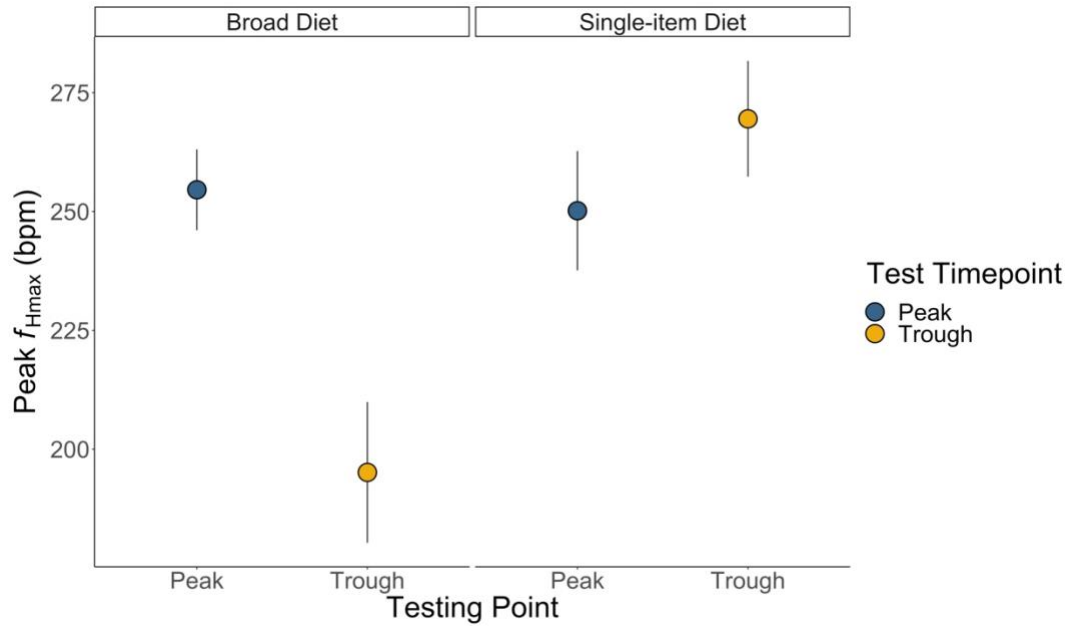
Figure 19. Mean SEM of f_{Hmax} for Hypothesis 3. *The dark blue circles represent fish tested at "peak" temperature (20°C); the yellow circles represent fish tested at "trough" temperature*

Table 6. ABT test metrics for Hypothesis 3. *Tests for rapid acclimation capacity were performed on 11-20°C fluctuating groups. Temperature for "Trough" = 11°C, while temperatures for "Peak" = 20°C. Differing letters indicate statistically significant differences across treatments (p<0.05).*

Diet	Single item		Broad	
Test temperature	11°C	20°C	11°C	20°C
Testing timepoint	Trough	Peak	Trough	Peak
n	8	6	8	7
n (T_{FAIL})	6	3	7	6
T_{ABT}	22.8 ± 1.2^c	25.6 ± 0.3^{bc}	17.5 ± 0.3^a	26.4 ± 0.5^b
T_{PEAK}	30.7 ± 0.6^c	28.8 ± 0.4^{bc}	26.3 ± 0.9^{ab}	30.6 ± 0.7^c
T_{FAIL}	34.2 ± 1.2^a	29.9 ± 0.5^a	28.9 ± 1.2^a	33.3 ± 1.0^a
Peak f_{Hmax}	269.5 ± 10.9^b	250.2 ± 8.2^{ab}	195.1 ± 13.2^a	256.5 ± 5.8^b
Thermal breadth	23.2 ± 1.5^b	9.9 ± 1.2^a	17.9 ± 1.5^{ab}	13.5 ± 1.6^a

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DISCUSSION

The effects of fluctuating temperature and diet on the cardiac thermal performance of aquatic ectotherms is largely unknown. The objective of this thesis was to explore the interaction between these two factors by using the California Killifish (*F. parvipinnis*), a resident of the environmentally variable Carpinteria Salt Marsh system that displays a generalist-feeding strategy, as a model species. Here, I report four key findings: (1) thermal tolerance of *F. parvipinnis* increased with warm acclimation, (2) a single-item diet conferred greater performance in *F. parvipinnis* compared to a broad diet at cold temperatures, (3) *F. parvipinnis* altered their thermal performance throughout the diurnal temperature phase and (4) fluctuating acclimations increased thermal breadth in *F. parvipinnis* when compared with static acclimations. These findings provide critical insight on the limits of cardiac performance when acclimated to ecologically relevant temperature regimes, and the nuanced role diet plays in cardiac function.

Thermal tolerance increases with warm acclimation

By examining ecologically relevant temperature regimes representative of summer (20-29°C) and winter (11-20°C) months along with corresponding static temperatures (11°C, 20°C, 29°C), I characterized the upper thermal limits of cardiac function in *F. parvipinnis*. I found that acclimation to summer temperatures (20-29°C and 29°C) shifted the acute TPC for f_{Hmax} to the right of the TPC measured at cooler, winter temperatures (11°C, 11-20°C and 20°C). This result provides evidence that acclimation to the higher temperatures commonly experienced by *F. parvipinnis* during the hotter, summer months may restructure the heart to allow for rhythmic function at higher temperatures (Eliason & Stecyk, 2021; Safi et al., 2019).

These findings are consistent with the growing literature that explores the relationship between thermal acclimation and thermal tolerance (Anttila et al., 2014; Drost et al., 2016; Hardison et al., 2021; Jayasundara & Somero, 2013; Safi et al., 2019; Schulte et al., 2011; Schwieterman et al., 2022). Similar responses in thermal limits have been found in a eurythermal relative (the Atlantic Killifish) that displays comparable levels of extreme plasticity. Safi et al. (2019) found acclimation to a static 33°C increased peak cardiac performance (peak f_{Hmax}) by an impressive 32% and supported rhythmic beating at temperatures 10°C greater than fish held in 5°C static acclimation. In my study, I found that acclimation to fluctuating temperatures increased f_{Hmax} by 44 – 49% from 186 bpm in the 11°C static group to 267 bpm in the 20°C group and 277 bpm in the 29°C static group. In the 20°C group, rhythmic beating was supported at temperatures 6°C greater than fish held at in 11°C static acclimation. Individuals in the 29°C static were found to continue rhythmic cardiac performance at an astounding 11°C greater than fish held in 11°C.

By measuring thermal limits across temperatures that represent the seasonality commonly experienced by fishes living in the Carpinteria Salt Marsh, I've found that cardiac thermal tolerance is highly plastic up to around 20°C, at which point the appear to hit a hard 'ceiling' and cardiac thermal performance can no longer increase with further increases in temperature. As *F. parvipinnis* are already living close to their thermal limits during the summer months – and regularly experiencing spikes in water temperatures greater than 30°C – their capacity for further acclimation may be constrained as temperatures continue to rise in this coastal salt marsh system.

Fluctuating acclimation increased thermal breadth compared to static acclimation

Empirical research that incorporates ecologically relevant acclimations (i.e., fluctuating temperatures) is critical as extrapolation from static acclimations may lead to erroneous conclusions (Morash et al., 2018; Niehaus et al., 2012). No clear differences in upper thermal limit metrics were present between fluctuating acclimation regimes and the corresponding static temperatures (i.e., 11-20°C compared to 11°C and 20°C; 20-29°C compared to 20°C and 29°C). By contrast, thermal breadth was found to differ between fluctuating and static groups. A wider thermal breadth suggests a greater span of temperatures for cardiac function, while a narrower breadth suggests a constrained thermal range for performance. The significant difference found between static vs fluctuating groups implies that fluctuating groups are able to effectively perform across a greater range of temperatures. A previous study on the effect of fluctuating vs static acclimations on upper thermal limits of cardiac function found no significant difference in thermal breadth ($T_{FAIL} - T_{ACC}$) between a fluctuating acclimation and the static acclimations that serve as the peak and trough temperatures for the fluctuating groups (Schwieterman et al., 2022). In contrast, my results provide evidence of a clear effect of fluctuating temperatures increasing thermal breadth when compared to the static acclimations that serve as the peak and trough temperatures (**Figure 16; Table 6**). In my study, I found that acclimation to fluctuating temperatures increased thermal breadth by 53% and 92% [comparing 11-20°C fluctuating group (breadth: 23°C) vs 11°C static group (breadth: 15°C) or vs 20°C static group (breadth: 12°C)]. Greater thermal breadth effectively increases the range of cardiac thermal performance, allowing *F. parvipinnis* to perform across a wider range of temperatures. Implications of this finding suggest that previous lab-based studies relying on static acclimations may be underestimating the effect of

temperature on measured traits. Ultimately, these results clearly suggest a critical need for more studies that incorporate fluctuating acclimations that are based on ecologically relevant temperature regimes.

Within the existing literature comparing fluctuating to static acclimations, it is clear fluctuating temperature has a varied influence on performance traits in ectotherms. In three species of tadpole, Kern et al. (2015) found an overall reduction in growth rate for fluctuating temperature acclimations and no effect on maximum burst swimming performance, however a 0.89 and 1.6°C increase was found on upper thermal limits between fluctuating groups and static groups, respectively, using CT_{max}. In a study similar to this thesis, da Silva et al. (2019) tested the effect of cold and warm fluctuating acclimations on thermal sensitivity for the eurythermal goby, *Bathygobius cocosensis*. Measuring burst swimming speed, maximum metabolic rate, resting metabolic rate, aerobic scope and ventilation rate, da Silva et al. found thermal sensitivity of burst swimming speed, maximum metabolic rate and aerobic scope to be affected separately by warm and cold fluctuating acclimations, and trends of increased performance for warmer acclimations. In a comprehensive study of trait variation in extreme thermal environments, Salinas et al. (2019) held Fathead Minnows (*Pimephales promelas*) in five fluctuating acclimations and four static temperature acclimation regimes and found evidence of a decrease in essential traits (including CT_{max}) as thermal stress increased. While this thesis has focused solely on the cardiac performance, the results of my work taken together with those of earlier studies provides additional context for how the incorporation of other performance traits can provide a more holistic understanding of the physiological mechanisms that allow *F. parvipinnis* to fare in an environment characterized by constantly fluctuating temperatures.

Single-item conferred greater performance compared to broad diets at cold temperatures

Nutrition has been found to support physiological responses to thermal stress, although the interactive effect of diet and temperature varies across levels of biological organization (Haller et al., 2015; Hardison et al., 2021; Lee & Roh, 2010; McKenzie, 2001; McLean & Todgham, 2015; Rodgers et al., 2019; Schwartz et al., 2016; Volkoff & Rønnestad, 2020; Zhao et al., 2022). A goal of this study was to understand the role diet plays in cardiac thermal acclimation capacity by comparing a restricted single-item diet consisting of brine shrimp to a broad diet which consisted of brine shrimp, bloodworms and arctic copepods.

I anticipated a broad diet would enhance cardiac performance as the diversity of nutrients and essential fatty acids available in the broad diet may support greater acclimation responses. However, I found the opposite. A broad diet decreased performance under winter conditions compared to a diet that consisted solely of brine shrimp. My results indicate that a simple diet of brine shrimp may satisfy metabolic demands for cardiac performance in collaboration with other plastic, adaptive mechanisms. Under winter temperature conditions, a brine shrimp diet was also found to be superior in supporting cardiac upper thermal performance compared to the broad diet. When contemplating the role marine FAs may play when in a thermally stressful environment, there is some evidence that heart fatty acid profiles may influence resilience to acute warming (Christen et al., 2020), although increased essential FA's (e.g., EPA, DHA) have been shown to decrease performance at extremely warm temperatures (Hardison et al., 2023).

Relative cost of digestion for *F. parvipinnis* under these two diet treatments may offer insight as a potential mechanism for the differences in cardiac performance. The metabolic cost of digestion (referred to as specific dynamic action, or SDA), quantifies the energy associated with the ingestion of a meal, the subsequent digestion, absorption and assimilation of nutrients (Bucking & Anderson, 2021). In fish, SDA can be influenced by species, size and quality of the meal, and environmental conditions (Beamish, 1974; Chakraborty et al., 1992; Volkoff & Rønnestad, 2020). For example, Bao et al. (2017) found a 1.3-2.7-fold increase in SDA after feeding two species of sea cucumbers diets that varied in protein, lipid content and energy composition, providing evidence that composition of diets can influence SDA and may explain the unexpected result of single-item diet outperforming broad diets. Indeed, Jackson et al. (1987) found the digestion rate of various prey to differ – with crustacean muscle requiring longer to process compared to fish, squid or cnidaria. Thus, a diet of solely brine shrimp may generate a smaller magnitude of SDA (i.e., more efficient digestion), and when acclimated to cold temperatures this surplus of energy (i.e., aerobic scope, **Figure 2**) can be diverted to support efficient cardiac performance. Another explanation for the superior cardiac performance for fish fed the single-item diet may lie in the digestion mechanisms present in the gut. To process a simple diet would require a less diversified gut, which necessitates less energy to support the breakdown of food molecules and creating a more efficient GI tract which allows more energy to be diverted to performance. Conversely in the warmer acclimations, one explanation for the lack of diet effect may be that the higher metabolic demand inherent in the warmer acclimation groups led to a decrease in aerobic scope available for cardiac performance (Dhillon & Schulte, 2011; Jayasundara & Somero, 2013) (**Figure 2**). If maintenance costs are greater in warmer temperatures, this will create a challenge for efficient digestion and effective

assimilation of nutrients, ultimately creating a limitation for cardiac performance – particularly as the warmer acclimation groups experience regular bouts of thermal stress during the temperature swing to 29°C.

In contrast to my findings, other studies on diet and cardiac thermal performance report differing results. Hardison et al. (2021) found that the effect of a carnivorous vs omnivorous diet on f_{Hmax} was present in the warmer acclimation group (20°C) while the colder acclimation group (12°C) remained unaffected. However, it should be noted that upper thermal limits were not found to differ between diets. Taken collectively, it is clear that there are species-specific responses to diet and temperature, and more research is required to better understand the complex relationship between diet and temperature on cardiac function in ectotherms.

Killifish exhibit rapid diel plasticity, altering thermal limits throughout diurnal phase

Rapid acclimation responses are critical for organisms in highly variable environments, moreover, an inability to meet the demands brought on by a change in environmental conditions may result in the organism's demise. Evidence of rapid cardiac plasticity has been found in fishes like Rainbow trout (*Oncorhynchus mykiss*) – an active species that experiences thermally heterogeneous environments in lake and stream systems. This species has been found to exhibit significant diel thermal compensation of f_{hmax} after only 24h of warm thermal acclimation (Gilbert et al., 2022a). In a separate yet similar study by Gilbert et al. (2022b), Arctic Char (*Salvelinus alpinus*) were found to exhibit a capacity for rapid cardiac plasticity after a short 72h warm acclimation. A deeper investigation into the rate of cardiac pacemaker resetting in warm acclimation found a remodeling after only 1h in *O. mykiss* (Sutcliffe et al., 2020). These findings contribute to the growing evidence for the tremendous acclimation

capacity that highly migratory species possess, however, the physiological demands of life in a highly variable salt marsh ecosystem necessitates this same capacity for rapid acclimation.

Eurythermal fish have been found to possess the capacity for rapid acclimation across various levels of biological organization. At the cellular level, Henderson and Small (2019) found evidence of a rapid acclimation of the cortisol stress response in an individual of *Nothobranchius furzeri* repeatedly stressed over one week. In this thesis, diet was found to have a significant influence on the maximum heart rate of *F. parvipinnis* individuals. In the 11-20°C fluctuating acclimation group, individuals fed a single-item diet outperformed fish fed a broad diet when tested at the trough of the temperature cycle (11°C). However, when comparing individuals tested at peak (20°C) vs trough (11°C) within this colder acclimation group I found that fish fed a broad diet had greater plasticity compared to fish fed a single item diet. Specifically, fish in the broad diet displayed varying cardiac thermal tolerance metrics (i.e., peak f_{Hmax}, T_{ABT} and T_{PEAK} add rest in here) across the thermal cycle while fish fed a single item diet had consistently high cardiac thermal performance whether they were tested at the peak or the trough of the thermal cycle. This suggests that *F. parvipinnis* possess a tremendous capacity for rapid cardiac acclimation that is highly affected by diet and acclimation temperature, with the broad diet group increasing peak f_{Hmax} by 31% higher when tested at peak temperature (20°C) compared to trough (11°). It appears that a broad diet may be able to better support rapid acclimations, while consuming a single-item diet constrains the capacity for *F. parvipinnis* to mount a plastic response in a thermally variable environment. To better predict how organisms living in highly variable environments will respond to such changes, it is essential to be cautious when attempting to predict how eurythermal fishes will respond to acute and diel temperature changes as cardiac upper thermal limits are likely tuned

to the change in tides and diet. Ultimately, we must incorporate the reality of rapid plasticity in lab-based acclimations and testing, and measure performance across a tidal cycle to determine such responses more accurately.

CONCLUDING REMARKS

This thesis provides insight as to how fluctuating acclimations and diet affect the cardiac thermal performance of the eurythermal California Killifish (*F. parvipinnis*). These salt marsh fish are champions of coping with environmental variability and often function in temperatures exceeding 30°C. However, individuals of *F. parvipinnis* were found to experience a limit in cardiac performance at a common temperature of 20°C, suggesting there is little to no plasticity for heart performance at warmer temperatures. This finding indicates that this species may experience a decreasing ability to effectively respond to the demands of life in the marsh as water temperatures in this system continue to increase, potentially reaching peaks that are far above this species' thermal ceiling. Lastly, this study contributes to the burgeoning field of research demonstrating the importance of dietary impacts on thermal tolerance in fishes – with diet indeed showing an influence on the capacity for the heart to perform across temperatures. My research outlines a clear need to incorporate more ecologically relevant temperature acclimations in lab-based research and sets the stage for more work on the influence of diet and fluctuating temperature on ectotherm performance.

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APPENDICES

Appendix 1. ANOVA outputs for Hypothesis 1. *One-way ANOVA outputs using treatment as the independent variable. The 29°C static group were excluded from the breakpoint analysis as the acclimation temperature was likely above the breakpoint and are not represented in the T_{ABT} output. Thermal distance ($T_{FAIL} - T_{ACC}$) as the independent variable for thermal breadth.*

	d.f.	F	p
T_{ABT}	3	5.302	0.00665
T_{PEAK}	4	9.333	5.65E-05
T_{FAIL}	4	9.123	0.000231
Peak f_{Hmax}	4	5.272	0.00257
Thermal breadth	4	11.1	6.59E-05

Appendix 2. ANOVA output for Hypothesis 2. *Two-way ANOVA outputs using diet and treatment as the independent variables.*

		d.f.	F	P
T_{ABT}				
	temp	1	87.6	8.2E-10
	diet	1	8.1	0.009
	temp x diet	1	11.9	0.002
T_{PEAK}				
	temp	1	42.0	7.2E-07
	diet	1	11.8	0.002
	temp x diet	1	5.8	0.02
T_{FAIL}				
	temp	1	29.009	2.4E-05
	diet	1	6.3	0.02
	temp x diet	1	2.7	0.1
f_{Hmax}				
	temp	1	6.7	0.02
	diet	1	3.9	0.06
	temp x diet	1	12.4	0.002

Appendix 3. ANOVA output for Hypothesis 3. Two-way ANOVA outputs using test time (i.e., peak vs trough in acclimation cycle) and treatment as the independent variables for upper thermal tolerance metrics, and diet and testing timepoint for thermal breadth.

		d.f.	F	P
T_{ABT}				
	time	1	43.9	7.5E-07
	trt	1	9.3	0.005
	time x trt	1	11.1	0.003
T_{PEAK}				
	time	1	2.5	0.1
	trt	1	3.1	0.09
	time x trt	1	13.0	0.001
T_{FAIL}				
	time	1	0.4	0.5
	trt	1	1.4	0.2
	time x trt	1	7.3	0.01
f_{Hmax}				
	time	1	3.1	0.09
	trt	1	9.3	0.005
	time x trt	1	11.0	0.003
Thermal breadth				
	diet	1	3.3	0.09
	testing timepoint	1	23.3	0.0001
	diet x timepoint	1	7.1	0.02

*Appendix 4. ANOVA outputs for fish morphometrics (**body size, body length and relative ventricular mass**). One-way ANOVA outputs using treatment as the independent variable.*

	d.f.	**F**	**p**
Body size	6	1.666	0.146
Body length	6	0.453	0.84
RVM	6	1.097	0.376