Phenotypic plasticity in response to thermal variability within and across generations

by

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Dedications

This thesis is about phenotypic plasticity, which is central to understanding "how organisms got to be the way they are[1]". If we consider my doctorate an expression of my phenotype, I have many environmental influences to thank for leading me down this path. This thesis is dedicated to my mentors, without whose effort and wisdom I would be a very different sort of organism.

In order of ontogenetic exposure, my thesis is dedicated to these mentors:

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[1] Mary Jane West-Eberhard, *Developmental Plasticity and Evolution*

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Abstract

In their natural habitats, organisms experience environmental variability throughhout their lives at multiple timescales. Yet, their ability to respond to realistic environmental challenges in the long-term is underappreciated in the field of experimental biology. This thesis seeks to address existing knowledge gaps in our understanding of organismal responses to ecologically relevant thermal variability through long-term experiments using zebrafish (*Danio rerio*), a model organism. Here, I examine multiple forms of phenotypic plasticity, or genotype-environment interactions that ultimately lead to phenotypic variation, both within and across generations in response to long-term thermal variability experienced from hatching through sexual maturity. Using an experimental framework that describes the contributions of both early and late life thermal environments to variation in within-generation physiology and reproduction, as well as offspring metabolism, I show that there are fundamental differences between organismal phenotypic responses to constant temperatures and thermal variability, even when thermal means are equal. I first show that zebrafish experience concurrent changes in multiple physiological traits when reared under thermal variability, including improved thermal and hypoxia tolerance, modifications to metabolic rate, and enhanced aerobic performance; these changes are largely mediated by developmental plasticity in response to early life environments. Next, I show that life-history is modified by both early and late life exposure to thermal variability, resulting in changes to spawning success, the relationship between egg quality and number, and body size in both males and females. Last, through investigating offspring metabolism of parents held under thermal variability, I show that offspring metabolism is modified based on parental early life experiences, such that early parental rearing under thermal variability reduces offspring metabolism independently of impacts on egg size. These changes may ultimately serve to enhance organismal performance in thermally variable environments, and my results suggest that phenotypic plasticity can mediate deleterious consequences of ongoing environmental change. Overall, my thesis illustrates the underappreciated capacity of organisms to plastically respond to environmental changes, given ecologically realistic conditions that span their full ontogeny.

List of Abbreviations and Symbols Used

α	Alpha; Maximum physiological oxygen supply capacity
°C	Degrees Celcius
BAH	Beneficial acclimation hypothesis
cm	Centimeter
CTmax	Critical thermal maximum
DO	Dissolved oxygen
dpf	Days post-fertilization
g	Gram
hpf	Hours post-fertilization
L	Litre
LOE	Loss of equilibrium
mg	miligrams
min	Minute
mL	Mililitre
mmol	Milimole
MO2	Metabolic rate
MS-222	Tricaine methanesulfonate
nm	Nanometer
Pcrit	Hypoxia tolerance: Partial (critical) pressure of oxygen in water at which organisms begin oxyconforming
q0.2	20th percentile (first quantile)
RMR	Routine metabolic rate
TGP	Transgenerational plasticity
TPC	Thermal performance curve
ZCF	Zebrafish Core Facility

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Chapter 1: General Introduction

"… There is hardly any question in biology of more importance than this of the nature and causes of variability."

- Charles Darwin, in a preface for *Studies in the Theory of Descent* (Weismann, 1882)

A crucial goal of biology is to understand the sources of the remarkable phenotypic variation we see both across and within species. We now understand that an organism's phenotype is the consequence of plasticity: The process of ongoing negotiations between the genome, existing phenotype, and environmental influences – including those that may have occurred in ancestral generations [1–4] (Figure 1).
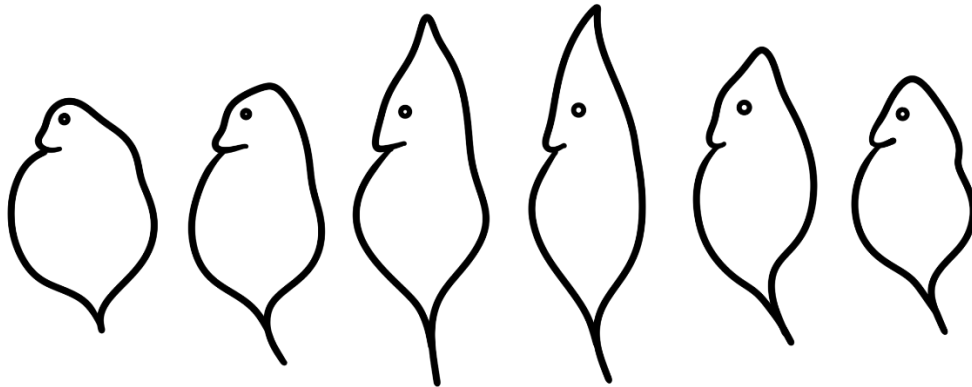
Figure 1: Illustration of phenotypic plasticity in 'helmet height' in Daphnia*. Woltereck (1909) [5] described intraindividual variation in defensive head morphology in response to varying predation pressure, in the first published example of adaptive phenotypic plasticity [2]. Image digitally recreated from original work [5].*

The late-20th Century saw a paradigm shift towards this view, moving away from a singular focus on genotype towards acknowledgement of the broader causes and consequences of phenotypic variation. Founded on the tenets of the Modern Synthesis, which reconciled Darwinian theories of natural selection with our understanding of genetics, a growing interest in the role of development as a modifier of phenotypic trajectories emerged [6,7]. Whereas development was previously considered an inconvenient 'black box' with little to no consequence for evolutionary dynamics, this

new wave of thinking led to the generation of theory and experiments on development's role in producing phenotypic variation that would ultimately be subject to selection [4,7].

Now, there is awakened interest in the myriad forms of plasticity and in how they will shape organismal phenotypes under contemporary climate change, especially in the field of ecology [8–13]. There is mounting empirical evidence supporting a role for plasticity in improving the resiliency of organisms to anthropogenic ecological stressors, especially with respect to critical changes in the means and variance of environmental temperature [8,12]. Yet, there are several long-standing limitations to existing work that hinder our ability to accurately forecast how plasticity may alter phenotypes under climate change conditions.

In this thesis, I address important limitations of existing plasticity research through a series of long-term experiments in a model organism (*Danio rerio*), with the goal of illustrating ontogenetically specific responses to ecologically realistic temperature variability within and across generations. I then discuss these findings in the context of organismal responses to contemporary climate change.

Delineating drivers of phenotypic plasticity within and across generations

Although plasticity is a key mechanism by which organisms can rapidly modify their phenotypes to cope with climate change conditions [8–10,13], there are multiple sub-processes that collectively contribute to plastic variation in phenotype. These are: within-generation developmental plasticity and acclimation [14], as well as across-generation transgenerational plasticity [3] (Figure 2). There is a great deal of ongoing debate on how these sub-processes are defined, particularly for within-generation plasticity [15,16] and between evolutionary and ecological research disciplines [17]. Here, I attempt to summarize existing generalizations and explain how I use these terms in the context of my work.
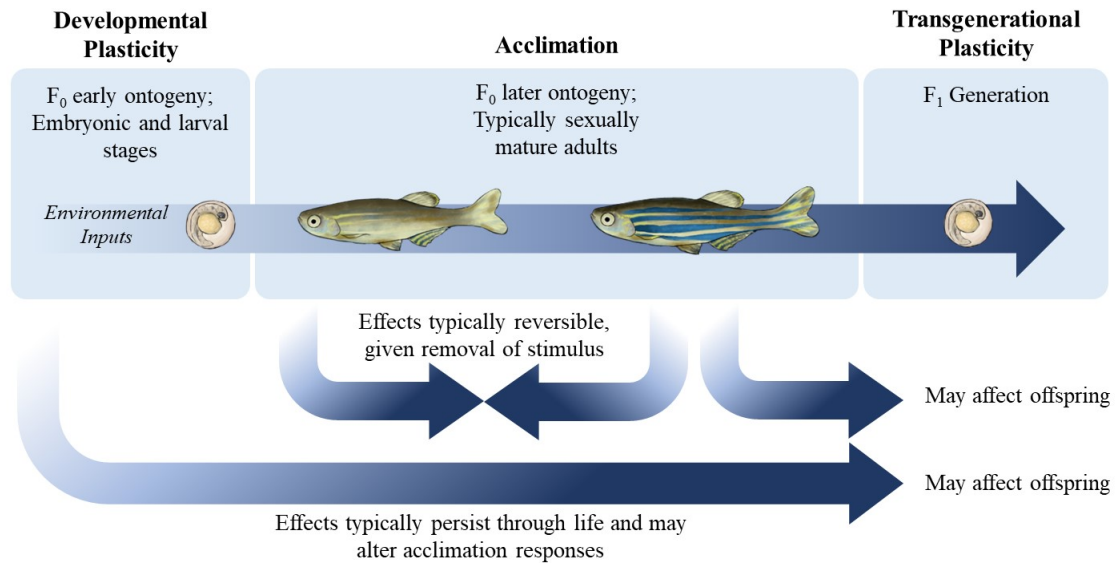
Figure 2*: Three types of phenotypic plasticity that occur in response to environmental inputs across ontogeny, and their consequences. Timing of environmental stimuli are denoted by light blue boxes. Developmental plasticity typically occurs in response to early life environments, e.g. embryonic and larval stages. Acclimation is typically studied in a physiological context in mature organisms. Transgenerational plasticity occurs when parental environmental inputs from any life-stage modify phenotypes of offspring. Blue arrows outside of boxes illustrate the effects of stimuli during each ontogenetic period; developmental plasticity often causes irreversible effects that persist through life and can influence later acclimation effects, whereas acclimation often involves reversible effects. Environmental stimuli experienced during any period of ontogeny may ultimately affect offspring phenotypes, through transgenerational plasticity.*

Developmental plasticity generally refers to phenotypic variation induced by early-life environments, which can lead to irreversible changes in phenotype that continue to affect developmental trajectories throughout life (Figure 2; [2,14]). However, this definition is not universal, because plasticity-induced phenotypes often revert, given changes in environmental conditions [16]. Moreover, organisms continue development throughout their lives and windows of ontogeny that are particularly sensitive to environmental conditions can differ intra- and inter-specifically, as well as between traits [4,16]. There is therefore no fixed timing for the study of developmental plasticity, and no evidence of universal persistence [4,18]. Methodologically, however, developmental plasticity is often studied in the context of immature individuals (Figure 2), likely because the earliest life-stages of organismal development (*e.g.*, embryogenesis) in ectotherms are environmentally sensitive [16].

In teleost fishes, like zebrafish, using early life-stages to define critical windows of developmental plasticity is also biologically reasonable; larvae transition into juveniles

through a metamorphic process that involves significant changes including remodeling of existing organ and sensory systems, fin development, and pigmentation [19]; at this point, sex is also already established [20]. Juveniles are therefore morphologically and physiologically similar to adults, and this period of their development is better defined by growth than by tissue differentiation, in contrast to embryos and larvae [19]. Regardless of unknown variation in these critical windows that generate developmental plasticity, authors have suggested that both the magnitude and persistence of developmental plasticity can be experimentally described by isolating early life environments from subsequent environmental conditions [21]; as such, in this thesis, I have made the explicit assumption that developmental plasticity occurs in response to embryonic and larval conditions (Figure *2*).

Acclimation, in contrast, is generally considered to be a reversible process that is studied in the context of adult organisms (Figure *2*), and often in physiological traits [22]. Yet physiologists have historically used the term broadly, including a range of responses that vary from rapid and acute (*e.g.*, a brief increase in breathing rate, which could also be described by a reaction norm [2,22]) to changes that occur after more prolonged environmental exposures (*e.g.*, a temporary shift in metabolic efficiency), and acclimation periods in physiology studies can range from minutes to months [17,22]. Whereas some authors have argued for a strictly physiological definition of acclimation (*e.g.*, changes in biochemical regulation) [17], others have included a greater range of traits, such as morphology, egg size, and reproductive output, which necessarily make no assumptions about reversibility [14].

Acclimation is nevertheless usually considered distinct from developmental plasticity [23,24], although the process of acclimation may itself be influenced by previous developmental plasticity [14,23], or produce opposing responses to those of developmental plasticity in the same environmental conditions [25]. It is likely that acclimation and developmental plasticity ultimately exist on a context-dependent continuum, sharing some, but not necessarily all, mechanisms [14,23,26]. As a result of uncertainty in defining acclimation, for my investigations of within-generation plasticity in Chapters 3 – 4, I place more focus on how developmental plasticity and acclimation responses are defined relative to the timing of stressors, describing phenotypic effects in

response to juvenile and adult environments as acclimation responses (Figure 2; *sensu* [27–29]). Focusing on stressor timing is also a useful approach in the context of climate change; if we can predict the timing of stressors, we can understand their biological consequences based on when they occur in the life cycle of organisms.

There is less debate in the literature on the definition of transgenerational plasticity, which describes phenotypic variation in offspring resulting from parental environments [1] (Figure 2). Transgenerational plasticity has received considerable attention for its role in promoting beneficial responses of offspring to climate change-related stressors (*reviewed in* [11,30]); for instance, hot acclimation of adult stickleback mothers improves the growth of their offspring under hot conditions [31]. Known mechanisms thus far include changes to maternal egg provisioning (*e.g.*, steroid hormone allocation, or variation in egg size [3]), as well as epigenetic mechanisms (*e.g.*, DNA methylation or histone modification [32]). However, we still have a poor understanding of *when* environmental inputs must occur during the life of parents to produce transgenerational effects [11,33]. In other words, there may also be a 'critical window' of ontogeny that promotes transgenerational plasticity within a given population of organisms.

An overwhelming majority of ecological experiments have looked only at the effects of environmental inputs during the reproductive life stages of parents (*e.g.*, sexual maturity, to breeding, to oviposition in egg-laying animals) [11,33]. Yet, theory makes no claims as to the specific relevance of this life-stage in producing transgenerational variation; instead, it is suggested that transgenerational plasticity should be favoured if parental environments provide some reliable 'cue' to offspring about future environments [1]. It thus follows that the importance or reliability of cue timing is dependent on the life-cycle and ecology of the study organism. For instance, in short-lived organisms, there is evidence that the adult reproductive environments are reliable fortune-tellers of the offspring early life environments that immediately follow, promoting adaptive transgenerational plasticity [11].

But in longer-lived organisms, such as those whose immature and adult life stages occupy different habitat niches [34,35], it is possible that the early life of parents are better predictors of the conditions their offspring will encounter [33]. Although this suggestion was championed decades ago by Taborsky [34,35], few studies have tested

these predictions in animals, and none have used temperature as the key environmental input; most studies test maternal nutrition (*reviewed in* [33]). In zebrafish – as in all ectotherms – temperature is a major driver of biological functions [24]. Moreover, in their natural floodplain and riverine habitats centred around the Ganges and Brahmaputra River basins, zebrafish experience their earliest life stages during the climatically unique monsoon season (June – August), characterized by high daily maximum temperatures and precipitation [36] (Figure 3). Spawning typically occurs just before the onset of monsoon season, with a fast period of embryonic and larval growth occurring during this time [37]. The shared early life environmental experience of zebrafish parents and offspring in nature is expected to promote adaptive transgenerational plasticity in this species, provided there is expected environmental autocorrelation between monsoon seasons [34,35,38].
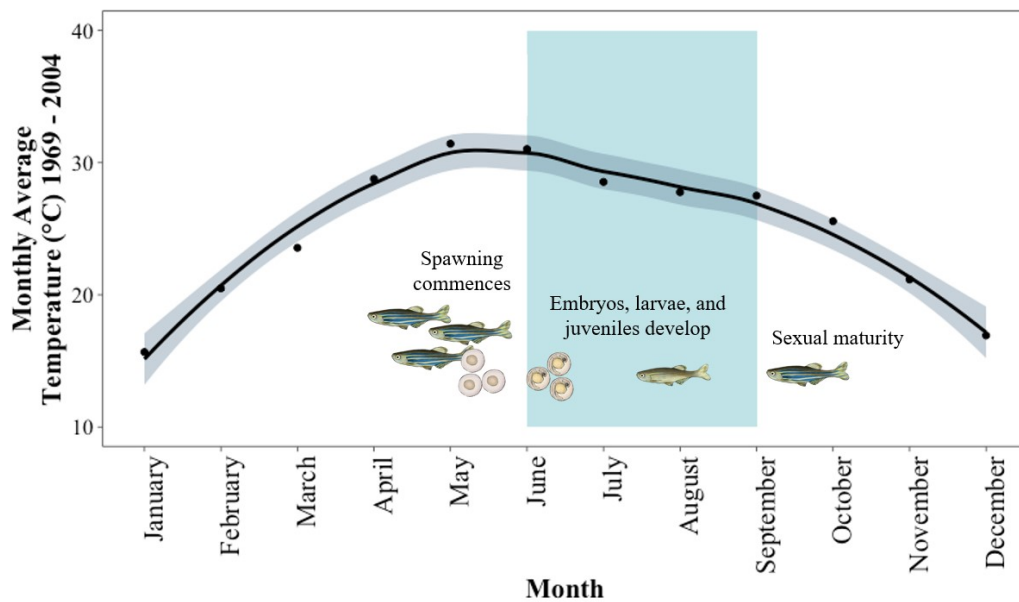
Figure 3: *Average monthly mean (dark line and points) and maximum/minimum (shaded interval around mean) temperatures of the Ganga Basin from 1969 – 2004; monsoon season is shaded in blue, with typical onset at the beginning of June. Zebrafish life cycle is superimposed. Spawning typically commences just before the onset of monsoon; embryonic, larval, and juvenile growth occur during monsoon [37]. In laboratory zebrafish, the presumptive onset of sexual maturity is around 3 months post-fertilization, and is included for illustrative purposes [39]. Thermal data accessed from the India Water Resources Information System.*

In Chapter 5, I explore this possibility. Using offspring of adult fish for which I described within-generation plasticity in Chapters 3 – 4, I make the first empirical test comparing the effects of early *vs*. late life parental thermal experiences on transgenerational plasticity in offspring.

The first major aim of my thesis is to describe and compare developmental plasticity, acclimation, and transgenerational plasticity in response to thermal experiences, considering specific 'critical windows' of exposure that may be susceptible to thermal environmental inputs. My long-term experimental approach follows zebrafish from fertilization to one year of age – the end of their natural lifespan in the wild – and into the next generation, allowing for a comprehensive illustration of how different forms of plasticity can collectively operate within a single species.

The external validity of experimental thermal conditions

The field of thermal biology is dominated by studies using constant thermal conditions to test the effects of temperature on organismal phenotypes [40–42]. The seminal insights of thermal biology were built on constant temperature studies, and are likely broadly reasonable reflections of organismal responses, including individual performance, population distributions and growth rates, and in some taxa, even sex determination [24,43–46]. Yet in nature, temperatures are not static. Temperatures instead vary in daily, seasonal, and stochastic ways, all of which have the potential to affect organisms differently than would constant average temperatures [47].

The "easily manipulated but ecologically arbitrary" ([48], p. 11) constant temperatures used in an overwhelming majority of thermal biology studies have been criticized for their inability to properly illustrate natural responses, and thus their limited utility in studies of plasticity [47,49]. For instance, developmental models built using empirical data from constant temperature studies almost always underpredict growth in the wild [50,51]. This underprediction is an artefact of time-dependent effects induced under constant exposure to challenging thermal extremes, such as prolonged overproduction of heat shock proteins, which would not otherwise occur when exposures are brief [52,53]. Indeed, studies often compound these issues by also selecting deleteriously hot treatment temperatures, leading to uncontrolled confounding bias [21,54]. For example, hot,

constant treatment conditions can cause serious mortality in experimental cohorts leading to an absence of data on responses [55], despite the possibility of organisms surviving brief exposures to those same temperatures. Given current limitations associated with experimental designs using constant temperatures, we are unlikely to accurately describe the extent to which organisms can alter their phenotypes to cope with stressful temperatures in nature.

In this thesis, I first explore the experimental literature on thermal variability in Chapter 2. Here, I conduct a systematic review on studies incubating ectotherm vertebrate eggs under thermally variable conditions, laying the groundwork for my three experimental chapters. Chapter 2 was developed in part as a reconciliation of taxonomic disparities in the discussion of thermal incubation; whereas there is a well-developed body of work focused on reptiles, at least in the constant temperature literature [41,44,56], little information has been critically synthesized on fish and amphibians. This synthesis was necessary to identify key areas of focus for my future chapters, especially given that within-generation plasticity is expected to be favored in organisms with fast life-histories [57] – such as small-bodied cyprinids like zebrafish, rather than long-lived chelonians or squamates.

I then use a shared experimental framework to investigate plasticity to variable temperatures in Chapters 3 through 5. To avoid the potentially confounding bias of deleteriously hot temperatures and improve the external validity of my experiments, I employ a range of sinusoidal diel thermal variability that is reflective of what could be encountered by zebrafish in the wild; *e.g.*, an annual range of 17 – 33 °C has been reported for zebrafish habitat in Bangladesh [36,37] (*see also*: Figure 3). The variable temperature treatment used in my experiments, which varied from 22 °C at midnight to 32 °C at noon (Figure 4) also has physiological relevance. This range encompasses the temperatures beyond which normal development is compromised in zebrafish; *i.e.*, leading to high rates of deformity and mortality [25,58].
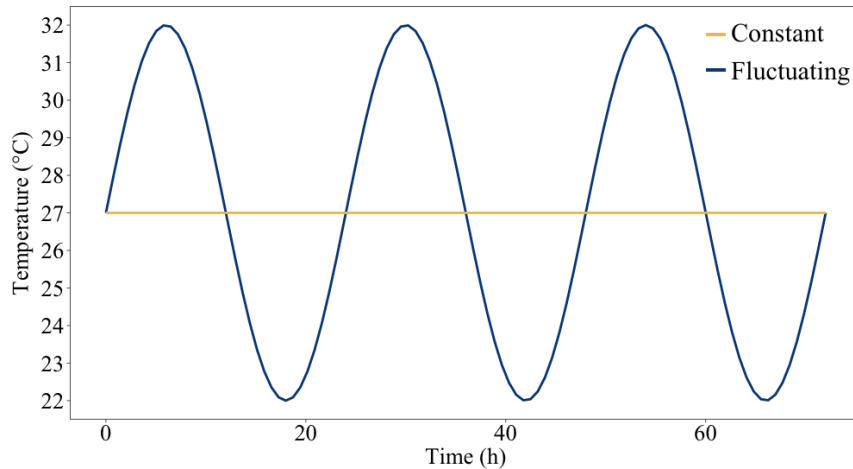
Figure 4*: Illustration of thermal treatments used in experimental Chapters 2 - 5. The 'Fluctuating' treatment varied from 22 to 32 °C on a diel a basis, and the 'Constant' temperature was a static 27 °C. Fluctuating temperatures reflect the range of temperatures permitting normal growth and development in zebrafish (Danio rerio). Constant temperatures reflect the thermal optimum for growth and development, and the average temperature of the Fluctuating treatment.*

I therefore specifically selected temperatures that were challenging and potentially even stressful, but still mild enough in terms of both exposure duration and thermal magnitude that I did not expect significant deleterious effects [21]. Moreover, this range's average temperature is equivalent to the optimal temperature for zebrafish growth and reproduction [25], a constant 27 °C, which I used as a control treatment (Figure 4). Because this constant temperature is used in zebrafish facilities worldwide, this treatment and the use of the ± 5 °C diel range around it allowed for a straightforward comparison with the prevailing conditions under which laboratory zebrafish are raised.

The second major aim of my thesis is to illustrate the plastic effects of thermal variability on phenotype. In Chapter 2, I focus on developmental plasticity across multiple taxa, through my review of thermally variable ectotherm egg incubation. Then, in Chapters 3 – 5, I investigate all three forms of plasticity experimentally, comparing within- and across-generation plasticity of zebrafish to variable *vs.* constant temperatures.

Experimental approaches

To achieve my two major goals, I employed a split-clutch, factorial experimental design exposing zebrafish to either constant or fluctuating temperatures (Figure 5). This design strictly delineates early and late ontogeny of zebrafish, and subsequent offspring environments (Figure 5). I based this design on the 'strong-inference approach' of Huey *et al.* [59], in which one clutch of organisms within a generation is iteratively split into a full-factorial combination of treatments at different timepoints (*i.e.*, in my experimental chapters, the resulting developmental plasticity-acclimation combinations would be Constant-Constant, Constant-Fluctuating, Fluctuating-Constant, and Fluctuating-Fluctuating).
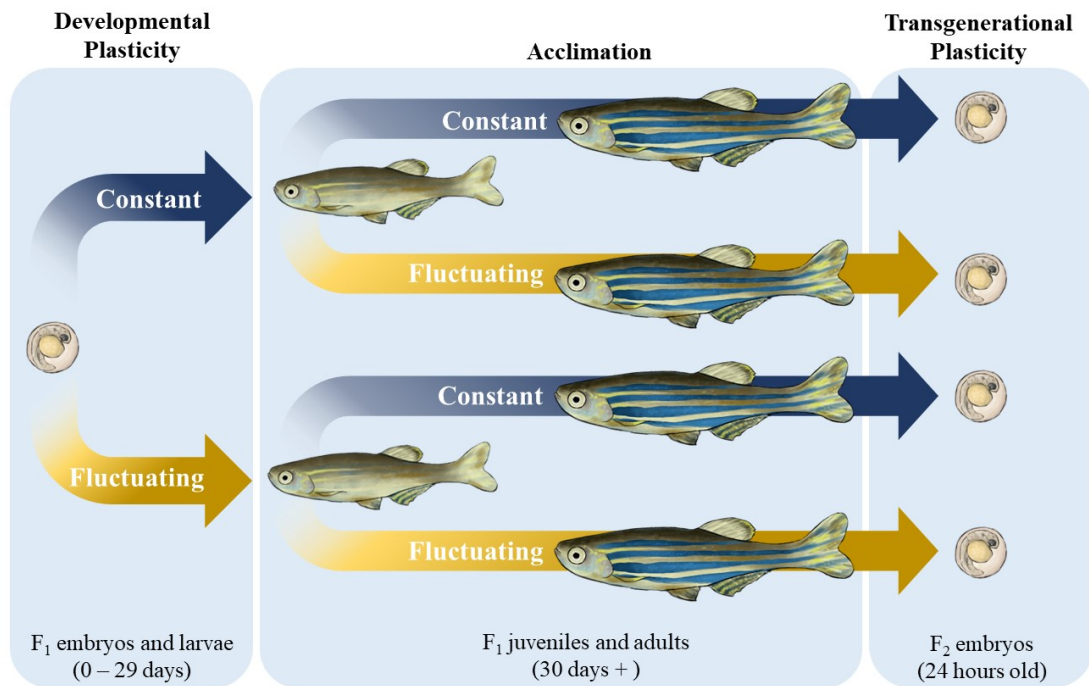
Figure 5: Factorial, split-clutch experimental design intended to delineate the effects of developmental plasticity in response to early life (F1 embryonic and larval stages; 0 – 29 days post-fertilization), acclimation in response to later life (F1 juvenile and adult stages; 30+ days post-fertilization), and transgenerational plasticity of F2 embryos in response to parental early or later lives. Constant temperature treatments (27 °C) reflecting the thermal optimum for growth and reproduction of zebrafish were contrasted with diel Fluctuating temperature treatments (22 – 32 °C, mean: 27 °C) reflecting ecologically realistic and physiologically challenging temperatures.

Huey et al.'s [59] original approach was designed to test multiple competing hypotheses about the adaptive benefits of plasticity; for example, if two temperatures (hot and cool temperatures in a 2 x 2 factorial design) were used, the subsequent hypotheses might be:

I. *Beneficial acclimation:* Organisms reared previously in one temperature perform better in that temperature when tested later in life.

II. *Hotter is better:* Organisms reared at hot temperatures always perform better than organisms reared at cool temperatures, no matter what condition they are tested in.

III. *Colder is better:* Organisms reared at cooler temperatures always perform better than organisms reared at hot temperatures, no matter what condition they are tested in.

I modify this strong-inference approach for my experimental chapters. Notably, the original approach outlined by Huey *et al.* [59] effectively tested the *cumulative* effects of both developmental plasticity and acclimation at a given acute test temperature: It is assumed that organisms are reared in their 'developmental' condition until acute testing during adulthood. Rather than directly testing the adaptive significance of combined plasticity, I instead divide treatments to span early and late ontogeny, leveraging the utility of the factorial design to explain the effects of each period of ontogeny on different metrics of performance, and de-emphasizing the focus on competing hypotheses and acute test temperatures.

With that said, the early work by Huey *et al.* [59] lent several early insights into proper experimental design that I have taken into consideration. First, highly stressful temperatures should be avoided because the costs of tolerance can overwhelm potential beneficial plasticity [59]. Although early work was concerned with how stress-induced "long-term decrements in organismal condition" [60] would obscure evolutionary conclusions about the adaptive value of plasticity, there are many additional reasons why the application of stressful temperatures (including static temperatures) is limited for ecological application, which I described above in 'The external validity of experimental thermal conditions.' For all these reasons – including avoiding experimental bias, illustrating ecologically realistic responses, and demonstrating potential effects of

anthropogenic thermal variability increases – it was important that variable temperatures were used in my experiments.

Second, Huey *et al.* [59] highlighted the point that fitness proxies are the most appropriate traits to study, and this sentiment was echoed by Woods & Harrison [60], as well as Wilson & Franklin [21], who wrote non-fitness correlates are "less than desirable." Like the suggestion to avoid stressful temperatures, the original intention behind this emphatic suggestion was that fitness proxies are necessary to properly infer the adaptive value of plasticity from an evolutionary perspective. In my own work, the use of ecologically relevant fitness proxies is also necessary to meaningfully examine how plasticity may influence important organismal responses under climate change conditions. In Chapter 3, I take a multi-trait approach to collectively investigate plasticity in thermal and hypoxia tolerance, which are important indicators of survival under acutely stressful climatic conditions [61,62], as well as metabolic rate and oxygen uptake capacity, which are linked to energy availability for growth and reproduction [63,64]. In Chapter 4, I test for plasticity in iterative reproductive performance, a direct fitness correlate. Then, in Chapter 5, I test for transgenerational plasticity of metabolism of offspring, which is directly linked to juvenile body size [65], an indicator of early survival in fishes [66]. Overall, across my experimental chapters, I measure numerous traits that either have a rational relationship to fitness or are otherwise direct fitness correlates.

Summary

This thesis is an integrative body of work, with influences from evolutionary theory on plasticity [1,4,16,21,59,60] that are applied to contemporary ecological issues associated with increased thermal variability under climate change. The overarching goal of my work is to holistically describe multiple long-term, fitness-associated, plastic responses of zebrafish to thermal variability, addressing key ontogenetic periods during which these changes are generated within and across generations. I then seek to place my findings in the context of organismal responses to anthropogenic climate change.

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Chapter 2: Thermal variability during ectotherm egg incubation: A synthesis and framework

Abstract

Natural populations of ectothermic oviparous vertebrates typically experience thermal variability in their incubation environment. Yet, an overwhelming number of laboratory studies incubate animals under constant thermal conditions that cannot capture natural thermal variability. Here, I systematically searched for studies that incubated eggs of ectothermic vertebrates, including both fishes and reptiles, under thermally variable regimes. I ultimately developed a compendium of 66 studies that used thermally variable conditions for egg incubation. In this review, I qualitatively discuss key findings from literature in the compendium, including the phenotypic effects resulting from different patterns of thermally variable incubation, as well as the ontogenetic persistence of these effects. I also describe a physiological framework for contextualizing some of these effects, based on thermal performance theory. Lastly, I highlight key gaps in our understanding of thermally variable incubation, and offer suggestions for future studies.

Student Contribution Statement

Melanie D. Massey conceptualized this piece with support from Jeffrey A. Hutchings. Investigation was conducted by Melanie D. Massey. Data curation, visualization, and formal analyses were conducted by Melanie D. Massey. Supervision was provided by Jeffrey A. Hutchings. Melanie D. Massey wrote the original draft and both authors edited and reviewed the final piece.

Introduction

Variability in temperature is a key feature of natural environments. For ectotherms, this variability typically results in a wide range of experienced body temperatures due to diurnal, seasonal, and stochastic thermal fluctuations. Ectotherms have consequently adapted to perform biological functions over ranges of inconstant temperatures, with the nature of performance changing between populations, and among and within life stages [1–3].

The embryo life stage is particularly sensitive to temperature, owing to the relatively small body size of embryos [4]. Differences in thermal incubation regimes influence myriad biological functions both during development and cascading into later ontogeny. Long-term effects of the incubation environment include changes to locomotor performance [5], growth [6], survival [7,8], behaviour [9], reproductive traits [10], and, for organisms with temperature-dependent sex determination, the outcome of sex [11,12].

Given the importance of the embryonic thermal environment to producing variation that impacts ecological and evolutionary dynamics in ectotherms [13,14], it is unsurprising that considerable attention has been devoted to thermal incubation experiments. Widespread discoveries of temperature-dependent sex determination (TSD) in reptiles in the 1970's began a snowballing fascination with the vertebrate embryonic thermal environment [15], ultimately encouraging further research on other elements of embryonic development and physiology. A recent review by Warner and colleagues [16] found that nearly 75% of 803 studies on reptile incubation focused on the effects of temperature, and the number of publications on the effects of embryonic thermal environment has been steadily rising since 1969.

Yet, under the basic assumption that the eggs of ectothermic vertebrates typically experience natural daily and seasonal variation in temperature, the vast majority of incubation studies continue to employ constant temperature incubation conditions [17,18]. Although constant temperature incubation experiments have inarguably led to important insights regarding the influence of developmental temperatures on many phenotypic measures, it is difficult to interpret these findings with respect to thermal fluctuations. One reason for this confusion is that expected phenotypic effects, based on the means of thermal variance, often poorly align with observed effects under thermal

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variation [18–23]. As a result, our understanding of the ecological relevance of fluctuating temperatures is limited [24], necessitating that we broaden our knowledge of how thermal variation affects organisms, and integrate these findings with the larger body of work on constant temperature incubation.

In the present review, I provide the first comprehensive compendium of incubation experiments that employ thermally variable regimes to incubate the eggs of all ectothermic vertebrates, including both herpetofauna and fishes. Notably, previous work on the topic has focused largely on reptiles [17,25], so I aimed to include additional insights from both fishes and amphibians, taxa which are frequently excluded from the broader discussion of egg incubation. Within the compendium, I also provide summaries of important findings from each study, report the temperature regimes used, and whether ecologically or physiologically relevant data informed the study. Ultimately, my goal for this compendium of studies is to act as an informative reference for authors studying thermal variability, and to facilitate taxonomic crosstalk between reptile, amphibian, and fish researchers.

I also sought to critically review several key concepts relating to the effects of thermal variation during incubation on embryos, hatchlings, and later life stages of ectothermic vertebrates, through a qualitative discussion of a selection of studies found in the compendium. First, I present a basic thermal performance framework for interpreting the phenotypic results of incubation under thermal variation, integrating what is known from constant temperature work. Next, I discuss findings from thermal variability studies not directly comparable with constant temperature work. Importantly, I also discuss the long-lasting effects of the thermal environment during incubation through developmental plasticity. I finish by discussing other biological or environmental sources of phenotypic variation, and present avenues for future research.

Summary of literature compendium

I conducted a systematic literature search, using the ISI *Web of Science* Core Collection database for vertebrate incubation studies in which variable temperature regimes were used in the artificial incubation of eggs. I applied combinations of synonymous terms for 'temperature' and 'fluctuation', and narrowed my search to the embryo life stage. The resulting advanced search query was: TS=(incubation AND (fluctuating OR variable OR seasonal) AND temperature* AND (embryo OR egg*)). The 561 results were then organized by 'Relevance', and included articles indexed until 28 February 2019. I examined abstracts for each study and followed these exclusion criteria:

(i) I excluded studies for which the study organism was not an ectothermic vertebrate, and for which egg incubation was not artificial (*i.e.*, in natural nests).

(ii) I excluded studies that were unable to incubate eggs for nearly the entire incubation period, specifically those that were unable to begin artificial incubation of eggs 72 h or more after oviposition.

(iii) I excluded studies of viviparous squamates (*e.g.*, those that incubated the mother rather than eggs), and squamates that undergo approximately one-half or more of development in-utero. Specifically, I imposed a cut-off of embryo oviposition at an average Dufaure and Hubert [26] Stage of 35; the majority of squamate species oviposit embryos when they have undergone one-third of development (Dufaure and Hubert Stages 25-33 [26–28]).

(iv) I excluded any articles that were not written in English.

(v) I excluded studies in which the focal measurements were of sex ratios, as incubation studies focusing on temperature-dependent sex determination are already the most heavily reviewed in the egg incubation field [25].

If these conditions were met, or if it was ambiguous whether these conditions were met, I further analyzed the full text of the article, removing studies that did not meet all conditions.

I cross-referenced my results with literature found in a recent review, as well as results from the Reptile Development Database [18,29], to capture additional studies that did not appear in my search; as a result, I added 8 studies to my pool from the Reptile Development Database [29].

From these studies, I recorded species, the temperatures used in incubation regimes, as well as the nature of thermal variability. I documented whether ecologically relevant nest temperatures for the population were recorded or cited, and whether physiologically relevant temperatures (*i.e.* the thermal minimum, optimum, or maximum for development rate) were mentioned. I also reported a brief summary of the effects of thermal variability for each study.

Using the search parameters and cross-referencing materials above, I collated 66 studies (58 from the Web of Science search, and 8 from the Reptile Development Database [29]). Most studies focused on squamates, followed by fishes, testudines, and anurans, with a single paper on urodeles (Figure 6), and spanned 48 species. Several types of fluctuating regimes were employed, and I classified them into six patterns (Figure 7). The most common was diel fluctuations in temperature, characterized by sinusoidal thermal fluctuations around a stationary mean (Figure 7A). Among other thermal regimes used were seasonal shifts in temperature, characterized by a changing thermal mean throughout incubation, in the absence of diel fluctuations (Figure 7B); ambient regimes, characterized by stochastic diel changes in temperature (Figure 7C); combinations of variable regimes (*e.g.* Figure 7D); and heat shocks, which involved brief exposures to high temperatures (Figure 7E). I termed the last category of variability 'idiosyncratic', characterized by large changes from one constant temperature to another at different points during incubation (Figure 7F). The magnitude of thermal variability ranged from 3 to 20 °C.
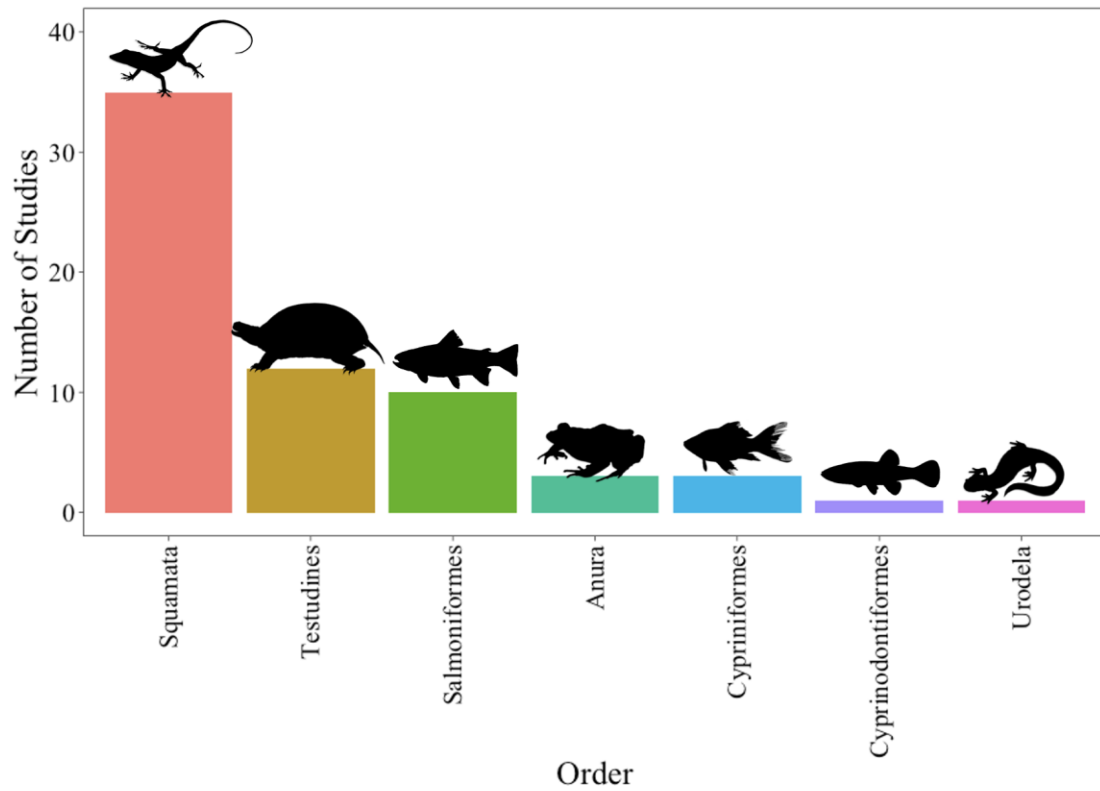
Figure 6*: Frequency of taxon appearance in 66 studies incubating ectothermic vertebrate eggs under thermally variable regimes. Reptiles are best represented in these studies (Squamates: 35 studies, Testudines: 12 studies), followed by fishes (Salmoniformes: 10 studies, Cypriniformes: 3 studies, Cyprinodontiformes: 1 study). Amphibians are the least represented group (Anura: 3 studies, Urodela: 1 study).*
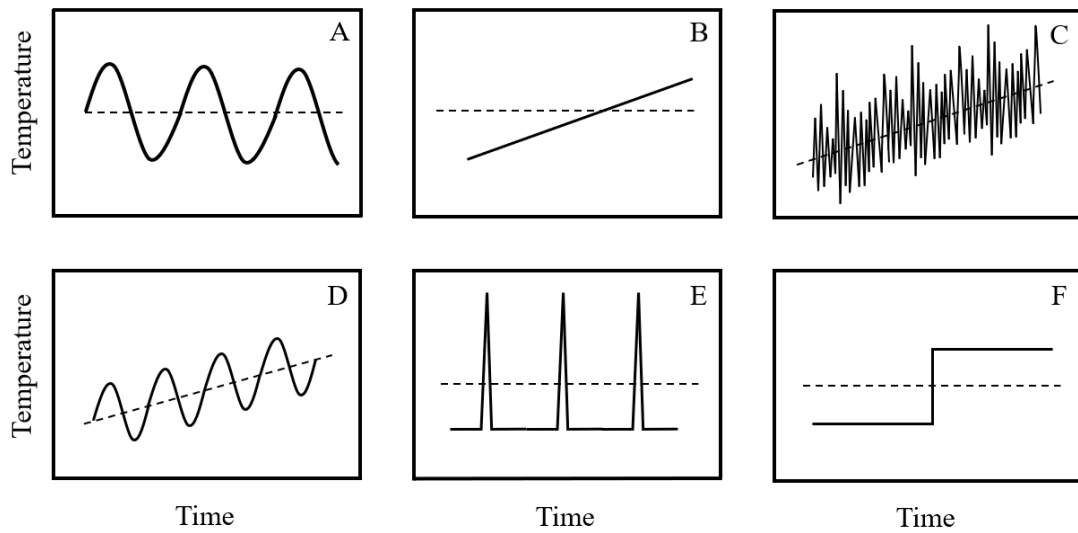
Figure 7: *Different regimes used in studies that incubate ectothermic vertebrate eggs under thermal variability. A. Sinusoidal fluctuations on a diel basis. B. Seasonally changing mean, accomplished by a slow change in temperature over a long period of time. C. Ambient temperatures, with stochastic changes in daily temperatures and seasonally shifting means. D. Any combination of A-C. E. Constant mean with heat shocks applied several times per week. F. Idiosyncratic, characterized by shifts from temperature to temperature at different stages of development.*

I also noted whether studies investigated or cited data relevant to the thermal physiology of the study organism (specifically: upper or lower temperatures for successful development, developmental thermal performance curves, critical thermal minima, maxima, and/or optima, and temperatures that result in high or low mortality and/or embryonic growth [from previously published or pilot experiments], at the species level) and the thermal ecology of the study population (natural nest temperatures from the focal environment, at the population level). Only approximately one in three studies (23/66) reported or cited data regarding thermal physiological parameters for their study species. In contrast, most studies (43/66) reported or cited natural nest temperatures for their study population.

It is important to note that that technical reports, government reports, and conference proceedings not published in books ('grey' literature) were not found in my search, and may represent valuable sources of information on thermal variability and egg incubation; thus, I acknowledge that my search design precluded these works from inclusion in the compendium.

The fallacy of the average: nonlinearity of thermal performance can result in differences between constant and variable temperature incubation

There is a long history of approaches to estimating organismal performance under thermal variability, that have been used to interpret the phenotypes produced under inconstant temperatures. Centuries ago, Réamur [30] described a relationship between plant development and temperature in which the sum of daily average air temperatures during development was always equal at maturity for a given plant species. This 'thermometric constant' represented a way of describing the amount of heat energy required for a plant to reach maturity, and necessarily assumes a linear relationship between growth and temperature. Through time, heat summation approaches were enhanced, with modifications that take into consideration biologically informed upper and lower thermal limits for growth [31–33] and the inclusion of daily minimum and maximum temperatures [34].

Contemporarily, heat units are often measured in growing degree-days (GDD), which are calculated by subtracting a base value (T_b), representing a temperature at which the process of interest cannot occur, from a daily average temperature (T_{avg}). Although traditional GDD approaches are still widely used in agriculture and food industries, there are long-standing criticisms of their underlying assumptions [35]. Notably, rates of biological processes respond non-linearly to temperature, especially at thermal extremes [36]. Under GDD (and other linear approaches), temperatures are used as a direct proxy for growth and, as a result, growth rates at thermal extremes can be over- or under-predicted. Ruel and Ayres [37] considered this phenomenon a consequence of Jensen's inequality, a mathematical principle stating that the average value of a portion of a curvilinear function (*e.g.* the average thermal performance under thermal variability) will differ from the value expected from the average temperature, depending on the portion's curvature (Box 1).

An alternative and common approach to accounting for the non-linearity of biological responses (*e.g.,* metabolism, locomotion, growth) leverages continuous thermal reaction norms, or thermal performance curves [38], which illustrate that the rate of biological processes increases nonlinearly with temperature to a particular optimum (T_{opt}), after which they descend sharply (Figure 8). These curves are bounded by critical thermal minima (CT_{min}) and maxima (CT_{max}), the minimum and maximum temperatures at which the rate of performance is zero. TPCs are typically developed by measuring performance of animals within one population at a series of constant temperatures and modeling a continuous reaction norm to fit these data. Cumulative performance under thermally variable conditions can then be estimated by integrating the TPC function, using experienced temperatures [39–45]. I note here that a recent nonlinear GDD approach has been developed which closely resembles a TPC approach [46]. In contrast to traditional GDD approaches, a higher degree of accuracy is expected from using nonlinear approaches because error resulting from Jensen's inequality is minimized.
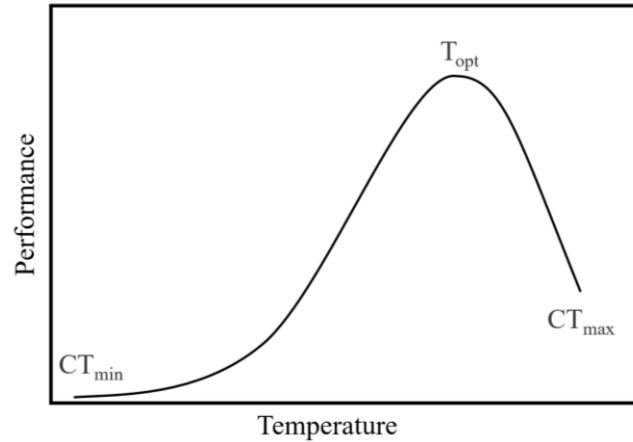
Figure 8: A thermal performance curve (TPC), showing that performance typically increases nonlinearly from a critical thermal minimum (CT_{min}) at which the biological process of interest cannot occur, to a thermal optimum (T_{opt}) at which performance is maximal, and then descends sharply back to a critical thermal maximum (CT_{max}) at which the biological process can no longer occur. TPCs are usually constructed by keeping organisms at a range of constant temperatures, measuring the traits of interest at each temperature, and then modelling the response curve to temperature.

Using integration of a TPC to predict performance results in three general expectations, and assists in understanding why fluctuating temperatures during incubation often do not produce the same phenotypic effects we see at constant temperatures sharing the same mean. I walk through examples of these expectations in Box 1.

First, if temperatures fall within a convex region of the TPC (for example, near CT_{min}), the realized average performance of an organism under a fluctuating temperature regime ($P_{avg}(T)$), is expected to be greater than the performance of the same organism under a constant temperature sharing the same thermal average ($P(T_{avg})$). In other words, we expect lower performance under fluctuating temperatures than at the constant mean of fluctuation (Box Figure 1). Second, if the temperatures fall within a concave region of the TPC (for example, near the optimum), realized average performance is expected to be lower than performance at a constant temperature sharing the same thermal average (Box Figure 2). Last, when variable temperatures fall within an approximately linear range of the TPC, realized average performance and performance at the constant average temperature should be similar (Box Figure 3). Within these regions, as the magnitude of thermal fluctuations increases, we also expect the difference between $P_{avg}(T)$ and $P(T_{avg})$ to increase. Of course, the exact relationship between $P_{avg}(T)$ and $P(T_{avg})$ will depend on the actual thermal experience of the organism(s) in question, and can be estimated by integrating the TPC function across the temperatures experienced.

Box 1: Jensen's inequality, or the "fallacy of the average": why incubation at variable temperatures sometimes produces different responses than incubation at the average.

In this hypothetical example, a biological rate (development) has been estimated for a population of organisms across different constant temperature regimes, and modeled using a gaussian function (for other common functions used in modeling Thermal Performance Curves (TPCs), *see* [47]) with the following equation:

$$D = 2e^{\left(-0.5\left(\frac{|T-30|}{6}\right)^2\right)}$$

where D is development rate (stages/day) and T is the temperature experienced by the organism (°C). The resulting function modeling thermal performance of development in response to temperature is shown in Box Figures 1-3

Example 1

If continuous thermal variability (blue box) from 12 – 18 °C around an average of 15 °C (T_{avg}) is experienced by an organism over a period of 24h, Jensen's inequality suggests that the realized daily performance will be the *average of the function* across the temperatures experienced ($P_{avg}(T)$), rather than a *function of the average temperature* experienced ($P(T_{avg})$). If we calculate $P(T_{avg})$, we find an estimate of 0.087 stages for this day. However, if we take the average of the development rate function from 12 – 18 °C, by taking the average of the integral beneath the curve, we find an estimate of 0.107 stages for this day. In this example, taking the function of the average temperature would result in a lower estimation of performance than taking the average of the function for all T experienced, which explains why, in this case, constant incubation at 15 °C (T_{avg}) should result in lower performance than thermally variable incubation from 12 – 18°C.
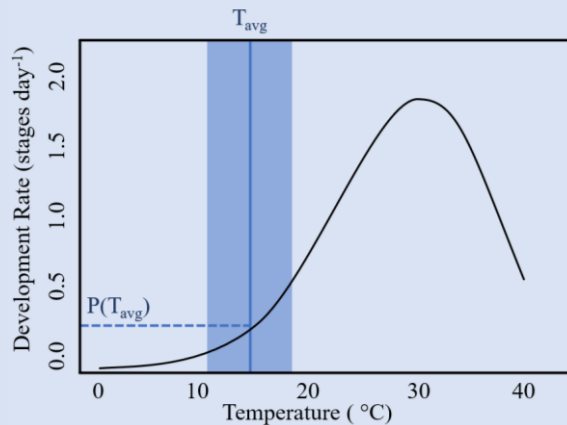
Box Figure 1: *Hypothetical thermal performance curve demonstrating a situation in which variable temperature incubation around a mean is expected to result in greater performance than a constant mean temperature.*

Example 2

In contrast, if we were to incubate the organism under the same magnitude of variability (Box Figure 2; red region), but in the concave portion of the curve (T_{avg} = 30 °C, range = 27 – 33°C, Box Figure 2), we estimate P(T_{avg}) as 2.00 stages for this day, but P_{avg}(T) as 1.92 stages. Therefore, we expect higher realized performance from incubation at a constant 30 °C.
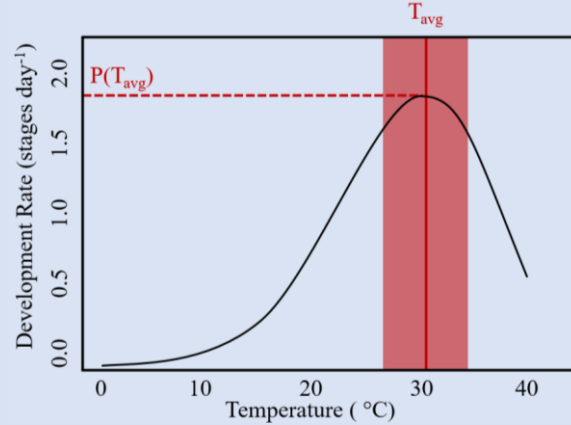
Box Figure 2: *Hypothetical thermal performance curve demonstrating a situation in which variable temperature incubation around a mean is expected to result in lower performance than a constant mean temperature.*

Example 3

In areas of the curve which are approximately linear (Box Figure 3, grey region), we expect P_{avg}(T) to approach P(T_{avg}). For example, if we incubate the organism at 21 – 27 °C with T_{avg} = 24 °C, both P(T_{avg}) and P_{avg}(T) are 1.21 stages. We would therefore expect realized performance to be similar in constant 24 °C and variable 21 – 27 °C regimes, because of the approximate linearity of this region of the TPC.
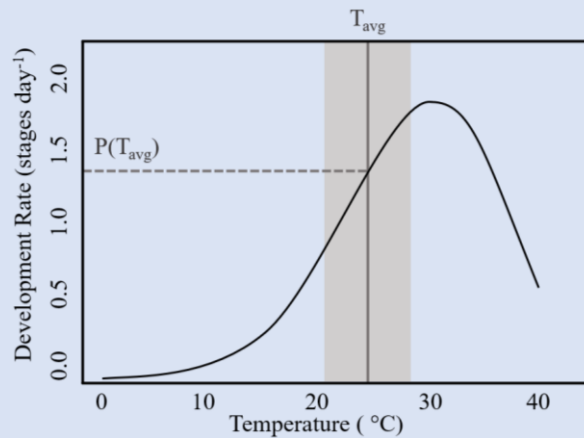
Box Figure 3: *Hypothetical thermal performance curve demonstrating a situation in which variable temperature incubation around a mean is expected to result in equal performance as a constant mean temperature.*

These generalizations are appropriate for regions of the TPC that are strictly concave, convex, or linear. However, if thermal variability occurs across a broad range encompassing more than one of these patterns, the relationship between P_{avg}(T) and P(T_{avg}) becomes more complex. To account for Jensen's inequality in these scenarios, P_{avg}(T) should be directly calculated by integrating the performance function across the temperatures experienced.

Interpreting thermal variability using TPCs

Studies incubating ectothermic vertebrate eggs under different thermal regimes have reported complex findings with regard to the relative effects of thermally variable incubation and constant temperature incubation. Overall, authors have found that the phenotypic results of incubation are largely dissimilar between regimes; that is, variability itself does not represent a single treatment. The 'fallacy of the average' provides a reasonable explanation for why these results differ in magnitude and direction of phenotypic effects, based on what might be expected from Jensen's inequality. Specifically, the fallacy of the average is useful for interpreting differences between results expected from a constant mean temperature, and the effects resulting from thermal variation around a mean (*e.g.*, Figure 7A).

For example, Les *et al.* [48] incubated painted turtle (*Chrysemys picta*) eggs at constant temperatures near the lower and upper limits for successful development (23 and 31°C; in the area of CT_{min} and CT_{max}, respectively), and regimes fluctuating 3 °C around those means. Based on theory, we expect that fluctuations around the convex area of CT_{min} should accelerate developmental traits, and those around the concave area of CT_{max} should decelerate them, relative to constant average temperatures (*as in* Box 1).

Indeed, *C. picta* embryos incubated near the lower limit experienced enhanced survival and development rates relative to constant temperatures, whereas those incubated near the upper limit developed slower and had higher mortality, as predicted [48]. Similarly, Warner & Shine [25,49] found that diel thermal fluctuations around a warm temperature mean near the thermal optimum decreased development rate in lizard (*Amphibolurus muricatus*) embryos, but development rate was enhanced when fluctuations occurred around a cool temperature mean, aligning with predictions. In a study incubating a lizard (*Sceloporus undulatus*) at a constant 28 °C, as well as 28 ± 5 °C, no difference in development rate was found [50]. Given that this range of temperatures falls between CT_{min} and CT_{max} for *S. undulatus* [51], it is likely that the temperatures experienced fall in an approximately linear portion of the TPC for development rate, which explains the lack of difference in incubation period between the two treatments.

Studies modifying the magnitude of variability around a constant thermal mean also demonstrate predictions stemming from the fallacy of the average. When eggs of Bynoe's

geckos (*Heteronotia binoei*) are incubated at a constant mean (32 °C), as well as treatments with fluctuations around that mean (32 ± 3, 5, and 9 °C), increasing fluctuations result in slower developmental rates, and the largest fluctuation results in significant mortality [52]. Given that the thermal optimum for development lies at 30 °C in this species [53], we assume that the temperatures experienced largely fall in the concave portion of the TPC for development rate in this species, and increasing thermal variability around a mean of 32 °C is expected to produce increasing reductions in performance than at a constant 32 °C. Similarly, in Chinese pond turtles (*Chinemys reevesii*) and Chinese softshell turtles (*Pelodiscus sinensis*), development rate decreases as the magnitude of fluctuations around T_{opt} increases [54,55], supporting the predictions of theory.

These experiments demonstrating the fallacy of the average show that variability should not be considered a monolithic treatment; the phenotypic response to variability appears to depend on the range of temperatures experienced, relative to the thermal sensitivity of the trait and organism in question. An existing difficulty in understanding the results of thermal variability experiments lies in the fact that thermal reaction norms for developmental traits are typically poorly characterized [17,25] resulting in a lack of physiological context when interpreting results. Future work would benefit, at the least, from qualitative knowledge of whether incubation temperatures are 'hot' or 'cold' relative to the thermal sensitivity of the trait and study organism [48], or by incorporating empirical frameworks describing thermal reaction norms derived at constant temperatures for the traits in question [41,56].

It is important to note, however, that TPCs have several limitations [22,57]. A key but rarely met assumption of TPCs is that thermal histories, both leading up to and during incubation treatments, do not affect resulting phenotypes through acclimation. Although we can eliminate the effect of the embryo's previous thermal history in the present review, as the studies discussed herein incubated embryos for the entirety of incubation, there is a possibility that transgenerational plasticity (*via* parental effects) or acclimation to temperature during incubation contributed to additional variation in phenotype. Further, given that TPCs are typically constructed under constant conditions, they may not accurately reflect instantaneous performance, especially at stressful temperatures. For

example, Niehaus *et al.* [44] incubated striped marsh frogs (*Limnodynastes peronii*) under two levels of diel thermal variation and found that TPCs underpredicted development rate when thermal variability was high. This finding suggests that development occurs more quickly at stressful temperatures when exposure periods are brief (*i.e.,* under daily sinusoidal variation), but development rates are slowed under chronic exposure to high temperatures (*i.e.,* the conditions under which TPCs are constructed). Nevertheless, given that TPCs have been able to explain the majority of variation in development rate in the wild [45], and modifications accounting for time-dependent effects are available [57], TPCs remain a useful avenue for explaining or predicting the effects of thermally variable incubation.

What have we learned from other thermally variable regimes?

Seasonal variation in temperature

In addition to experiencing diel thermal variation, embryos in seasonal environments can experience increasing or decreasing mean temperatures as incubation progresses (illustrated in Figure 7B). Seasonal thermal variation is particularly relevant to embryos that undergo long incubation periods: northern Australian saltwater crocodiles (*Crocodylus porosus*), for example, have a mean incubation period of 101 days, and mean nest temperatures decrease seasonally throughout development [58,59]. In combination with environmental sources of heat, both large-bodied testudines and crocodilian embryos can also generate metabolic heat throughout incubation, adding considerable heat to nests during the latter half of development [60–63].

Seasonally variable incubation regimes appear to influence embryonic development differently than do stable temperatures when thermal means are identical. For example, Shine [64] simulated three realistic seasonal incubation scenarios for *Bassiana duperreyi* lizards, all with the same thermal mean: stable, increasing throughout incubation, and decreasing throughout incubation. These three treatments generated significant differences in developmental dynamics and hatchling phenotypes, with faster development occurring in both seasonal regimes, and high deformity levels resulting in poor locomotor performance under seasonally decreasing temperatures [64]. These

results suggest that seasonal changes in temperature, in spite of mean temperatures, are sufficient to generate significant phenotypic variation, and may be of particular relevance to species that have phenologically or geographically dispersed nesting [64].

Because many organisms with long incubation periods experience gradual seasonal thermal changes, it is also possible that embryonic thermal sensitivities can be locally adapted to seasonality, *i.e.*, that thermal sensitivity changes as development progresses. Indeed, in Arctic char (*Salvelinus alpinus*), populations that spawn in relatively warm autumn temperatures and incubate throughout winter appear to have much higher hatching success and fewer deformities when warm temperatures are used during early incubation, and subsequently fall [65]. In fact, the reversal of natural seasonal temperatures, *i.e.,* moving from cool to warm incubation temperatures, results in significantly higher mortality and deformity rate in embryos [65]. These results highlight the importance of understanding how natural thermal variability in study populations relate to the temporal dynamics of development. For long-incubating species in seasonal environments, the question of whether embryonic thermal sensitivities change as development progresses has yet to be explicitly explored.

Heat shock

Heat shock refers to short exposures to sublethal, high temperatures, often resulting in increased thermal tolerance upon subsequent exposures [66]. If an organism experiences temperatures near the upper range of its thermal tolerance, denatured and abnormal proteins are produced, triggering the production of heat shock proteins (hsps). Hsps, among other cellular changes, ameliorate negative effects of heat on cellular proteins [67–69]. Ultimately, brief exposures to hot temperatures in adult organisms have been shown to produce an effect known as 'heat hardening' [70] in which there is a transient increase in thermal tolerance.

For embryos, however, it appears that very brief exposures to high temperatures (*i.e.,* heat shocks, example illustrated in Figure 7E) throughout development have minute effects on embryonic and post-hatching phenotypes, at least for many of the traits investigated so far. For instance, Lim *et al.* [71] incubated lake whitefish (*Coregonus clupeaformis*) under a near-constant 2 °C regime, with 1 h temperature spikes to 5 °C

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twice-weekly, and found that development proceeded at the same rate as in constant 2 °C treatments. A slight difference in body length emerged at the pre-hatch stage, wherein embryos from the heat-shock regime were slightly (5%) longer. Another study on whitefish found similar results, where weekly 1 h temperature spikes of varying magnitude (from 2 – 5 °C and from 2 – 7 °C) had no effect on incubation period, survival, or size at hatch relative to a constant 2 °C regime [72]. In future experiments, it may prove interesting to increase the frequency of heat shocks as body size of whitefish embryos in these experiments was affected by twice-weekly heat shocks, but not by once-weekly heat shocks of the same magnitude, suggesting as others have [44,57] that performance responds differently as the duration of exposures changes.

The severity of the heat shock relative to the organism's thermal sensitivity may also play a role in determining whether phenotypic effects are significant. Overall [73] incubated canyon lizards (*Sceloporus merriami*) at both moderate (31 °C) and hot (34 °C) constant temperatures with brief daily exposures to 37 °C. Heat shocks accelerated development rate and increased hatchling body size relative to constant temperatures, but there was low embryo survival in the hot (34°C) heat-shocked treatment. In canyon lizards, constant incubation at 37 °C is lethal to embryos, but short exposures to severe temperatures appear to enhance phenotype (larger body size, earlier hatch date), increasing mortality only when mean incubation temperatures are already stressful [73].

There are still many questions that remain unanswered regarding temperature shocks during development. Firstly, to my knowledge, no experiments have tested the effects of cold shocks on oviparous ectothermic vertebrate embryos. Given that cold temperatures and hot temperatures illicit different gene expression responses in adult ectotherms [74], cold shocks may result in different phenotypic responses than those observed as a result of heat shocks. Next, it is important to note that these studies generally tested whole-organism level responses to heat shocks (*e.g.*, body size, locomotor performance). Given that acute heat shocks to embryos have been shown to illicit significant transcriptomic changes in expression of developmental and hsp genes [75,76], and that isolated incidents of thermal stress can transiently affect embryonic heart rates [77], it is likely that temperature shocks during development can significantly – and perhaps, persistently – affect other traits that have not yet been tested.

Idiosyncratic thermal regimes

I termed regimes involving temperature changes at various timepoints during development 'idiosyncratic' (Figure 7F). Often, these experiments aim to isolate temperature effects that occur at discrete developmental stages; for example, to determine temperature-sensitive periods for sex determination under TSD [78], but they can also be applied to developmental studies investigating time-sensitive effects of temperature during development.

Thus far, critical windows of physiological sensitivity to temperature have been identified by temperature-switching experiments. By changing *C. clupeaformis* embryos between cool, moderate, and warm constant thermal regimes at key milestones during development, Eme *et al.* [79] found that organogenesis represented a particularly sensitive period through which strong plasticity acts on heart rate and oxygen metabolism. Ultimately, the significant physiological changes occurring during organogenesis persisted into hatchlings. Identification of critical windows, such as organogenesis, may further elucidate mechanisms by which developmental plasticity (*see* 'Irreversible developmental plasticity', below) occurs.

Other studies that changed temperatures at various timepoints throughout development have specifically investigated embryonic acclimation capacity. Booth [80] found no evidence for metabolic acclimation in Brisbane river turtles (*Emydura signata*), showing through a temperature-switch experiment that embryonic metabolism during the latter half of development was not dependent on embryos' previous thermal experience in early development. Likewise, Angilletta [81] reported that, although embryos exhibit metabolic temperature sensitivity throughout development, *S. undulatus* embryos did not undergo metabolic acclimation after being temperature-switched during development. Interestingly, these results directly conflict with the identification of organogenesis as a plastic or acclimatory window in *C. clupeaformis* [79], perhaps suggesting that taxonomic differences in embryonic metabolic acclimation capacity exist.

Irreversible developmental plasticity: how persistent are the effects of thermal fluctuations?

Previously, I discussed the effects of thermal variation primarily as they affect embryos and hatchlings. However, the developmental environment can continue to shape the phenotype of organisms beyond the incubation period, *via* developmental plasticity. Developmental plasticity is often considered a permanent change to phenotype as a result of early developmental environments ("irreversible nongenetic adaptation"; Kinne [82]), albeit there are many exceptions to this irreversibility (*e.g.,* [83–85]). At constant temperatures, developmental plasticity to temperature is well-described for numerous traits, but what happens when eggs are incubated at fluctuating temperatures?

In an elegant experiment in zebrafish (*Danio rerio*), Schaefer & Ryan [86] attempted to disentangle the relative contributions of reversible and developmental plasticity to diel fluctuating temperature regimes to thermal tolerance. After rearing fish from eggs for 100 days across a range of constant and fluctuating conditions, the authors acclimatized fish to constant temperatures for 12 - 15 days. They then tested the thermal tolerance of fish, and determined that the critical thermal maximum, or temperature at which opercular spasms occur, was significantly higher in fish incubated and reared at fluctuating temperatures when compared to constant temperatures, regardless of acclimation temperature (reversible plasticity). The ages of fish at the time of testing (112 – 115 d) represent a significant portion of the lifespan of zebrafish, suggesting that their persistent physiological plastic response to fluctuating temperatures can affect long-term fitness. Likewise, long-term morphological differences arising from incubation regime have been detected in other fishes. Nathanailides *et al.* [87] incubated Atlantic salmon (*Salmo salar*) at a constant temperature of 11 °C and at natural, seasonal temperatures that gradually rose throughout incubation (5 - 10 °C). Although fish from the constant regime hatched sooner and had larger muscle fibres, by 3 weeks post-feeding, fish from the ambient thermal regime exhibited significantly faster growth rates and larger muscle fibre area. Again, these effects have significant ecological importance: the post-hatching growth rates of salmonids are strongly linked to fitness [88].

Several experiments in reptiles have also examined long-term responses of traits to variable developmental temperatures, finding mixed results with respect to the

persistence of developmental plasticity. For example, Pearson & Warner [24] incubated the lizard *Anolis sagrei* at fluctuating temperatures with different thermal means and found that differences in running performance between treatment groups at hatching disappeared at 3 weeks. Conversely, in the lizard *B. duperreyi*, running performance was consistently higher in individuals incubated in a warm fluctuating regime for 20 weeks post-hatching relative to those incubated in cold fluctuating regimes, although morphological differences apparent at hatching disappeared within 6 weeks [89]. In a study on Western fence lizards (*Sceloporus occidentalis*), some morphological characters (body size, hindlimb length) persist after incubation, while others (forelimb and tail lengths) do not [90]; it is possible that body size and hindlimb lengths, which are directly linked to sprint speed in this species [91], exhibit a higher degree of developmental canalization than traits for which links to fitness are unclear [90], although more trait-focused studies are needed to resolve this puzzling inconsistency.

The studies mentioned previously in this section described long-term effects measured in captive animals, but how do measures change when animals are reared in natural habitats? Dayanada *et al*. [92,93] conducted experiments on velvet geckos (*Oedura lesueurii*) in which eggs were incubated at fluctuating temperature regimes reflecting current (cold) and future (warm) conditions. After releasing hatchlings into the field, they determined that cold-incubated hatchlings had significantly higher survival and growth rates *in-situ* than did warm-incubated hatchlings 10 months after release. Furthermore, the authors found differences in post-hatching growth rates of geckos between two release sites, suggesting that post-hatching environmental conditions interact with incubation conditions to produce significant effects on phenotype. Similarly, Andrews *et al.* [50] found that cold fluctuating incubation temperatures resulted in higher survival 7 – 9 months post-hatch in lizards (*Sceloporus undulatus*), when compared to high fluctuating incubation temperatures. Interestingly, in this experiment, post-hatching growth rates in the field ultimately were not influenced by incubation regime; it is possible that the degree of developmental plasticity in response to temperatures was low for growth rates, or that developmentally plastic growth rate differences could not be fully realized under natural conditions (*e.g*., due to low food availability). Although long-term studies under natural conditions are scarce, they raise interesting questions about

whether phenotypic differences caused by incubation regime can be masked by natural conditions, and about which traits are ultimately relevant to fitness in the wild.

Long-term changes in gene and protein expression in response to developmentally variable temperatures have not been widely explored. Nonetheless they may represent an interesting avenue for future research. Recent literature has revealed that, during acclimation to fluctuating thermal regimes, adult fish exhibit large-scale changes in mRNA levels for genes that regulate cell growth and proliferation, molecular chaperones, and cellular membrane integrity [74]. Furthermore, different genes appear to be activated at fluctuating *vs.* constant temperature conditions [74]. With regards to proteins, increases in the concentration of hsp70 molecular chaperones in response to fluctuating temperatures have been linked to improved thermal tolerance in adult fishes [94,95], and seasonal changes in heat shock protein levels occur in frogs [96]. Given the persistent effects of thermal variability during incubation on ectothermic vertebrate thermal tolerance, morphology, growth rates, and locomotor performance mentioned herein, it is not difficult to imagine that developmentally plastic molecular changes would also be detectable. Future work should leverage established molecular techniques such as mRNA and heat shock protein assays to illuminate the proximate mechanisms of developmental plasticity in response to thermal variability.

Other sources of phenotypic variation

Although temperature overwhelmingly governs the development of oviparous ectothermic vertebrates, there are other major factors that can influence incubation and phenotype of larvae and hatchlings. First, theory predicts that population-level differences between the thermal sensitivity of traits should emerge due to adaptation to local thermal regimes [38], and indeed this prediction is founded within the studies of thermal variability herein. For example, Buckley *et al*. [90] incubated *S. occidentalis* eggs from four populations under variable thermal regimes and found significant population-level effects on several traits relevant to fitness (*e.g.*, body size and hindlimb length). Further differences that arise because of differences between clutches of eggs have also been heavily discussed. Numerous studies in reptiles [50,97–100] have estimated significant clutch effects on the phenotypic outcomes that were measured, although the

extent to which these differences arise from genetic differences between families, maternal effects, or combinations thereof have yet to be explored in detail.

Recent studies that illustrate significant effects of transgenerational plasticity, or the ability of environmental influences during a parent's lifetime to affect offspring as well, also present interesting avenues through which to explore the effects of thermal variability. In fishes, parental acclimation to high temperatures enhances growth [101] and size [102] of offspring. Transgenerational effects are particularly strong at high temperatures, suggesting they may ameliorate negative physiological consequences of climate change [102]. Given predicted rises in both mean and variability of future temperatures [103] and the potential of thermal variability to significantly enhance thermal tolerance [86], studies that examine transgenerational plasticity in a variable temperature context could yield much-needed insights about the resilience of ectothermic vertebrates to climate change.

Conclusions and future directions

It has long been acknowledged that incubation at constant temperatures poorly reflects what organisms encounter in nature [24,41,104]. Despite this empirical reality, constant temperatures continue to dominate incubation studies [17]. Variable thermal regimes experienced during incubation have resulted in unanticipated and complex effects, producing phenotypic effects that differ from what would be expected from constant thermal means (*e.g.* [48,49]), and permanently enhancing thermal tolerance [86], morphology [90], and growth rates, even after release into the wild [92,93].

Existing studies that employ variable temperature incubation suggest fruitful avenues for future research. Firstly, the field would benefit significantly from incorporating physiological frameworks that assist in explaining the phenotypes produced under variable temperature regimes (*e.g.*, the use of TPCs). Incorporation of physiological knowledge opens up possibilities that integrate our vast knowledge of incubation under constant regimes with thermal variability, and is especially important because the majority of existing studies do not use physiologically relevant data — that is, data that are empirically anchored in what the organism is likely to experience under its own, often population-specific, natural conditions — to inform the experimental design or analyses.

To facilitate this integration, authors could aim to quantify thermal reaction norms for their traits of interest, especially because broadly characterized reaction norms are uncommon in the literature [17].

Most of the attention in studies of thermal variation has been, thus far, devoted to reptiles, and currently there is a relative paucity of studies on amphibians and non-commercial fish species. This fact is puzzling, considering that many amphibians and fishes are short-lived organisms with fast life-histories, and are thus predicted to experience strong developmental plasticity to temperature [99]. Researchers could consider directing future studies of fluctuating temperature incubation towards taxa that might be ideal for informing a generally applicable predictive framework for understanding organismal responses to climate change-driven thermal fluctuations. In particular, tropical stenotherms with narrow margins of thermal functionality may be more heavily impacted by climate-driven increases in thermal variation, and are thus prime candidates for further investigation [23].

Importantly, more studies of proximate mechanisms that lead to phenotypic changes under variable temperatures are needed. Constant temperature studies have already utilized genetic tools such as transcriptomics to explain thermal acclimation [105], and studies in adult organisms have investigated gene expression changes in response to variable thermal regimes in adult organisms [74]. However, there is currently a considerable gap of knowledge about the mechanisms that impart phenotype under thermal variability.

Taken together, these recommendations suggest the promise of exciting future work on thermal variability. The breadth of studies incubating ectothermic vertebrates under thermally variable conditions is much more robust than previously acknowledged, and these studies have significantly advanced our understanding of natural thermal regimes and their relationship to organismal development and ontogeny.

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Chapter 3: Developmental plasticity facilitates enduring physiological tolerance under ecologically realistic temperatures

Abstract

Aquatic ecosystems are more frequently facing lethal hypoxic events, warmer average temperatures, and increased thermal variability. Within-lifetime phenotypic plasticity can act to buffer the negative impacts of these environmental stressors, but data from experiments that track ecologically relevant variation across an organism's lifetime are needed to better understand and reasonably predict long-term organismal responses. In this 8-month experiment following zebrafish (*Danio rerio*) from fertilization through sexual maturity, I use a factorial design to isolate the effects of developmental plasticity in early ontogeny (0 – 29 days post-fertilization) from effects of later life acclimation (30+ days post-fertilization) on multiple physiological traits, in response to ecologically realistic diel thermal variability (27 °C ± 5 °C) or a constant optimal temperature (27 °C). I show that developmental plasticity in response to thermal variability can facilitate enduring, beneficial plasticity in multiple physiological traits, including thermal and hypoxia tolerance (CT_{max} and P_{crit}), resting metabolic rates, and oxygen supply capacity (alpha). However, only thermal tolerance was additively improved by experiencing acclimation to thermal variability later in life. These results renew appreciation for the early developmental environment as a modulator of adult phenotype, and emphasize the importance of long-term studies in understanding the gamut of phenotypic variation achievable though plasticity.

Introduction

Aquatic ecosystems are more frequently facing lethal hypoxic events, warmer average temperatures, and increased thermal variability [1–3]. The persistence of organisms under these conditions will depend on their ability to migrate to more favorable environments, undergo adaptive evolution, or nongenetically alter their physiological tolerances to suit prevailing environmental conditions *via* phenotypic plasticity [4]. In particular, plasticity is implicated as a key driver of rapid phenotypic responses to environmental stressors [5], especially for aquatic fishes, which may face barriers to dispersal [6] and limited capacity for evolution [7]. Indeed, fishes display remarkable plasticity across multiple levels of biological organization, allowing them to cope with changing within-lifetime environments [8].

In the context of climate change, a focus in the study of plasticity are the largely reversible phenotypic changes that occur in mature organisms exposed to short-term stressors, or acclimation [9]. Yet, fishes must cope with the reoccurrence of stressful environments throughout their lives, from the earliest embryonic stages through adulthood. Alongside acclimation in mature fishes, early life experiences may also confer beneficial responses to environmental stressors through developmental plasticity [10,11], and compelling evidence suggests developmental conditions can lead to physiological changes that endure through an organism's lifetime [10,12]. However, our understanding of developmental plasticity's role in promoting resilience to environmental stressors is limited by a paucity of long-term studies investigating fitness-related physiological traits [13,14].

Most studies investigating plastic responses to climate change focus on isolated traits [15], but physiological processes are inherently environmentally sensitive and plasticity can occur in multiple traits simultaneously [9]. Moreover, some physiological traits are pleiotropically or ecologically linked, promoting tolerance to multiple stressors after exposure to a single stressor (*e.g.*, 'cross-tolerance' [16]). For instance, the release of heat shock proteins in response to thermal stress can also promote hypoxia tolerance through shared regulatory pathways [17,18], and cause overarching changes to stress response and aerobic metabolism [19,20]. In aquatic fishes, these co-occurring responses are particularly relevant because environmental heat and hypoxia stressors are

physiochemically and ecologically linked, such that oxygen solubility decreases and the metabolic demands of living organisms increase as water temperatures rise [21,22]. Fishes, under warming scenarios, will therefore likely be coping with the combined influence of thermal stress, hypoxic environments, and increased metabolic demands [1,23]. It is therefore increasingly important that we study plastic responses to stressors in multiple, ecologically relevant traits to better anticipate future performance of fishes.

Temperature acts as an overarching driver of biological functions in aquatic ecosystems [24], and beneficial plasticity of thermal tolerance in response to thermal stress is well-documented in fishes, with thermal tolerance generally increasing after exposure to warm temperatures [25]. Less explored are the co-occurring responses of other traits, such as hypoxia toleranec or metabolism, to thermal stressors; in these, there appears to be is no unequivocal signature of beneficial plasticity following heat exposure. For example, authors have generally found that short-term exposures to hot temperatures (*e.g.,* from hours-long heat shocks to several weeks of exposure), generally reduce the capacity for hypoxia tolerance and metabolic compensation of fishes, even if thermal tolerance is improved (*e.g.*, [26–30]). These results are somewhat surprising, given our understanding of the shared mechanisms governing thermal tolerance and cardiorespiratory capacity in fishes [17,18,20].

But, it is possible that the timescales on which plasticity operates extend beyond common experimental timelines. Indeed, longer-term acclimation periods to warm temperatures in mature fishes (*e.g.*, one month or more) have often been shown to improve hypoxia tolerance [26,31], potentially *via* modifications to the cardiorespiratory system including gill remodeling [31,32] and increases in aerobic scope [33]. It is therefore likely that early developmental thermal environments can significantly influence adult physiological tolerance. For example, long-term exposure of young coral reef fish to hot temperatures can induce metabolic compensation in adults [34]. Yet, thermal developmental plasticity in physiological traits is underexplored [11,14] and can be difficult to delineate from later-life acclimation responses, if authors do not isolate developmental periods [35]. To fully understand the sources of adult physiological tolerance in fishes, we must better understand the effects of both early developmental and adult environments.

A remaining challenge in understanding if plasticity can benefit fishes in changing climates is that our foundational knowledge is largely derived from experiments exposing animals to constant temperatures [14]. However, thermal variability is the norm in nature, and thermal stressors are unlikely to occur in the form of hot, static temperatures [36]. Constant and variable temperatures elicit different responses, even when they share thermal means [37]; this can lead to underpredictions of performance metrics [38,39] and deleterious pathological effects that bias experiments, especially at hot temperatures [40,41]. Therefore, in addition to understanding the importance of timing of thermal stressors, there is a also critical need to select experimental conditions that more accurately reflect current or future thermal environments to make more robust predictions of adult tolerance.

In the present experiment, I address these challenges by comparing plasticity of four related physiological traits in response to ecologically relevant thermal variability: heat tolerance, hypoxia tolerance, resting metabolic rate, and oxygen supply capacity. I test these traits in 8-month old zebrafish (*Danio rerio*) that experienced thermal variability (27 ± 5 °C) during early development (embryonic and larval stages; $0 - 29$ days post-fertilization), late development (juvenile and adult stages; 30+ days post-fertilization), or both, contrasting these treatments with a constant optimal control temperature (27 °C). This modified 'strong-inference' (factorial design) approach [42] allowed for isolation of Developmental Plasticity from later Acclimation effects in response to thermal variability [26,43]. I selected a variable temperature regime to better reflect natural environmental conditions, in which fish may be transiently exposed to stressful temperatures, while reducing the experimentally confounding influence of static heat-induced pathologies [40]. The primary objectives of this study are thus: (i) to determine whether beneficial plasticity in heat and hypoxia tolerance occurs in response to ecologically relevant thermal variability and (ii) to identify the contributions of Developmental Plasticity and Acclimation on potential beneficial plasticity responses in adult fish.

Methods

Fish rearing and thermal regimes

The fish used in this experiment were from the same cohort as those used in Chapter 4, using the same experimental design and timeline, but different clutches of embryos. I began these experiments in February 2021 with three clutches of recently (0 – 2 h) fertilized eggs from three separate wildtype-AB zebrafish pairings, collected from the Dalhousie Zebrafish Core Facility (ZCF). The ZCF fish are kept in standard conditions at a constant temperature (27 – 28 °C) and use the same husbandry protocols described herein. I transferred embryos to my laboratory in the Dalhousie Aquatron facility within 3 hours post-fertilization (hpf), and randomly and equally divided them into our experimental thermal treatments by 4 hpf, keeping embryos within original clutches (*i.e.* with siblings).

I contrasted two thermal treatments, a Constant and diel Fluctuating temperature regime. The Constant thermal treatment was set to a static temperature of 27 °C, representing the commonly used temperature in zebrafish laboratories, and within the optimal range of temperatures for zebrafish growth and development [44,45]. The Fluctuating treatment was set to follow a sinusoidal diel cycle, smoothly alternating between 22 °C at 24:00 h and 32 °C at 12:00 h (*see* Figure 4 in Chapter 1). I specifically selected a thermally variable regime in order to transiently expose fish to high temperatures in a naturalistic manner, minimizing damaging and ecologically irrelevant pathologies that could be introduced by hot constant temperature regimes [40]. The Fluctuating temperatures in this treatment spanned the range of temperatures that zebrafish in early life-stages can tolerate without causing high degrees of mortality and deformity [44]. Therefore, the Fluctuating regime represents a physiologically challenging level of thermal variability, but still falls within a natural range of daily variability zebrafish experience in the wild [46]. Experimental fish were kept in 14 hour light : 10 hour dark photoperiod conditions, and fed size-appropriate commerical feed (Gemma Micro, Skretting, Tooele) twice-daily.

Fish were kept in their initial thermal treatment throughout embryonic and larval development (0 – 29 days post-fertilization), creating two Developmental Temperature

treatment groups (Figure 9), in which there were three replicates (clutches) of approximately 60 larvae each. At day 30, representing the onset of the juvenile life-stage [47], fish were split once more into either Constant or Fluctuating thermal treatment groups for the remainder of juvenile and adult development (30 days – 8 months), creating an Acclimation Temperature treatment group, until physiological trails began at 7 months post-fertilization (Figure 9). In total, there were 12 tanks of 12 – 15 fish, comprised of three replicates each of the four combined Developmental and Acclimation temperature treatment groups.
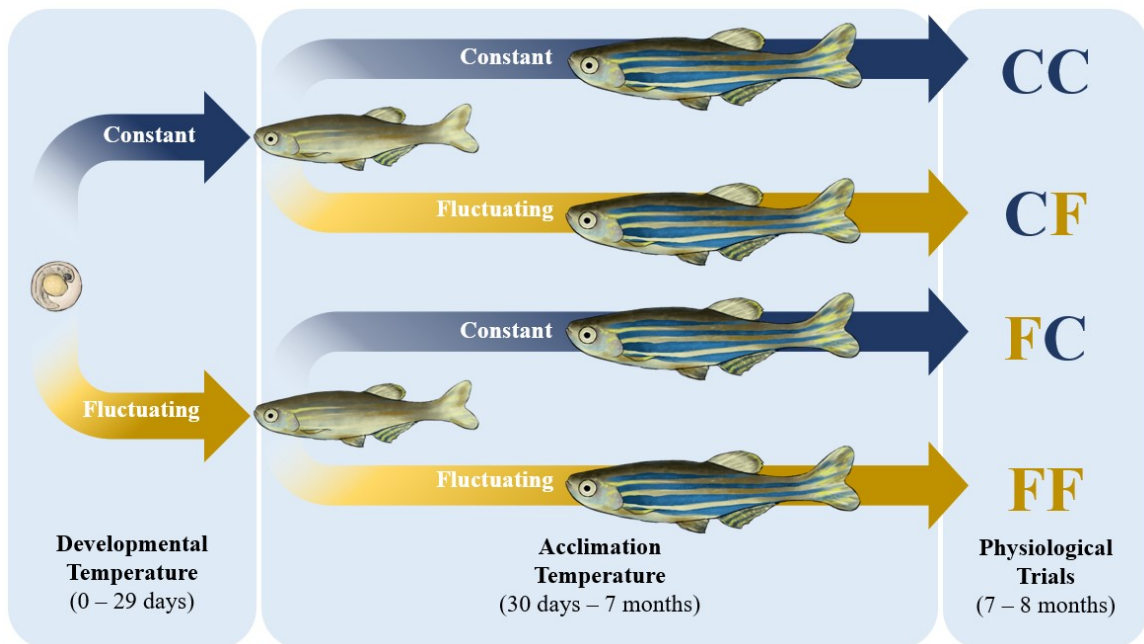
Figure 9: *Flowchart illustrating split-clutch experimental design testing the effects of Constant (27 °C; C) or diel Fluctuating (sinusoidal 22 – 27 °C; F) experienced during a Developmental Temperature treatment (embryonic and larval stages, 0 – 29 days post-fertilization) and an Acclimation Temperature treatment (juvenile and adult stages, 30 + days post-fertilization) in zebrafish (Danio rerio). This experiment was designed to contrast the effects of developmental plasticity vs. later acclimation on traits of adult zebrafish. Physiological trials (thermal tolerance, hypoxia tolerance, resting metabolic rates, and oxygen supply capacity) were performed at 7 – 8 months post-fertilization. Final combined Developmental and Acclimation Temperature treatment groups are denoted by respective letter combinations CC, CF, FC, and FF.*

Critical thermal maxima (CT$_{max}$) trials

To measure thermal tolerance, I used the critical thermal maximum (CT$_{max}$) method [48,49]. CT$_{max}$ is a widely used, repeatable, and physiologically meaningful measure of maximum thermal tolerance, defined as the temperature during thermal ramping at which locomotion becomes disorganized [48,49]. For all CT$_{max}$ trials, I used the recommended thermal ramping rate of 0.3 °C/min, which is slow enough to allow body temperatures to match water temperatures, while fast enough to minimize within-trial thermal acclimation [49]. I defined the CT$_{max}$ endpoint as loss of equilibrium (LOE) for a minimum of two seconds of uncontrolled and disorganized swimming.

To test CT$_{max}$, I built a custom assay chamber from a 10 L fish tank insulated in a styrofoam container. I used two 300 W immersion heating coils encased in a protective metal enclosure to heat water, and fish were separated from the heating coil compartment by an inserted mesh screen. I included an aquarium circulation pump to ensure water mixing and oxygenation of water, and measured water temperatures with a resolution of 0.02 °C in the fish compartment using a TSUB21 temperature probe (PyroScience GmbH, Aachen, Germany).

In August 2021, I began CT$_{max}$ trials. I took groups of five fasted (~24 h) adult tankmates, carefully placing them in the trial tank with fresh system water set to 27 °C, avoiding prolonged air exposure. Groups of fish, rather than individual fish, were used, because zebrafish are a highly social shoaling species [46]. Therefore, group CT$_{max}$ assays were expected both to reduce stress and to increase the realism of the test; further, this allowed my results to be comparable to other studies of zebrafish using group CT$_{max}$ assays and improve throughput [50,51]. Trials were conducted over four days until each of the 12 tanks had ~10 fish sampled; the order in which CT$_{max}$ trials were conducted each day was randomized with respect to both treatment and clutch (replicate).

Once all fish were inside the tank, I turned on the heating coils, which warmed the water at a rate of approximately 0.3 °C/min. M. Kate Fredericks monitored water temperatures while I monitored fish for signs of distress or LOE. Trials took approximately 45 min; fish were removed from the trial when they displayed LOE for 2 s. At this point, temperature was recorded, and fish placed in individualized aerated recovery tanks set to 27 °C. After at least 30 min of recovery, fish were anesthetized,

measured for standard length (SL), gently blotted with a clean paper towel, and weighed with a precision of 0.001 g. Visual sex identification was made using established macroscopic sexual characteristics (males were identified by their shallow abdomens, yellow-to-pink abdominal and caudal coloration, and highly pigmented fins). Fish were subsequently returned to their tank of origin and rested for one month before further trials.

Routine metabolic rate, hypoxia tolerance, and oxygen supply capacity trials

I began routine metabolic rate trials in September 2021, to measure routine metabolic rate (RMR), hypoxia tolerance (P_{crit}), and the maximum physiological oxygen supply capacity (α). P_{crit} is is the critical oxygen concentration in water (here, expressed in units of % dissolved oxygen (DO)) at which organisms begin to 'oxyconform', *i.e.*, can no longer maintain their metabolic rate under increasingly low dissolved oxygen levels [53] (*see below*). α represents a biologically meaningful measure of an organism's maximum physiological ability to extract oxygen from its environment (*i.e.*, maximum achievable oxygen consumption, reaching a limit at maximum metaoblic rate) at a give DO [54].

I set up four 180 mL glass chambers as respirometers for individual fish. Each respirometer had a mesh dome fitted to its interior base to separate fish from a 6 mm magnetic stir bar included for water circulation. An oxygen sensor spot on the inside of the chamber detected dissolved oxygen levels, and readings were taken non-invasively by a fiber optic cable (PyroScience GmbH, Aachen, Germany) which was affixed to the outside. These chambers were then placed into a water bath set to 27 °C, sitting atop four magnetic stir plates, for the duration of the trial. Test chambers were sealed tightly with a rubber stopper, ensuring no air bubbles were present in the chamber, and two holes in the rubber stopper permitted water to be pumped in and out of the respirometer during acclimation to ensure water was fully oxygenated before tests. Within the water bath, opaque plastic dividers were used to prevent fish in respirometers from seeing one another and reduce stress.

I fasted test fish for 24 h before trials began to reduce the metabolic requirements of digestion and buildup of nitrogenous wastes in test chambers. Oxygen sensors were

recalibrated each test day according to manufacturer's instructions, and chambers were sterilized before trials using 70% ethanol, then rinsed thoroughly with system water.

Blank measurements (30 min each) using 27 °C system water were made before and after taking fish measurements for each of the four respirometers. The respiration during these two 30 min intervals were averaged for each respirometer, then subtracted from fish MO_2 measurements to account for background (microbial) respiration. 8 mm magnetic stir bars were activated in each respirometer throughout measurements for water mixing.

To take metabolic measurements, individual fish were placed into a test chamber filled with water using a clean, wet net, minimizing air exposure to < 2 s. Each trial consisted of an individual fish from each of the four combined thermal treatments, and respirometers were randomzied with respect to treatment each trial.

Although others have found that an acclimation period of 2 h is sufficient to allow metabolic rate to stabilize [52], pilot trials indicated that the lowest, stable routine metabolic rates tend to occur after 3 h of acclimation. Therefore, I acclimated fish to test chambers for 3 h, flushing fresh 27 °C water from our flow-through system into chambers during this period. After the 3 h acclimation period, I removed flow-through tubes and sealed the chamber completely using small stoppers, careful to minimize fish disturbance. Respirometer volume was estimated using the difference between empty test chambers and chambers filled with water, subtracting fish mass, assuming 1 g of mass was equivalent to 1 mL of volume.

I monitored oxygen levels inside the chambers using the Pyro Oxygen Logging Software (PyroScience GmbH, Aachen, Germany), set to record at 2-minute intervals in units of mg O_2 per L. Recordings of metabolic rate at low ambient oxygen are required to accurately estimate P_{crit} and α, but to reduce mortality and stress to individuals, I did not allow respirometers to reach anoxic levels. I therefore halted experiments if oxygen levels reached anoxic levels (5% dissolved oxygen) or if fish lost equilibrium.

In sum, I aimed to test each combined Developmental and Acclimation temperature treatment group 10 times each, for a total N of 40 individuals. Each replicate (clutch) therefore had 3 – 4 individuals measured per combined treatment group. I accounted for the combined influence of intraclutch and intratank variation on physiological traits statistically (*see below*).

Statistical analyses

Routine metabolic rate (RMR) was estimated using the quantile ($q_{0.2}$) method, such that all recorded oxygen consumption (MO_2) values at normoxia (>80% DO) were split into quantiles and RMR was taken as the value which falls above 20% of recorded values [55]. The $q_{0.2}$ method is expected to produce conservative estimates of RMR and is broadly applicable to most fishes [55].

P_{crit} was estimated using the alpha (α) method [54]. Briefly, RMR was estimated as described above, and a line from the origin to the average of the three highest MO_2/DO values (α-line) was estimated (Figure 10). The α-line therefore represented the maximum physiological oxygen supply capacity at any DO (until maximum MR is reached). Averaging these three values is a more conservative approach than using the single highest MO_2/DO value [54]. The intersection of the α-line and RMR was taken as P_{crit}; in other words, an estimate of the minimum ambient DO level at which RMR can be maintained [54] (Figure 10).
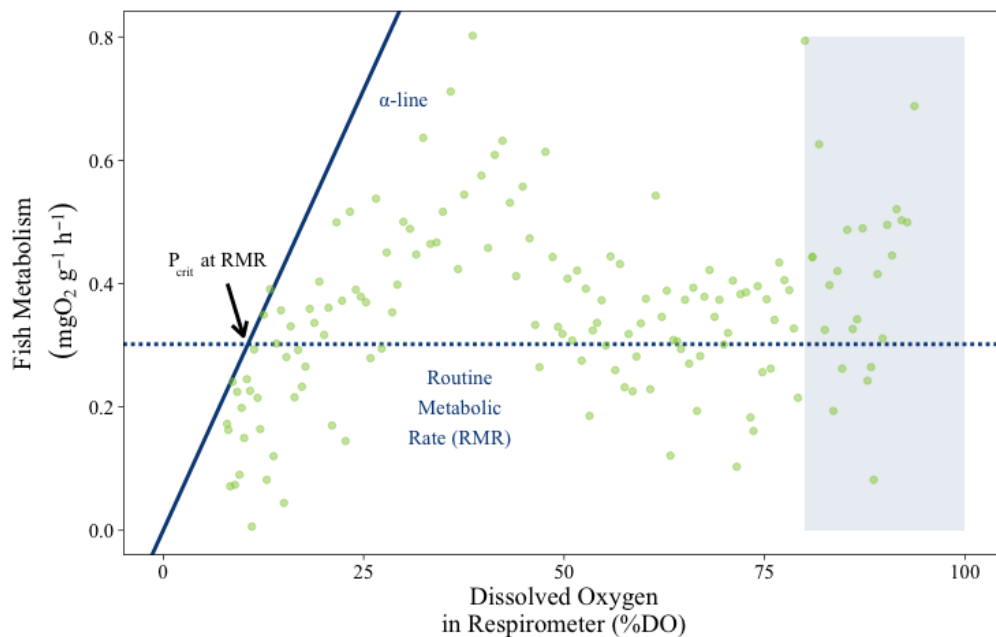
Figure 10: *Example of the estimation of hypoxia tolerance (P_{crit}) using the alpha method, measured at routine metabolic rate (RMR). Data are taken from a single chamber in this experiment; method follows Seibel* et al. *[54]. RMR (dashed line) is first estimated for an individual in normoxia using the lowest quantile method [55]; the α-line (solid line) is then estimated by creating a line from the origin to the average of the three greates metabolism (MO_2) over dissolved oxygen (DO) points [54]. The intersection of these two lines represents P_{crit} α (at RMR),* i.e., *the lowest level of ambient dissolved oxygen at which RMR can be maintained.*

Although P_{crit} can be estimated using a variety of different methods (*summarized in* [54]), I chose the α method because it avoids arbitrary selection of a linear or nonlinear function describing the relationship between ambient DO and MO_2 values, and reduces difficulty associated with achieving model convergence for nonlinear or breakpoint estimates of P_{crit} [54]. Using the alpha method allowed for estimation of both P_{crit} (hypoxia tolerance, taken in the traditional sense) and α (physiological oxygen supply capacity). I used the 'respirometry' package [56] in the R environment to estimate α. P_{crit} at RMR was then estimated by multiplying RMR by α.

I analyzed data using Bayesian generalized linear mixed models describing the effects of adult sex and thermal treatments during two different ontogenetic periods (a 'Developmental' plasticity period from 0 – 29 dpf, and an 'Acclimation' period from 30 dpf – testing at 8 months) on CT_{max}, RMR, P_{crit}, and α.

In a first set of models, Developmental Temperature and Acclimation Temperature (Constant or Fluctuating) were included as fixed predictors. Mass measured from fish in this experiment was included as a continuous covariate in metabolic models (RMR, P_{crit}, and α) to account for metabolic scaling differences that occurred as a result of variation in body size among fish, a best-practice method of standardization [55]. As such, to avoid overcontrol bias [41], causal Acclimation Temperature effects should be interpreted as direct, and not cumulative, effects on responses, because Acclimation Temperature mechanistically impacts adult mass (previous work has shown negligible effect sizes owing to Developmental Temperature; see Chapter 4 [43]).

To isolate estimates of the effect of Sex on responses, I fitted a second set of models for each response variable, including Developmental Temperature as a control for Sex, given that temperatures during larval development are known to influence zebrafish sex ratios [57]. Models estimating the effect of Sex also included mass for metabolic scaling, as above. In all models, I included random intercepts for Tank to account for non-independence between tankmates. Clutch of origin was not included as a covariate because clutches were equally represented among treatment groups (*i.e.,* all three clutches were pooled, but Tank-level dependencies were accounted for). In metabolic models, I also included random intercepts for Day of testing to account for non-independence of measurements due to a combination of differences in daily respirometer calibration and

increases in the age of fish throughout the ten trial days; this was not included in CT$_{max}$ models, because trials took place over four days and CT$_{max}$ is repeatable across the lifetime of zebrafish [50]. All models used a Gaussian response distribution and identity link function. The final models took the form:

1. $(CT_{max})_{ij} = \beta_0 + \beta_0 DT_i + \beta_1 AT_i + u_{0j} + \varepsilon_{ij}$
2. $(CT_{max})_{ij} = \beta_0 + \beta_0 DT_i + \beta_1 Sex_i + u_{0j} + \varepsilon_{ij}$
3. $Metabolic\ Traits_{ijk} = \beta_0 + \beta_1 DT_i + \beta_2 AT_i + \beta_3 Mass_i + u_{0j} + w_{0k} + \varepsilon_{ijk}$
4. $Metabolic\ Traits_{ijk} = \beta_0 + \beta_1 DT_i + \beta_2 Sex_i + \beta_3 Mass_i + u_{0j} + w_{0k} + \varepsilon_{ijk}$

Where β_0 is the intercept, DT is the Developmental Temperature, AT is the Acclimation Temperature, u is the adjustment term for the random effect of Tank, w is the adjustment term for the random effect of Day, and ε is error. 'Metabolic traits' include all of RMR, P$_{crit}$, and α.

I ran all models using the 'brms' package in the R environment (4.2.2), using weakly informative default priors to provide moderate regularization. Each model was run for 4 chains with 2000 iterations, following a 1000 iteration warmup. Model fits were further validated through visual joint and pointwise (median and skew) posterior predictive checks to ensure real data fit reasonably within simulated model predictions. Model results for predictors are presented visually in the form of posterior predictive distributions, with means and uncertainty intervals (UI's, 50% and 90%) highlighted. Means can be interpreted as average effect sizes such that those further from 0 imply stronger effects and are analogous to parameter coefficient estimates in frequentist statistics. UIs illustrate certainty of effect size estimates and can be interpreted analogously to frequentist confidence intervals; certainty of the effect decreases as UIs cross 0.

Animal ethics

This study was approved by the Dalhousie University Committee on Laboratory Animals (Protocol 20-087).

Results

Experimental metadata and model information

Early embryonic mortality before 3 days post-fertilization was similar in both Constant (C) and Fluctuating (F) Developmental Temperature treatments (22% *vs.* 25%, respectively, data not shown). 'Early embryonic mortality' rates of 10 – 30% are common for zebrafish, and are generally the result of either fertilization failure or embryonic death [58].

In adults, hereafter referred to by letter combinations denoting their respective Developmental and Acclimation temperature treatment groups (*e.g.*, FC is Developmental Fluctuating and Acclimation Constant), only one fish died due to unknown causes during the adult stage (CF); a second female fish (CC) was removed from the experiment and humanely euthanized before testing began due to a large abdominal cyst. During CT_{max} trials, one fish (FF treatment) jumped from the trial tank and was removed from the experiment. A total of 107 fish ($n = 24 – 28$/treatment) were tested for CT_{max}, and there were no mortalities following trials.

Sensor tears were discovered upon inspection of two different respirometers on two different days of testing, likely the result of damage during prior sterilization. These chambers were not used until sensors were replaced and recalibrated, resulting in no recordings being made for two of the 40 planned measurements. A large air bubble was also discovered in one chamber post-trial, concomitant with exceptionally low oxygen consumption in that chamber; this result was thus removed from the experiment, though no air bubbles were noted in any other respirometer across trials. As such, I was able to obtain RMR measurements for 37 fish ($n = 9 – 10$/treatment) out of 38 fish tested. Subsequent estimates of P_{crit} failed to converge for 3 fish (2 fish from FF treatment and one fish from CC treatment), and I was unable to obtain an estimate of α for one fish (FF treatment) for which P_{crit} failed to converge. Therefore, *N* for P_{crit} and α are respectively 34 ($n = 7 – 10$/treatment) and 33 ($n = 8 – 10$/treatment). Visual posterior predictive checks indicated high skewness in RMR models, but this issue was resolved by log-transforming RMR; transforming positive data is commonly applied to improve normality, and interpretation of results simply changes to multiplicative rather than

additive (*i.e.*, percent differences) [59]. All other models passed checks, and both visual model checks and numeric model results are presented in Appendix A.

Effects of Developmental Temperature, Acclimation Temperature, and Sex on Thermal Tolerance

Both Developmental (0-29 dpf) and Acclimation (30+ dpf) Temperature affected the thermal tolerance (CT_{max}) of adult fish (7 mo post-fertilization; Figures 11A, 12A). Acclimation to Fluctuating temperatures had a strong, positive effect on CT_{max}, increasing thermal tolerance on average by 1.18 °C (Figure 12A; 90% UIs: [0.85, 1.52]). Developmental plasticity to Fluctuating temperatures likewise had a positive, but lesser, effect on thermal tolerance, increasing CT_{max} on average by 0.30 °C (Figure 12A; 90% UIs: [-0.06, 0.65]). The effect of Sex was similar in magnitude to that of Developmental temperature, with males experiencing on average a 0.29 °C reduction in CT_{max} compared to females (Figure 12A; 90% UIs: [-0.60, 0.01]).

Effects of Developmental Temperature, Acclimation Temperature, and Sex on Routine Metabolic Rate

Of the three predictors, routine metabolic rate was only meaningfully impacted by Developmental Temperature (Figures 11B, 12B). Note that because RMR was log-transformed, interpretations of effect sizes are multiplicative (expressed in percentage differences). Results have been back-transformed. On average, Developmental plasticity to Fluctuating temperatures decreased RMR by 15.0% (Figure 12B; 90% UIs: [-30.4%, 5.2%]) relative to Constant temperatures. In contrast, Acclimation to Fluctuating temperatures caused a negligible decrease in RMR of 1.5% on average, and the posterior predictive distribution broadly overlapped zero, suggesting low confidence in these estimates (Figure 12B; 90% UIs: [-22.3%, 29.9%]). Sex had relatively minor effects on RMR, such that the RMR of males was increased by 5.8% relative to females, though there was low certainty around these effects (Figure 12B; 90% UIs: [-20.0%, 39.6%]).

Effects of Developmental Temperature, Acclimation Temperature, and Sex on Hypoxia Tolerance

P_{crit}, estimated at RMR, was also only meaningfully affected by Developmental Temperature treatment (Figures 11C, 12C). Developmental plasticity to Fluctuating temperatures improved hypoxia tolerance by reducing P_{crit} on average by 5.9 % DO (Figure 12C; 90% UIs: [-10.4, -1.6]) relative to Constant temperatures. There were negligible effects of both Acclimation to Fluctuating temperatures and Male Sex on P_{crit}, increasing P_{crit} by 1.0 and 0.8 % DO respectively, with low certainty around both effects (Figure 12C; 90% UIs: [-3.9, 6.9]; [-5.3, 6.8], respectively).

Effects of Developmental Temperature, Acclimation Temperature, and Sex on Oxygen Supply Capacity

Last, oxygen supply capacity (α) was most strongly affected by the Developmental Temperature treatment (Figures 11D, 12D). Developmental plasticity to Fluctuating temperatures increased α by 4.3×10^{-3} $mgO_2g^{-1}h^{-1}DO^{-1}$ on average (Figure 12D; 90% UIs: [-3.7×10^{-4}, 9.4×10^{-3}]). Acclimation to Fluctuating Temperatures had a minute effect, decreasing α by 1.2×10^{-3} $mgO_2g^{-1}h^{-1}DO^{-1}$ on average, with low certainty of these effects (Figure 12D; 90% UIs: [-7.1×10^{-3}, 4.4×10^{-3}]). Sex also impacted α, such that Males had an average increase of 3.3×10^{-3} $mgO_2g^{-1}h^{-1}DO^{-1}$ (Figure 12D ; 90% UIs: [-3.1×10^{-3}, 9.7×10^{-3}]).
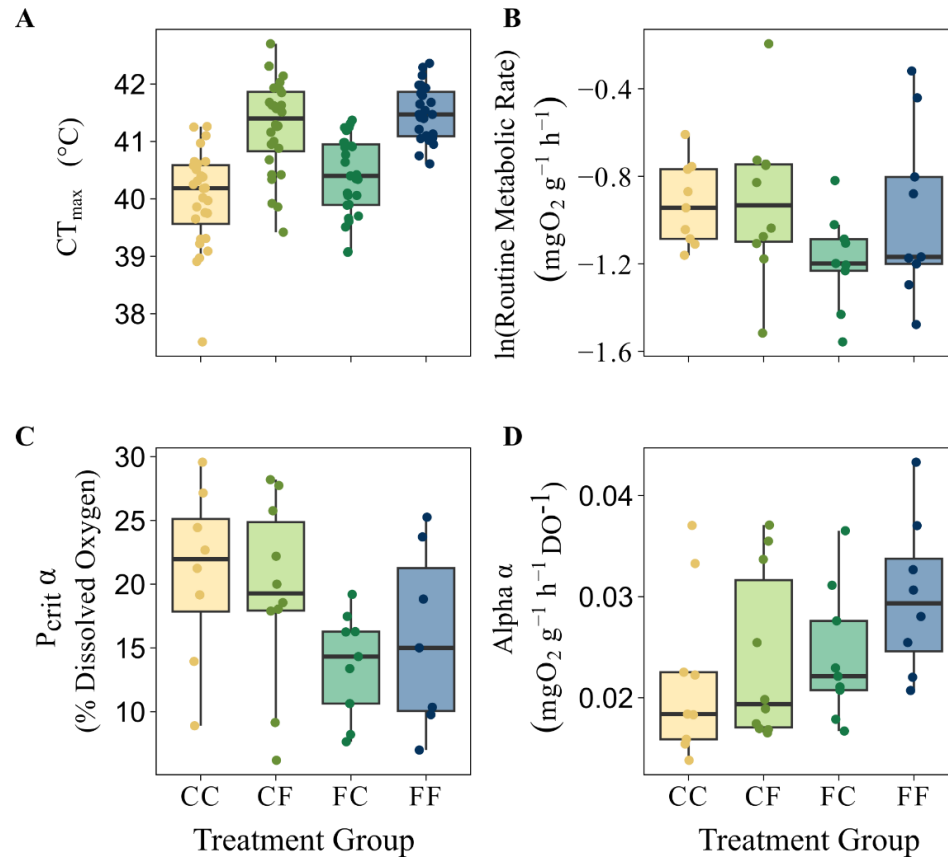
Figure 11: *Effects of factorial thermal treatments using Fluctuating (F; 22 – 32 °C diel) and Constant (C; 27 °C) Temperatures applied during early development (0-29 days post-fertilization; embryonic and larval stages, first letter) and later life (30+ days post-fertilization; juveniles and adults, second letter) in zebrafish (*Danio rerio*). Boxplots show median responses with individual data included as circles. A: Critical thermal maximum (CT$_{max}$). B: Log-transformed routine metabolic rate (RMR). C: Partial pressure of oxygen at which organisms begin to oxyconform, a measure of hypoxia tolerance (P$_{crit}$). D: Physiological oxygen supply (α).*
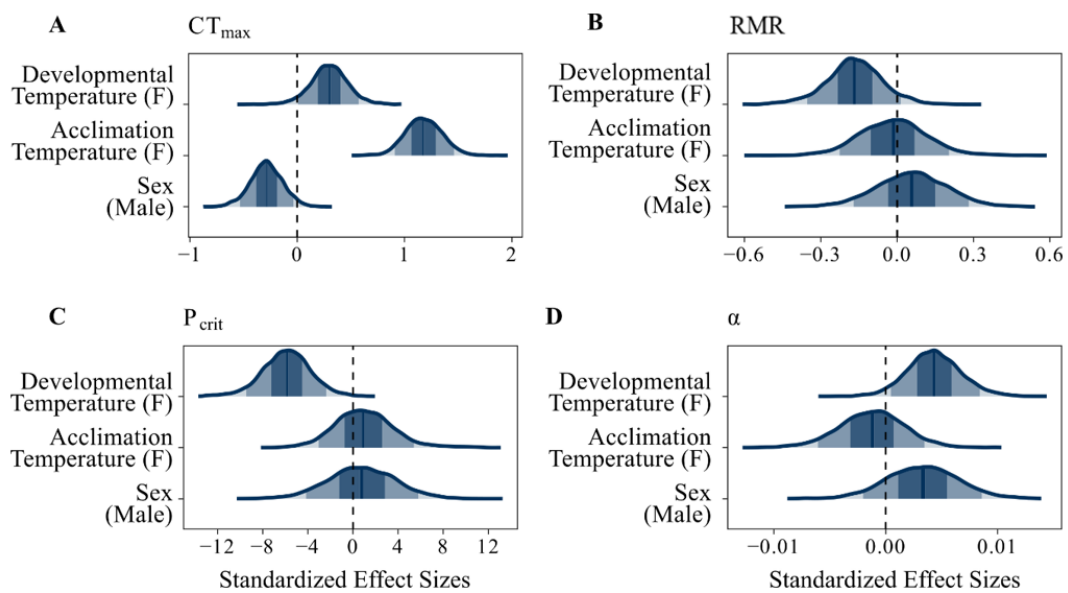
Figure 12: *Posterior distributions of effect sizes describing effects of Fluctuating (22 – 32 °C, diel) Developmental Temperature, Fluctuating Acclimation Temperature, and Sex on four physiological responses in zebrafish (*Danio rerio*), from causal Bayesian hierarchical models. Reference categories are Constant (27 °C) Temperature treatment and Female Sex. Developmental Temperatures were experienced during embryonic and larval life stages (0 – 29 days), whereas Acclimation Temperatures were experienced during juvenile and adult life stages (30 days – 8 months). A: Critical thermal maximum (CT$_{max}$). B: Routine metabolic rate (RMR). C: Partial pressure of oxygen at which organisms begin to oxyconform, a measure of hypoxia tolerance (P$_{crit}$). D: Physiological oxygen supply capacity (α). Posterior means are represented by thin dark lines at the mean of each distribution, 50% uncertainty intervals are represented by the inner dark blue shading, and 90% uncertainty intervals are represented by the outer blue shading. Means further from zero indicate stronger effects, and credible intervals overlapping zero indicate less certainty in effect size predictions.*

Discussion

A minority of studies investigating plasticity to in response to ecologically realistic environmental conditions investigate organismal responses across development, and fewer still examine concomitant effects in multiple ecologically relevant traits [13,14,26,60]. Here, using a variable thermal regime reflective of naturally occurring temperatures, I demonstrate long-term physiological improvements to thermal and hypoxia tolerance, metabolic rate, and oxygen supply capacity in zebrafish. Remarkably, the majority of these changes co-occur in response to variable temperatures experienced during early ontogeny – the first 29 days of life – and persist through sexual maturity well into adulthood. These results highlight developmental plasticity's role as a significant mechanism for producing beneficial phenotypic variation in adults, and suggest that these effects may be underappreciated when study durations are short [13,26], or unnatural thermal treatments are used [40].

Effects of ontogenetic exposure and temperature treatment on adult thermal tolerance, hypoxia tolerance and metabolism

I found improved thermal tolerance in fish that experienced variable temperatures during both early and late ontogeny, where the latter had a stronger effect. These results were unsurprising, given similar improvements to thermal tolerance have previously been documented under long-term thermal variability exposure [35] and, broadly, constant temperature heat acclimation in other fishes [10,61]. However, the sole previous study incubating fish under thermal variability in the long-term was not able to delineate persistent effects stemming from developmental plasticity from those of acclimation, as fish were kept in the same thermal treatments from fertilization through maturity [35]. Here, I built on the seminal insight of this work by showing adult variation in CT_{max} depends on the additive influences of both developmental plasticity and later acclimation to thermal variability across ontogeny, highlighting that nearly 20% of this improvement stems from the earliest developmental stages. It is interesting to consider that the 1.48 °C total increase in CT_{max} achieved within one generation herein is nearly seven times greater than that afforded through six generations of artificial selection in a recent

zebrafish study by Morgan *et al.* [7]. Indeed, developmental plasticity to thermal variability alone led to greater average increases in CT_{max} than experimental evolution (0.30 *vs.* 0.22 °C [7]), supporting the argument that plasticity will be a critical factor determining species persistence in the face of climate change [62].

Yet, in contrast to the strong influence of acclimation to variable temperatures on thermal tolerance, later life acclimation appeared to have negligible effects on hypoxia tolerance, metabolic rates, and oxygen supply capacity in mature adults. Instead, improvements in these traits were facilitated through developmental plasticity. It is thus possible that previous studies investigating multiple trait tolerance in fishes were limited because the ontogenetic timeframes of exposure were not long enough to facilitate plastic changes [26]. Studies using heat shocks and short-term warm acclimation (less than four weeks) have described no significant plasticity in hypoxia tolerance [28], and even negative effects on both hypoxia tolerance [27] and metabolic rates [29,30,63,64]. However, studies that used longer exposures have often demonstrated beneficial plasticity in hypoxia tolerance [65] and even metabolic compensation [20,66]. For instance, in wild adult killifish, McBryan *et al.* [31] found that six-week acclimation to warm, constant temperatures also improved hypoxia tolerance, but acute warm exposure did not. Indeed, metabolic compensation in response to hot temperatures has been shown to be time-dependent, improving over the course of weeks of exposure [67], suggesting that physiological plasticity may occur on longer timescales than previously anticipated.

A second explanation for variation between studies may lie in the nature of thermal treatments themselves. Constant temperature experiments, especially those using stressful temperatures, have been criticized for their potential to illicit deleterious effects on organisms that overshadow adaptive plasticity [40,41]. For instance, incubation under unnaturally hot, constant temperatures can lead to overproduction of heat shock proteins, siphoning resources from growth, reproduction, and other physiological processes – including tolerance mechanisms [38]. This may have occurred in an experiment demonstrating reduced hypoxia tolerance after a one-month acclimation of Amazonian fishes to hot temperatures, as most experimental fish did not survive acclimation itself [27], as well as a study in coral reef fishes that found detrimental metabolic effects under semi-lethal acclimation treatments [64]. In contrast, salmon acclimated to a warm

temperature (18 °C) within their thermal tolerance range (15 °C optimum) improved hypoxia tolerance after only two weeks [68]. In the present experiment, it is possible that beneficial cross-tolerance between heat and hypoxia was revealed because stressful temperatures were only experienced transiently during thermal fluctuations, as they would be in nature [14,38]. Notably, a recent study by Ridgway and Scott [69] showed that six-week acclimation to naturalistic fluctuating temperatures reduced the thermal sensitivity of hypoxia compared to constant temperature acclimation, highlighting the differential effects of constant *vs*. ecologically relevant fluctuating treatments.

Last, there is also evidence to suggest the existence of beneficial thermal plasticity across traits varies inter- and intra-specifically. Hilton *et al.* [70] found differences in hypoxic acclimation capacity between sister species of subtidal fish exposed to warm acclimation temperatures, and Anttilla *et al.* [65] noted that salmon were able to improve hypoxia tolerance after warm acclimation, whereas brook char could not. Different populations or species often display adaptive variation in their capacity for plasticity [31,71], and given sufficient genetic variation, plasticity is expected to be favored when there environmental heterogeneity is present [72,73]. Here, zebrafish likely represented a good model system in which to test for beneficial plasticity of multiple traits, because in their natural habitats they experience high thermal heterogeneity [46].

The ecological relevance and mechanisms of plastic changes to thermal tolerance, hypoxia tolerance, metabolic rate, and oxygen supply capacity

There are clear and likely beneficial impacts of across-lifetime plasticity in response to thermal variability in the four physiological performance traits studied herein. First, CT_{max} is widely considered to be an ecologically relevant metric of upper thermal tolerance [74], explaining both aquatic species range distributions [75] and tolerance to both slow and rapid warming [76]. I thus expect that the additive plasticity-induced increases in heat tolerance will confer within-generation benefits to fish under warming climate scenarios, especially in light of predicted anthropogenic increases in thermal variability [77].

The ecological relevance of changes to other metabolic traits is more nuanced. P_{crit}, a measure of the lowest oxygen partial pressure at which an organism can maintain a given

metabolic rate, was used as our measure of hypoxia tolerance. P_{crit} was traditionally thought to represent a useful measure of an organism's viability under hypoxic stress [78]. It is also a useful comparative metric [53] that is associated with the ecology and lifestyle of fishes; for example, species that inhabit areas with drastic dissolved oxygen cycles tend to have lower P_{crit} values, as they can maintain their metabolic rate at relatively lower environmental oxygen concentrations [79]. In this traditional sense, these results suggest that fish may be more tolerant of acute hypoxic events if exposed to thermal heterogeneity during early life. However, there is ongoing debate about true ecological meaning of P_{crit}, which has been widely criticized as a poor predictor of survival in natural hypoxic conditions [54,80]. Alternately, Seibel *et al*. and Seibel and Deutsch [54,81] suggest P_{crit} is expected to be inversely proportional to factorial aerobic scope, or an organism's metabolic capacity to support activities including reproduction, growth, and movement. Therefore, developmental plasticity of P_{crit} in response to thermal variability may instead be reflective of improvements to metabolic efficiency, which offset costs associated with frequent exposure to stressful temperatures [38,66,67]. This interpretation is further supported by the concomitant reduction in routine metabolic rate we noted in fish that experienced thermal variability during early life; these changes are ultimately expected to increase available energetic resources for other functions [66].

Under the alpha metric framework, the developmental plasticity-mediated improvement we saw in oxygen supply capacity (α) is actually a better indicator of performance at low oxygen levels than P_{crit} [54]. I was therefore still able to see improvements in hypoxia tolerance owing to early exposure to thermal variability, which were revealed through increases in oxygen supply capacity [54]. A second interpretation of the oxygen supply capacity results is that they reflect developmental plasticity in athleticism (*i.e.*, maximum metabolic rate), which was not measured in this study. It may prove interesting for future work investigating plasticity in aerobic traits to test these competing interpretations though metrics such as swimming performance. Nevertheless, the developmental plasticity we saw in these four traits (thermal and hypoxia tolerance, metabolic rates, and oxygen supply capacity) collectively illustrate a holistic improvement to physiological performance, which we expect could confer tolerance benefits to adults in hot and hypoxic conditions.

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There are several molecular and morphological mechanisms that may have contributed to improved cross-tolerance in CT_{max}, alpha and P_{crit}. Thermal experience during early development of zebrafish can persistently modify expression of genes associated with metabolism, cellular stress, and the cardiovascular and skeletal systems [45], which may explain the enduring developmental plasticity seen in this experiment. A future goal will be to determine the mechanisms underlying the physiological traits reported here, connecting them to whole-organism performance; for example, in zebrafish larvae, the release of the protectant hypoxia-inducible-factor-1 leads to developmental plasticity in hypoxia tolerance [82] and is upregulated during heat shock [83]. At greater levels of biological organization, warm acclimation has been shown to increase oxygen transport capacity in fish via increases in hemoglobin-oxygen affinity [69], increases in gill respiratory surface area [31,69,84], and improved cardiac efficiency [20]. In the context of this experiment, enduring developmental plasticity in physiological traits may have occurred because foundational morphological structures develop in early life, and early development is especially environmentally sensitive [20,85]. Long-term that take into account the conditions experienced during early life, rather than acclimating wild-caught adult organisms, will therefore offer greater utility in understanding how real populations will respond to local environmental changes.

Life-history changes and costs

Although the improvements to thermal tolerance, hypoxia tolerance, and metabolism I describe here may have appreciable benefits for organisms under changing climatic conditions, they were not 'cost-free'. Long-term exposure to thermal variability in zebrafish led to smaller adult body sizes in this experiment and others [35,43], and has been shown to reduce reproductive output [43]. These costs echo the shift towards smaller body sizes in fishes seen worldwide under warming temperatures [86,87], caused by increased metabolic demands, as well as sweeping changes to life-history patterns that include earlier maturity [88] and reallocation of resources towards reproduction [87]. Using relevant current or future thermal regimes, future investigations should aim to connect overarching life-history patterns, such as maturity rates and lifespan, to plasticity-driven changes in physiology.

Conclusions

Aquatic systems are increasingly experiencing lethal hypoxic events [89], warmer average temperatures, and increased thermal variability, including heatwaves [3,90]. Although within-lifetime phenotypic plasticity is an oft-cited mediator of the negative impacts of these stressors [5,62,91], existing research lacks both the ontogenetic breadth and external validity required to reasonably predict long-term organismal responses [14,26,41]. Here, I show that developmental plasticity in response to ecologically realistic thermal variability can facilitate enduring, beneficial changes to multiple physiological traits, including heat and hypoxia tolerance, metabolism, and oxygen supply capacity. These results renew appreciation for the early developmental environment as a modulator of adult phenotype, and emphasize the role of long-term studies in describing the gamut of phenotypic variation achievable though plasticity.

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Chapter 4: Differential reproductive plasticity under thermal variability in a freshwater fish (*Danio rerio*)

Abstract

Human-driven increases in global mean temperatures are associated with concomitant increases in thermal variability. Yet, few studies have explored the impacts of thermal variability on fitness-related traits, limiting our ability to predict how organisms will respond to dynamic thermal changes. Among the myriad organismal responses to thermal variability, one of the most proximate to fitness – and, thus, a population's ability to persist - is reproduction. Here, I examine how a model freshwater fish (*Danio rerio*) responds to diel thermal fluctuations that span the species' viable developmental range of temperatures. I specifically investigate reproductive performance metrics including spawning success, fecundity, egg provisioning, and sperm concentration. Notably, I apply thermal variability treatments during two ontogenetic timepoints to disentangle the relative effects of developmental plasticity and reversible acclimation. I found evidence of direct, negative effects of thermal variability during later ontogenetic stages on reproductive performance metrics. I also found complex interactive effects of early and late-life exposure to thermal variability, with evidence of beneficial acclimation of spawning success and modification of the relationship between fecundity and egg provisioning. My findings illuminate the plastic life-history modifications that fish may undergo as their thermal environments become increasingly variable.

Student Contribution Statement

Melanie D. Massey conceptualized and developed methodology for this piece with support from Jeffrey A. Hutchings and David Malloy. Investigation was conducted by Melanie D. Massey and suppored by M. Kate Fredericks and David Malloy. Data curation, visualization, and formal analyses were conducted by Melanie D. Massey and supported by Suchinta Arif. Supervision was provided by Jeffrey A. Hutchings. Melanie D. Massey wrote the original draft and all authors edited and reviewed the final piece.

Introduction

Phenotypic plasticity is a universal property of living organisms, facilitating both acute and long-term nongenetic responses to changes in environmental conditions [1–3]. The nature of plastic responses is complex and dependent on numerous factors, including the species and phenotypic traits under study, as well as the ontogenetic timing and duration of exposure to environmental signals [3]. As organisms undergo early development, they may alter their ontogenetic trajectories through developmental plasticity, a process which is often considered irreversible [2]. Throughout their lifetimes, organisms may also mount reversible responses to environmental inputs through the process of acclimation [4]. Moreover, developmental plasticity and acclimation are mechanistically linked and can interact to produce a range of phenotypic responses [5]. An existing goal of physiologists is to disentangle and describe the relative and interactive contributions of these two forms of plasticity, especially given recent suggestions that plasticity will play a critical role in mediating the biological impacts of climate change [6,7].

A growing body of literature has examined the differential and interactive effects of developmental plasticity and acclimation in a variety of traits, particularly in the context of thermal acclimation. Many of these studies sought to explore whether early exposure to one environment resulted in improved performance under those conditions later in life, as tests of the Beneficial Acclimation Hypothesis (BAH; [8]) in the broadest sense of the term [4,9–11]. For example, both developmental plasticity and adult acclimation of fruit flies to cool temperatures result in increased cold tolerance during assays later in life [12], and female seed beetles display higher fitness when developmental and adult reproductive temperatures match [10]. Furthermore, recent studies in fish species have suggested that beneficial developmental acclimation of metabolism [13,14] and reproduction [15,16] can occur in response to warm temperatures. With that said, examples of beneficial acclimation tend to be the exception rather than the rule [4]; more often than not, there appears to be a developmentally optimum environment which results in the strongest performance later in life across a range of adult conditions [4,9,17]. Moreover, authors have occasionally detected opposing responses of developmental plasticity and acclimation to the same environmental conditions [9,18], highlighting the importance of ontogenetic timing of exposure to environmental conditions. Taken

together, these equivocal results suggest that developmental plasticity, acclimation, and their interaction may significantly affect phenotypic outcomes, but that the nature of these outcomes can be context-dependent and unpredictable.

Another major challenge of generalizing conclusions across acclimation experiments is that the environmental treatments used often do not reflect ecologically realistic conditions. In studies of thermal plasticity, much of what is currently known is derived from studies that employ simplified constant temperature regimes ([19]; *but see* [20]). Although constant temperature experiments have generated remarkable and seminal biological insights [19], they likely reflect evolutionarily novel environments, and their applicability to natural conditions are limited [21,22]. It has also been suggested that stressful static conditions commonly chosen to examine the effects of temperature may themselves impose detrimental pathologies, obfuscating the consequences of thermal acclimation [4,23]. For this reason, authors have advocated for the use of thermal variability over constant temperature conditions, to mitigate the confounding effect imparted by stressful or ecologically irrelevant temperatures [4,23].

Indeed, there are few studies that leverage thermal variability to investigate the contributions of different forms of plasticity to phenotypic variation [24,25]. Schaefer and Ryan [24] determined that developmental plasticity, acclimation, and their additive interaction in response to a broad range of diel fluctuating temperatures significantly increased the heat tolerance of zebrafish (*Danio rerio*). Bilcke, Downes, and Büscher [25] likewise found evidence of developmental beneficial acclimation of locomotor and predation performance in common garden skinks using ecologically realistic treatment temperatures. These studies suggest that there may be appreciable plastic responses to thermal variability, and in some cases, these responses impart important benefits to organismal performance.

In the present study, I estimate the relative and combined influences of developmental plasticity under thermal variability, comparing these effects with constant optimal temperatures. I implement a factorial experimental approach (*sensu* [26]), applying thermal treatments during early ontogeny (embryonic and larval stages) and late ontogeny (juvenile and adult stages) in zebrafish, a model organism. Specifically, I measure key elements of reproductive performance, including spawning success,

fecundity and egg provisioning, and sperm quality. I chose reproductive performance because it is strongly influenced by temperature-mediated plasticity in ectotherms [6,27–31], and fitness correlates represent the most pertinent metrics for investigating the importance of plasticity [4,10,23].

Here, I test for evidence of beneficial acclimation of reproductive traits, which I describe as a positive interaction between early and late ontogenetic environments, such that fish exposed to thermal variability during early life may perform better under those same conditions later in life. I consider this an extension of the BAH [10,11,26]. Aside from the BAH, I also examine both positive and negative responses to thermal variability, teasing apart the contributions of developmental plasticity and acclimation. Last, because fecundity and egg allocation are correlated through trade-offs in a life-history framework [32], I examine their plastic responses jointly to test whether life-history can be modified by thermal variability [10].

Methods

Parental fish rearing and breeding

I initiated my experiment in February 2021 with 600 freshly laid (<4 h post-fertilization) F_0 zebrafish eggs from an ancestral stock of three wild-type AB lineages acquired from the Dalhousie University Zebrafish Core Facility (ZCF). For a detailed explanation of rearing conditions, see Appendix B.

Thermal treatments during 'Early' and 'Late' ontogeny

I manipulated temperature during 'Early' (embryonic and larval stages; 0 – 29 days post-fertilization) and 'Late' (juvenile and adult stages; 30+ days post – fertilization) ontogeny of F_0 fish, employing a full-factorial, split-clutch design (Figure 13). The Early period ultimately represented the developmental plasticity treatment, whereas the Late period represented the acclimation treatment.
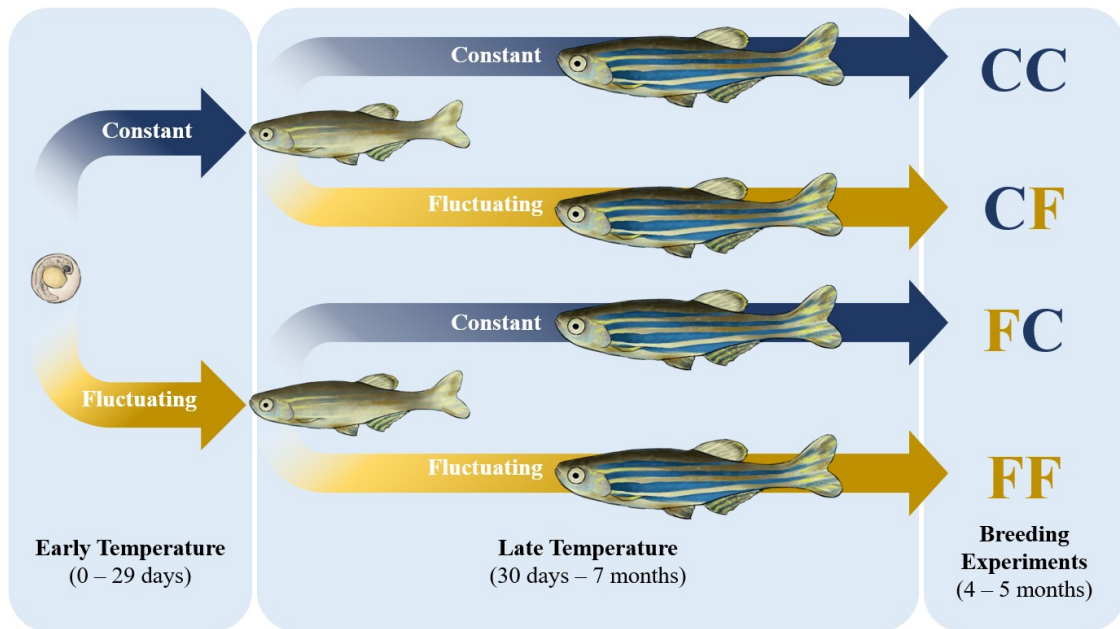
Figure 13: *Flowchart illustrating split-clutch experimental design testing the effects of Constant (27 °C; C) or diel Fluctuating (sinusoidal 22 – 27 °C; F) experienced during an Early Temperature treatment (developmental plasticity; embryonic and larval stages, 0 – 29 days post-fertilization) and a Late Temperature treatment (acclimation; juvenile and adult stages, 30 + days post-fertilization) in zebrafish (*Danio rerio*). Breeding experiments were performed at 4 -5 months post-fertilization. Final combined Early and Late Temperature treatment groups are denoted by respective letter combinations CC, CF, FC, and FF.*

I randomly and evenly split clutches of freshly laid eggs from three lineages (pairings) of F_0 zebrafish into Constant or Fluctuating treatments, representing the developmental plasticity phase. Fish remained in these treatments until the onset of the Late ontogenetic period, then I once again split groups into Constant and Fluctuating treatments, representing the acclimation phase. This factorial design resulted in four groups that experienced a combination of Early and Late thermal treatments (Figure 13): Constant-Constant (CC), Constant-Fluctuating (CF), Fluctuating-Constant (FC), and Fluctuating-Fluctuating (FF).

I selected 27 °C as a Constant temperature treatment, and a diel fluctuation from 22 – 32 °C as a Fluctuating treatment. Warm temperatures were set to peak at 12:00 pm, and cool temperatures were set to peak at 12:00 am. These thermal treatments were designed to have different magnitudes of variability while maintaining an equal thermal mean (~27 °C). Whereas temperatures ranging from 26 - 28 °C are often considered constant 'optimal' temperatures for laboratory zebrafish (*e.g.*, promoting growth, fecundity, and immune responses [33,34]), 22 – 32 °C represents the maximum range of temperatures under which zebrafish develop normally, representing the extreme developmental thermal boundary beyond which high levels of mortality, deformation, and thermal stress occur [35,36]. Yet, during reproductive season in natural habitats, temperatures tend to vary from ~23 – 31 °C [34]. As such, the Fluctuating regime represents a physiologically challenging [35] but ecologically realistic range of temperatures. My temperature treatment system is described in detail in Appendix B.

Size and spawning success in fish exposed to thermal treatments during 'Early' and 'Late' ontogeny

In May 2021, when fish were sexually mature (120 d old), I began breeding experiments. Zebrafish are seasonal batch-spawners, with the ability to spawn continuously after reaching sexual maturity [34], though reproductive effort is largely expended during monsoon season, characterized by high environmental variability, in their natural habitats [37]. Further, in the wild, they generally exhibit an annual life cycle, typically experiencing only one reproductive season [34]. To reflect natural conditions, I

conducted breedings once per week over the course of five weeks for each lineage to attain an estimate of female fecundity. A one-week rest period between spawnings has been shown to be sufficient to allow zebrafish to recuperate their reproductive investment [38].

To measure spawning success, which I defined as the production of any eggs by a breeding pair, I randomly selected pairs of sibling males and females from the same treatment tank. Sibling pairs, rather than between-family crosses, were selected so that I could later delineate family-level effects from treatment effects. I placed breeding pairs in a zebrafish breeding box connected to the flow-through system the afternoon before breeding. I separated males and females using a clear plastic divider and placed identical sterilized plastic plants in each female's compartment to stimulate egg production [39]. The next morning, immediately after the onset of the light period at 08:00 h, I disconnected the flow-through system from breeding tanks and removed dividers. I elevated one end of each tank by 5 cm to create a gradient of water depth to stimulate breeding, and allowed pairs to breed for 3 h. After this period, fish were sedated *via* inhalation of buffered MS-222 [80mg/L], weighed, and measured for standard length (SL) as my metric of body size, before being returned to their tank of origin.

Female reproductive traits: Egg counts and measurements

Female zebrafish will occasionally produce necrotic, 'non-viable' eggs, as the result of resorption of mature ova; these non-viable eggs are identified by an opaque and asymmetric appearance [34]. I collected and sorted F_1 eggs from each spawning event, separating out non-viable eggs. I placed viable eggs in petri dishes filled with E3 embryo medium, and photographed them under a dissecting microscope, using a 0.001 cm micrometer for size calibration. I took the production of any eggs (viable or non-viable) to indicate that spawning took place (*i.e.*, breeding conditions stimulated the female to produce eggs), but only included counts of viable eggs in my estimates of fecundity.

To estimate egg provisioning, I measured equatorial yolk diameter for a random subsample of up to 10 viable eggs per spawning using ImageJ (National Institutes of Health, Bethesda, MD). Yolk volume is a common and suitable proxy for maternal

provisioning of eggs [40], and is relevant given its correlation with offspring fitness in oviparous ectotherms [41].

Male reproductive traits: Sperm concentration

Sperm concentration and volume are both significantly correlated with sperm quality in zebrafish, and are positively associated with higher rates of fertilization and lower rates of offspring deformity [42]. I thus used sperm concentration as a proxy for male reproductive quality. In September 2021, two weeks after the last breeding, I measured sperm concentration and volume from 8 - 12 randomly selected F_0 males per treatment. I collected and measured sperm from anesthetized males taken directly from home tanks, using 10 µl glass microcapillary tubes. A visual assessment of the collected sperm was then made to minimize urine or faeces contamination in samples [43]; poor quality samples indicated by low opacity or fecal content were removed from analyses. Known volumes of sperm from each male were then diluted with 4 microlitres of E400 medium. The absorbance of the resulting sample was measured at 400 nm using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and sperm concentration (sperm cells/mL) was estimated by using a hemocytometer-calibrated standard curve [44].

Statistical analyses

I applied Bayesian linear mixed models to estimate 'Early' and 'Late' thermal treatment effects (two level factors) and their interaction on parental body sizes, spawning success, fecundity, and yolk volume. Additionally, I used a Bayesian linear model to estimate thermal treatment effects on sperm concentration. In all models I included Family (three-level, categorical) as a covariate to investigate the variability in effect sizes owing to family-level effects, with the exception of sperm concentration, for which males from different families were pooled due to small sample size. Finally, I included paternal size as a covariate for breeding success and sperm concentration models, as paternal size is expected to influence both metrics [45], and I sought to estimate direct effects of thermal treatments. Likewise, I included maternal size as a covariate for both fecundity and yolk

volume models as female size is expected to influence both metrics [46], and to isolate direct treatment effects. Because measurements on individual tanks were repeated weekly over five weeks (for all metrics except sperm concentration), I included *tank* as a random intercept term to account for repeated measures [47].

I used a binomial distribution to model breeding success, a negative binomial distribution with a log link function for fecundity (to account for overdispersion detected in pilot Poisson models [48]), and gaussian distributions with identity link functions for parental body size, yolk volume, variation in yolk volume, and sperm concentration models. I ran all models using the 'rstanarm' package in the R environment (4.1.0), using weakly informative default priors to provide moderate regularization [49]. Model fits were further validated through joint and pointwise (median and skew) posterior predictive checks to ensure real data fit reasonably within simulated model predictions. I also cross-validated models using Pareto-smoothed importance sampling (PSIS-LOO) cross-validation (visual model checking is detailed in Appendix B).

I visually described model results using plots of posterior median values from Bayesian models, which are analogous to parameter estimates in frequentist models, and can be directly interpreted as 'effect sizes'. These effect sizes intuitively represent the relative strength of each parameter's effect on response variables. I further included 50% and 90% uncertainty intervals (UIs), which illustrate certainty in parameter estimates based on the posterior generated from each model. UIs can be interpreted analogously to frequentist confidence intervals.

Animal ethics

This study was approved by the Dalhousie University Committee on Laboratory Animals (Protocol 19-105).

Results

Thermal treatments

The mean temperature of the Constant treatment throughout the experiment was 27.58 (± 1.23 SD) °C, and the mean temperature of the Fluctuating treatment was 28.18 (± 3.64 SD) °C (Figure 14). The Constant treatment was slightly more variable than anticipated due to constraints on the water supply system, and both treatments were subject to slight seasonal variation in ambient water temperatures provided to the facility.
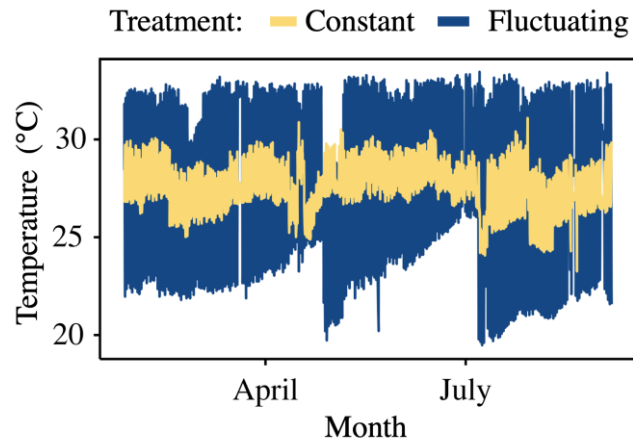
Figure 14: *Temperature logs of thermal treatments from January to September 2021. The constant temperature treatment (yellow) is plotted in the foreground, and the diel fluctuating treatment (blue) lies behind; the two treatments were designed to share a common thermal mean.*

Effect of thermal variability on male and female body sizes

There were differences between Early and Late thermal treatment groups on maternal and paternal body sizes (Figure 15A, B). There was a negative effect of Early Fluctuating temperature on maternal body size, with moderate certainty such that 95% uncertainty intervals (UIs) crossed zero, but 50% UIs did not (Figure 16A, B). Late Fluctuating temperature exhibited a stronger negative effect on both maternal and paternal body sizes (Figure 16A, B). Both maternal and paternal body sizes were influenced by family lineage (Figure 16A, B).

Effect of thermal treatments on spawning success

I conducted 174 breedings in total, and of those, females spawned (viable and/or non-viable) in 132 (75.9% of trials). There were differences between Early and Late thermal treatment groups in breeding success (Figure 15C). The Bayesian mixed model for spawning success revealed a relatively strong, positive interaction between Early and Late thermal treatments, such that experiencing the same temperature regime during both life-stages strongly enhanced spawning success (Figure 16C). Further, there was a negative influence of Early Fluctuating temperature on spawning success (Figure 16C). Paternal length also had a modest positive effect on spawning success (Figure 16C). The effects of week of the experiment, family lineage, and exposure to Late Fluctuating temperatures on spawning success were negligible (Figure 16C).

Effect of thermal treatments on female reproduction: fecundity

The number of viable eggs produced across trials ranged from 0 to 389, and there were differences in the average fecundity across the Early and Late thermal treatment groups (Figure 15D). The Bayesian mixed model for fecundity revealed a negative influence of Late Fluctuating temperatures on fecundity (Figure 16D). At the same time, there was a nonnegligible, positive influence of Early Fluctuating temperatures on fecundity, albeit with moderate certainty (95% UIs overlap zero, but 50% UIs do not; Figure 16D). There was a modest positive effect of Week on fecundity, but maternal length was of negligible influence (Figure 16D).

Effect of thermal treatments on female reproduction: yolk volumes

We estimated yolk volumes for a total of 1194 eggs. Volumes ranged from $6.2*10^{-2}$ to $2.1*10^{-1}$ mm^3, and there were differences between Early and Late thermal treatment groups on yolk volumes (Figure 15E). The Bayesian mixed model suggested yolk volumes were relatively strongly and negatively affected by Late Fluctuating temperatures (Figure 16E). There was also a significant negative interaction between Early and Late Fluctuating temperatures, that resulted in an additively negative impact on eggs from parents in FF treatments (Figure 16E). Maternal length had a relatively weak negative influence on yolk volumes (Figure 16E). The week of breeding had a positive effect on yolk volumes, and there were differences between family lineages (Figure 16E).

Effect of thermal treatments on male reproduction: sperm concentration

We attempted to collect sperm samples from 36 individuals but were only able to collect 23 samples. I experienced difficulty collecting sperm from 12 males (*i.e.*, were unable to collect sperm by the third attempt) and did not proceed with collection to prevent undue stress or mortality to the individuals. One sample was removed before spectrophotometry due to contamination with feces. Average sperm concentrations across Early and Late thermal treatment groups are illustrated in Figure 15F. The Bayesian linear model suggested paternal mass had a comparatively strong and positive effect on sperm concentration, but neither Early nor Late thermal treatment affected sperm concentration (Figure 16F).

Additional information

Model results and further data on non-viable eggs are described in detail in Appendix B.
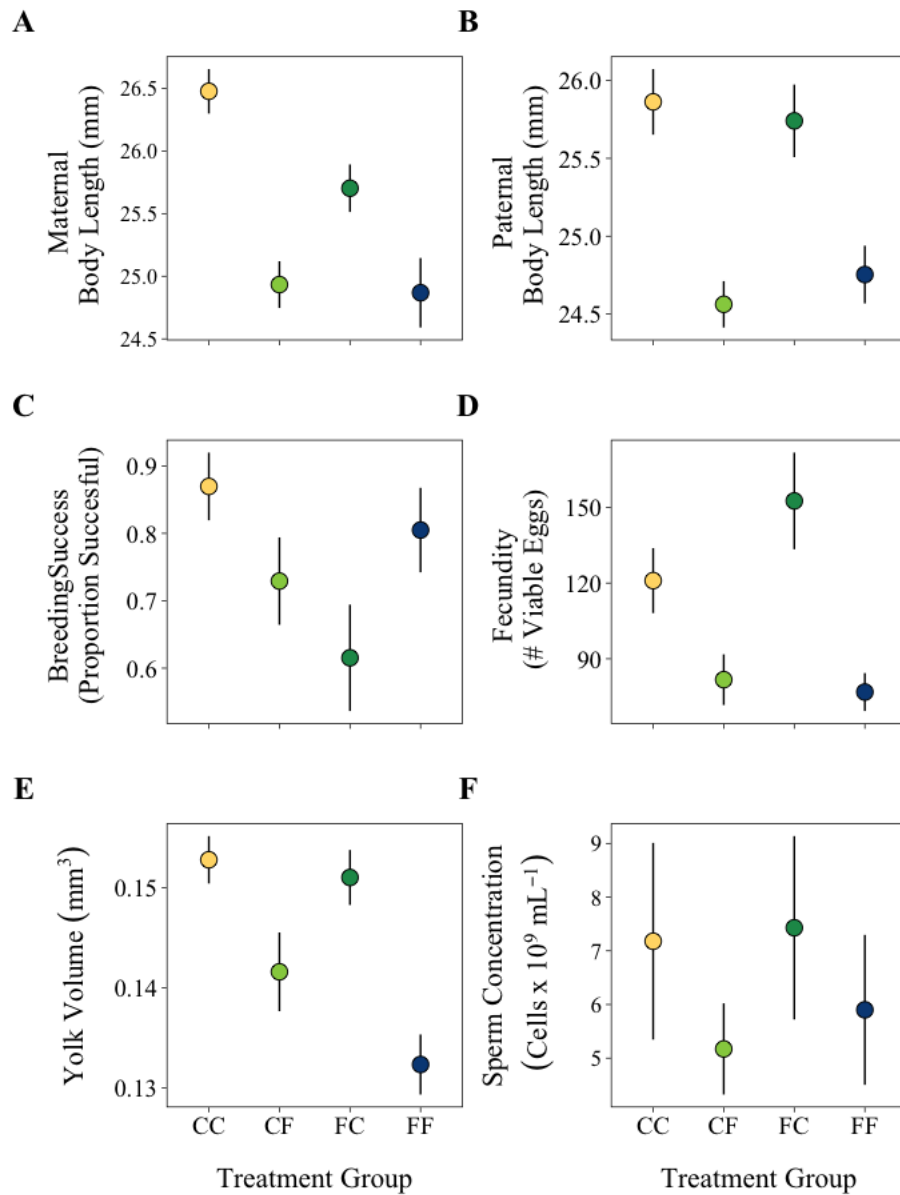
Figure 15: *Means (±SE) of male and female body sizes and reproductive traits (A–F) of adult zebrafish (Danio rerio) exposed to a combination of Constant ('C'; 27 °C) or Fluctuating ('F'; diel, 22 – 32 °C) thermal regimes during an 'Early' developmental plasticity period (0 – 29 days post-fertilization, first letter) and a 'Late' acclimation period (30+ days post-fertilization, second letter).*
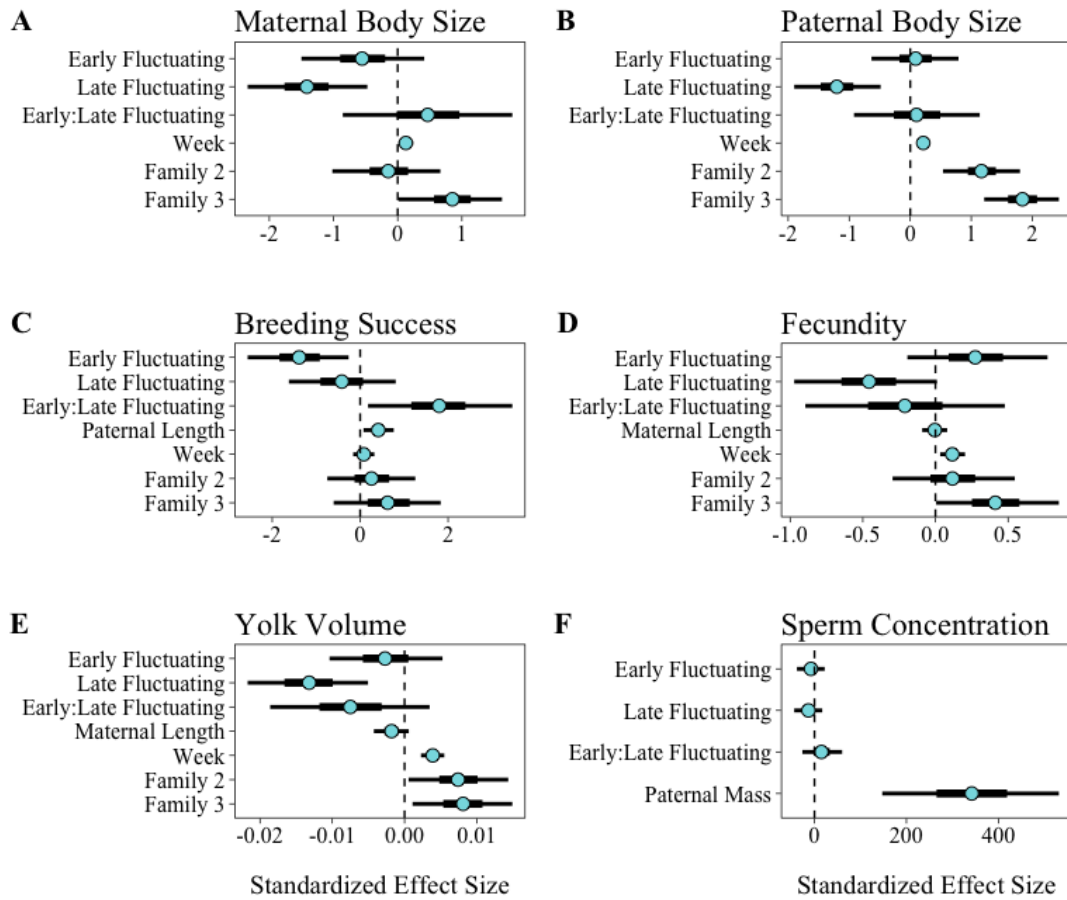
Figure 16: *Standardized effect sizes (posterior medians) of covariates and predictors (y-axis labels) for Bayesian mixed models (A – E) and Bayesian linear model (F) of body sizes and reproductive traits. Blue dots indicate effect sizes, thick black lines indicate 50% uncertainty intervals (UIs), and thin black lines indicate 90% UIs. Briefly, effect sizes that are further from 0 suggest stronger effects, and UIs that cross 0 suggest less certainty in estimates.*

Discussion

Developmental plasticity and reversible acclimation may act alone or in tandem to shape the phenotypes of organisms in variable environments, but their contributions to variation in reproduction under ecologically relevant thermal variability are largely unknown. Here, I applied simulated diel thermal variability spanning early and late ontogenetic periods, with the goal of investigating the singular and interactive effects of developmental plasticity and reversible acclimation on reproductive traits in zebrafish. I found the ability of pairs to spawn was enhanced when their late ontogenetic environment matched that of early development. I also found complex interactions between early and late thermal experiences that ultimately shaped the fecundity-egg size relationship; early developmental exposure to thermal variability enhanced fecundity while concomitantly decreasing egg size, whereas later acclimation to thermal variability represented a constraint on both. Last, temperature did not exert direct effects on male fertility, but thermal variability's negative influence on male body sizes, the realized effect was a reduction in sperm quality. Overall, my results indicate that experiencing thermal variability largely led to decreases in reproductive metrics, but there was evidence to suggest plasticity significantly altered these effects.

Among the reproductive traits studied, the only one that exhibited clear beneficial acclimation was spawning success. Fish reared in the same environment throughout life showed greater spawning success, and notably, this positive effect still occurred regardless of whether the thermal regime was constant or variable. Interestingly, although male body size is expected to be a significant contributor to mating success [45], I detected only a minute positive effect of paternal length, which was dwarfed by that of thermal beneficial acclimation. It is possible that this beneficial acclimation of spawning success is due to individual mating preferences acquired during early development [50]. In this scenario, desirable phenotypes, including appearance, chemical signaling, and behaviour, may have been conditioned by the Early ontogenetic environment in anticipation of maturity [38,50]. This scenario would be both plausible and possibly adaptive if the temperatures experienced during Early ontogeny act as reliable indicators of future breeding conditions, to the benefit of parental fitness [51]. Alternatively, it is possible that beneficial acclimation of performance traits such as aerobic capacity or

swimming speed indirectly influenced males' chance of success during courtship, as others have found [52]. Although tests of these two possibilities were beyond the scope of this study, future behavioural observation or metabolism studies may elucidate the mechanisms behind beneficial acclimation of spawning success.

In contrast to spawning success, male fertility did not appear to exhibit beneficial acclimation. Instead, there was a significant detrimental effect of male body sizes on sperm quality [42], which was associated with thermal variability's negative impact on male body size during late ontogeny through acclimation. Other authors have likewise found developmental rearing temperatures do not impact fish sperm counts [53], which is unsurprising given that spermatozoa are regenerated daily in sexually mature fish [54]. Consequently, it would be prudent to consider both the timing- and sex-specific effects of temperature on body sizes of male fish going forward, given the cascading impact that temperature-driven changes in body size have on male fertility.

In response to variable temperature regimes, I detected evidence of plastic life history trade-offs in fecundity and egg provisioning of females owing to developmental plasticity. My results suggested that exposure to thermal variability during the first 30 days of development had a lasting, enhancing effect on fecundity, concomitant with a negative effect on yolk volumes when fish were exposed for their entire lives. Interestingly, these results are contrary to existing theoretical predictions and some empirical studies, which often support the expectation that variable or stressful conditions should lead to decreased fecundity and larger eggs [55–57].

However, these expectations of fecundity-egg provisioning relationships often assume that 'bigger is better' – or, specifically, that well-allocated offspring are advantaged in unideal environments [57]. For conditions under which smaller offspring are favored, this assumption is violated [58–62]. Under the thermally variable regime used in my study, it is possible that smaller offspring may ultimately experience metabolic advantages, especially given the lower oxygen solubility and higher energetic demands associated with increases in water temperature [58]. Further, in zebrafish, both fertilization and hatching success are significantly lower for larger eggs at hot constant temperatures (30 °C; [40]), suggesting a reduction in egg size may have adaptive benefits under diel thermal variability that crosses hot temperature thresholds. However, further experiments

investigating fertilization rates and offspring survival, which were not studied herein, are needed to determine whether this unexpected life-history trade-off is indeed beneficial for parental fitness.

In contrast to developmental plasticity to thermal variability, acclimation had a negative effect on both fecundity and yolk volumes. It is likely that these effects are facultative; *i.e.,* a direct and negative consequence of chronically experiencing suboptimal temperatures [63]. Given the metabolic demands required to cope with temperature fluctuations beyond thermal optima, fish exposed to the fluctuating regime for the latter portion of their lives likely expended greater portions of their energy budget towards somatic maintenance [64]. As a result, these fish had fewer total resources available to invest in reproduction [65]. This suggestion is supported by the fact that negative effects of fluctuating temperatures later in life were found on female body sizes, fecundity, and yolk volumes alike, signaling a constraint. Overall, it is important to recognize that female fish reared in fluctuating treatments during late ontogeny still had appreciably lower total reproductive output than those reared in optimal conditions. Although developmental plasticity slightly enhanced fecundity, total compensation was not achieved; multiple generations of plastic changes or evolution may be needed to optimize reproductive output under increased thermal variability [66,67].

A notable, though unintended, feature of this study is that the Constant temperature treatment was somewhat thermally variable. Thus, my results more closely represent a comparison between regimes with low *vs.* high thermal variability. Interestingly, I note that estimates for spawning success, fecundity, yolk volume, and sperm concentrations in my lifetime Constant treatment group were similar to previously reported results in wildtype zebrafish held in explicitly optimal constant thermal conditions [68–70]. These results suggest that small fluctuations in temperature may have minor impacts, but the magnitude of response is dependent on the magnitude of variability, as others have found [20,22,63]. Ultimately, as the climate changes, we must address both thermal means and magnitudes of variability to accurately predict organismal responses [22,71].

It was beyond the scope of this study to investigate growth rates, age at maturity, and reproductive lifespan of both parents and offspring, but these measures play important roles in the suite of connected life-history characteristics that can vary in response to

environmental conditions, and collectively shape parental fitness [32]. For example, recent evidence suggests that temperature itself can modify the onset of sexual maturity independently of temperature-induced changes in somatic growth [72], and that temperature differentially influences lifespan and fecundity [73]. Future experiments examining fitness explicitly – including the onset of sexual maturity, breeding successes or failures, and subsequent offspring survival – will unequivocally address if and how developmental plasticity and acclimation influence fitness through life-history trade-offs.

The world is becoming both warmer, on average, as well as more thermally variable [74]. In this study, I show that thermal variability results in myriad changes to fundamental reproductive traits in zebrafish, and that these effects are a result of a complex interplay between developmental plasticity and reversible acclimation. My results support a significant role for developmental plasticity in the alteration of life history and awakened support for the beneficial acclimation hypothesis under ecologically realistic thermal regimes. Further tests into the adaptive value of these plastic changes will benefit our understanding of organismal performance under changing climatic conditions.

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Chapter 5: Parental early life environments drive transgenerational plasticity of offspring metabolism in zebrafish (*Danio rerio*)

Abstract

Parental experiences can lead to changes in offspring phenotypes through transgenerational plasticity (TGP). TGP is expected to play a role in improving the responses of offspring to changes in climate, but little is known about how the early lives of parents influence offspring TGP. Here, I use a model organism, zebrafish (Danio rerio), to contrast the effects of early and later life parental thermal environments on offspring routine metabolism. To accomplish this, I exposed both parents to either constant optimal (27 °C) or environmentally realistic diel fluctuating (22 – 32 °C) temperatures during early (embryonic and larval) and later (juvenile and adult) life in a factorial design. I found significant reduction of routine metabolic rates (>20%) at stressful temperatures (22°C and 32°C) after biparental early life exposure to fluctuating temperatures, but little effect of later life parental temperatures on offspring metabolism. This reduction reflects metabolic compensation and is expected to enhance offspring body sizes under stressful temperatures. These changes occur over and above the effects of parental environments on egg size, suggesting alternate nongenetic mechanisms influenced offspring metabolic rates.

Student Contribution Statement

Melanie D. Massey and Anne C. Dalziel conceptualized and developed methodology for this piece. Investigation was conducted by Melanie D. Massey and suppored by laboratory assistant M. Kate Fredericks. Data curation, visualization, and formal analyses were conducted by Melanie D. Massey and supported by Anne C. Dalziel. Supervision was provided by Anne C. Dalziel. Melanie D. Massey wrote the original draft and both authors edited and reviewed the final piece.

Introduction

The experiences of parents can impact their offspring through a suite of mechanisms that transcend genetic inheritance, including parental care, offspring provisioning, and epigenetic facilitation [1,2]. These nongenetic parental effects, collectively termed 'transgenerational plasticity' (TGP), have drawn considerable attention for their role in shaping offspring phenotypic variation and fitness [3,4] and potential to facilitate rapid, beneficial responses to changing environments [2,5,6].

Temperature is a key driver of phenotypic plasticity in ectotherms [7], and continued increases in global thermal means and variance are expected to negatively impact many aspects of organismal performance, including body size via increased metabolic demands as temperatures rise [8–10]. Yet, through TGP, parental exposure to warm temperatures can improve outcomes for offspring experiencing warm temperatures themselves, relative to offspring whose parents received no such cue ('anticipatory parental effects' [11]; reviewed in [2]). For example, lifelong or reproductive parental acclimation to warm, constant temperatures increases the relative growth rates and body sizes of warm-incubated offspring in fishes [12–17] and is associated with epigenetic increases in offspring metabolic efficiency [12,18]. However, no studies to date have examined TGP in response to variable temperatures, which better-reflect natural or predicted thermal conditions. Thermal variability and periodic heating events are expected to intensify with climate change [19,20], and constant vs. variable temperatures often produce different phenotypic effects, even when sharing thermal means [21,22]. There is therefore an urgent need to understand how TGP may manifest under ecologically realistic thermal variability [2,23].

The occurrence and strength of TGP can also be influenced by the ontogenetic timing during which parents experience thermal stressors, and most studies have investigated parental exposures during sexual maturity and reproduction [2,24]. However, embryos and larvae may be especially environmentally sensitive, and cellular/molecular changes during early development can cascade into large effects on parental and subsequent offspring phenotypes [6,25,26]. Moreover, beneficial TGP is expected to occur when cues experienced by parents supply accurate information about offspring environments; the windows of parental sensitivity should thus vary depending on the ecology of focal

organisms [1,3,11]. In some cases, such as when juvenile and adult habitat use differs, parental early life environments may be better predictors of offspring environments [27,28].

Few studies have compared the transgenerational effects of pre- and post-maturity parental thermal experiences ([2,24]; but see: [29]). In fishes, pre-maturity parental exposure to warm, constant temperatures appears to have beneficial effects on offspring in warm conditions, enhancing body size [30] and aerobic scope [31,32]. Although these studies support the existence of early ontogentic critical windows for TGP, they do not delineate the influence of the earliest parental life stages from later juvenile development. The sensitivity of parental development between fertilization and the juvenile stage is thus of particular interest, especially as these stages are highly responsive to environmentally induced epigenetic modifications [2,25].

Here, I use zebrafish (*Danio rerio*) as a model to investigate how the timing of biparental thermal exposure influences offspring metabolism, by comparing the effects of constant vs. challenging diel variable thermal treatments in a one-year long factorial experiment. I delineate parental 'early life' as premetamorphic embryonic and larval stages (day 0 to 29), in contrast to 'later' postmetamorphic juvenile and adult stages (day 30+). I chose this delineation because premetamorphosis is a candidate window for epigenetic effects [25], defined by significant tissue differentiation in teleost fishes [33]. Moreover, the earliest life-stages of wild zebrafish occur during the distinctly thermally variable monsoon season (~12 – 31 °C) [34,35]. Zebrafish parental early life environments may therefore act as better cues for young offspring than later life environments (*i.e.,* given environmental autocorrelation between monsoon seasons) [1,28]. I reared parents in thermal treatments for one year, simulating their annual life cycle [34], and tested for TGP in routine metabolic rate (RMR) of one-day old offspring at stressful temperatures (22°C and 32°C). I measured this trait because plasticity-induced reductions in RMR can improve subsequent juvenile growth in stressful thermal conditions, [12,18,36], and juvenile size is a fitness-related trait in teleost fishes [37].

Methods

Fish husbandry and thermal treatments

I collected parental generation (F1) zebrafish embryos from three nonsibling matings ('families') of wildtype-AB fish within three hours post-fertilization (hpf) from the Dalhousie Zebrafish Core. Grandparental source fish (F0) were kept in control standard zebrafish laboratory conditions at 27 °C (Appendix C).

F1 embryos were brought to the Dalhousie University Aquatron and each family was divided into two thermal treatment groups (Fig. 17 A, B): a Constant 27 °C, which reflects a thermal optimum for reproduction and growth in zebrafish [34,35], and a Fluctuating temperature that varied sinusoidally on a diel basis (22 – 32 °C; Fig. 17 A, B). This regime was intended to reflect natural thermal variability [34], while exposing fish to transient stressful temperatures, minimizing constant temperature-induced pathologies [21,34,38–40].

F1 embryos and larvae were reared in their initial 'Early Parental Temperature' treatment (Constant or Fluctuating) for 29 dpf (days post-fertilization). At the onset of the juvenile stage (30 dpf), fish were equally split into Constant or Fluctuating temperatures in groups of 12-15, and held in these conditions through sexual maturity (90 dpf) until one year of age, establishing a 'Later Parental Temperature' treatment (Fig. 17 A, B). In total, there were four groups representing factorial combinations of Early and Later Parental Temperature treatments, with six replicate tanks per group, each with two replicates per family. At one year of age, sibling F1 fish were bred within each treatment to produce F2 embryos, which were kept at 27 °C for 24 h (Fig. 17 A, B; Appendix C).

Embryo metabolism

I measured routine metabolic rates (RMR) of 24 hpf F2 embryos at two acute Test Temperatures (22 and 32 °C) representing the thermal extremes at which zebrafish develop normally [35]. I used a Microplate Respirometry System (Loligo Systems) with two 24-well, 80 μL microplates run in parallel. Microplates were calibrated to manufacturer specifications the previous night. I photographed individual embryos under a dissecting scope and placed embryos into wells randomly assigned by MicroResp software (Loligo Systems), with four blanks (Appendix C). Microplates were then set to

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the two Test Temperatures, and the entire system was placed on an orbital shaker on the lowest setting, reducing oxygen stratification. I recorded oxygen consumption in each microplate for at least 3 h. I repeated measurements across six trials, with each trial day consisting of offspring from one of the three F1 families, and each of the four parental thermal treatment combinations.

Statistical analyses

I used MicroResp software to estimate embryo RMR in normoxia (80 – 100% oxygen saturation). I selected an r2 value of ≥ 0.8 for linear estimations of oxygen consumption (MO2) rates, and applied automatic correction for background respiration from blanks.

I measured F1 embryo egg diameter from photographs as a proxy for offspring size. I could not consistently measure yolk diameters, as yolks were occasionally obscured by embryos; however, we previously showed a strong positive correlation between yolk and egg diameters across thermal treatments [41]. Upon reviewing images, we excluded embryos that exhibited deformity (e.g., kyphosis) or had visible tears in their chorions.

I used Bayesian generalized linear mixed models to analyse data using the *brms* package (v. 2.19.0 [42]) in R (v. 4.2.2); details are in Appendix C. These models estimated the causal effect of predictors on offspring RMR. For Model 1, I estimated the effects of 'Early' (0-29 dpf) and 'Later' (30+ dpf) Parental Temperatures (Constant or Fluctuating) as well as the acute 'Test' Temperature (22 or 32 °C) on log-transformed embryo RMR [43]. I included interactions between 'Test' Temperature and each Parental Temperature treatment, to test for differences in TGP thermal sensitivity. I included 'Trial' as a random intercept to account for within-trial interdependence (e.g., due to the sum of differences in daily calibration and family-level effects). To test whether changes in offspring RMR were mediated via changes to egg size, I also fitted Model 2, correcting for 'Egg Size' (diameter). Using two separate moels allowed us to avoid overcontrol bias [40] in our estimation of 'Later' Parental Temperature in Model 1 as this treatment impacts egg size [41]. I interpreted nonnegligible effects of Early or Later Parental Temperatures on offspring RMR as evidence of transgenerational plasticity (TGP), and interactions between Parental Temperatures and Test Temperatures as differences in the

thermal sensitivity of TGP between Test Temperatures. Visual model checks were made and are presented in Appendix C.

Animal ethics

This study was approved by the Dalhousie University Committee on Laboratory Animals (Protocol 22-005).
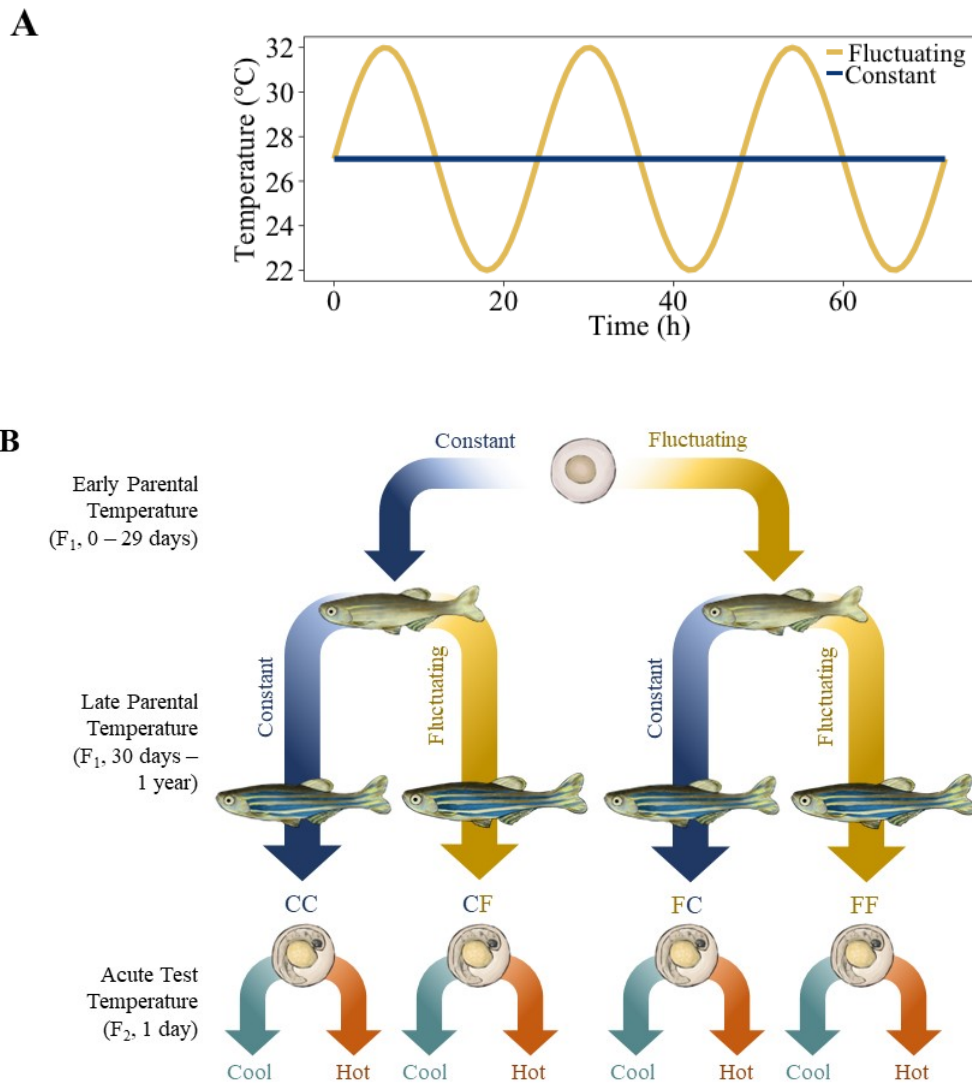
Figure 17: *Split-clutch, factorial design of a long-term transgenerational plasticity experiment. A) Constant ('C', blue; 27 °C) and diel Fluctuating ('F', gold; 22-32 °C) thermal treatments for F_1 fish. B) F_1 zebrafish (Danio rerio) were exposed to thermal treatments during Early development (0 – 29 days; first letter) and Later development (30 days – 1 year; second letter). Offspring (F_2) routine metabolic rates were then tested at Cool (teal; 22 °C) or Hot (red; 32 °C) temperatures.*

Results

Visual model checks revealing adequate model specification and numeric detailed model results are presented in Appendix C. Median results for metabolism and egg size, with individual data circled, are illustrated in Figure 18 (A, B), and posterior effect sizes for Model 1 are illustrated in Figure 18C. Briefly, mean effect sizes further from 0 indicate stronger effects of predictors on offspring RMR, and the greater the overlap of uncertainty intervals (UIs) with 0, the less certainty in estimates.

As expected, I found large differences in F2 offspring RMR at the two acute Test Temperatures, with 28.4% (90% UIs: [13.93%, 44.8%]) higher metabolic rates at 32 °C compared to 22 °C (Fig. 18A, C). Early Parental Fluctuating Temperatures led to a decrease in embryo RMR, with 21.3% lower RMR when compared to offspring from Early Parental Constant Temperatures (Fig. 18A, C; 90% UIs: [-28.3%, -14.0%]). In comparison, Later Parental Temperature had a weak effect on offspring RMR, with 2.7% lower RMR in offspring of Fluctuating vs. Constant temperatures (Fig. 18A, C; 90% UIs: [-11.1%, 6.5%]).

I also detected a weak interaction between Early Parental Temperature and the acute Test Temperature, such that the offspring of parents exposed to Early Fluctuating Temperatures were 7.0% less sensitive (90% UIs: [-5.8%, 21.7%]) at the acute 32°C Test Temperature, when compared to 22 °C (Fig. 18A, C). I detected no significant interaction between Later Parental Temperature and Test Temperature (Fig. 18A, C; median: 1.0%, 90% UIs: [-13.2%, 12.7%]).

There was a strong influence of egg diameter (Fig. 18B) on RMR, such that a 1 mm increase in diameter was predicted to lead to a 70% increase in RMR, with wide variation in the posterior distribution (Model 2; 90% UIs: [4.7%, 178.3%]; details in Appendix C). However, the inclusion of this predictor did not meaningfully impact estimates of Early Parental Temperature's effects, though the lowering effect of Later Parental Fluctuating Temperature on offspring RMR disappeared (median: 1.4%, 90% UIs:[-8.0%, 11.3%]).
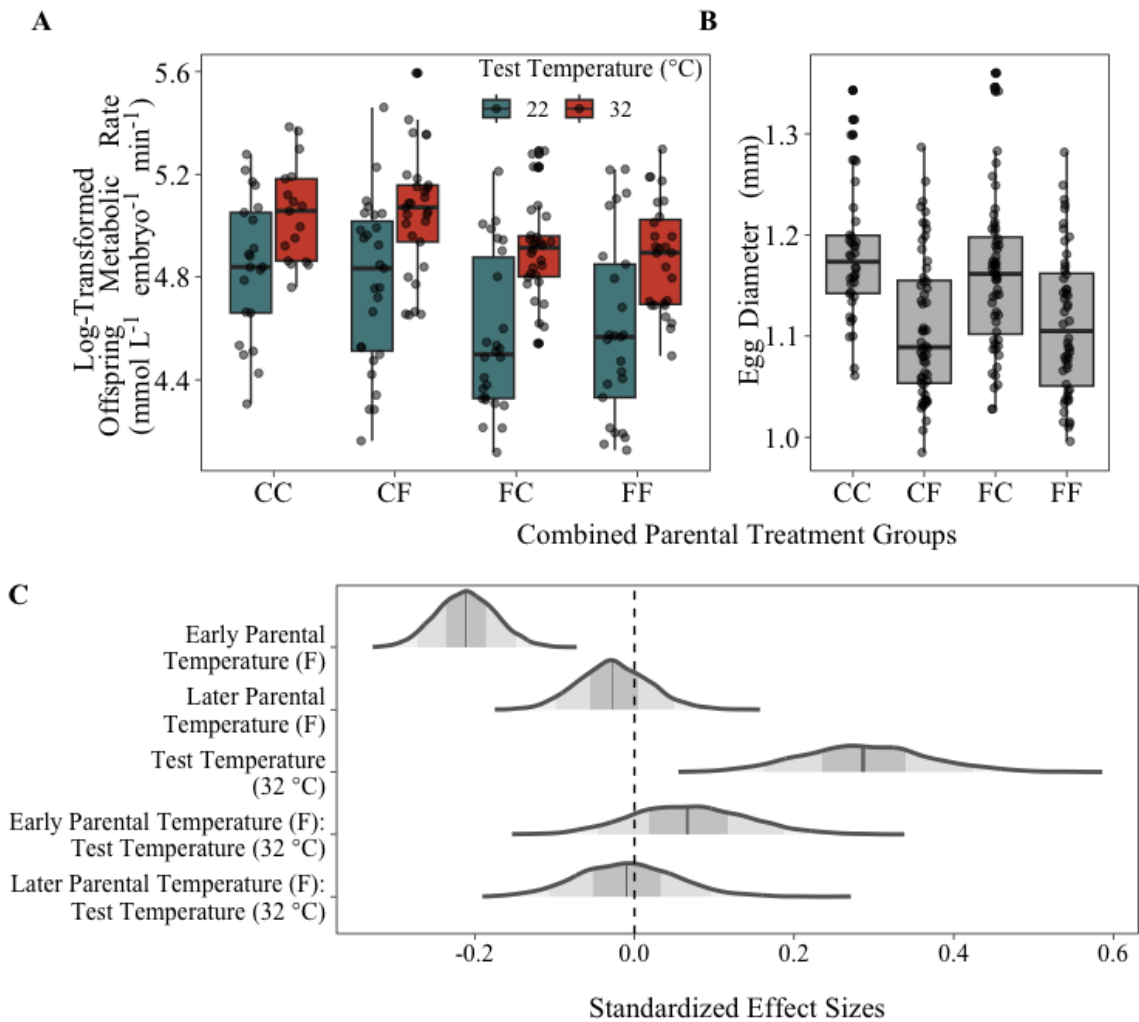
Figure 18: *Transgenerational plasticity in offspring metabolism of F₁ zebrafish (Danio rerio) exposed to Constant ('C', 27 °C) or diel Fluctuating ('F', 22 – 32 °C) temperatures during Early Parental development (0 – 29 days, first letter), Later Parental development (30+ days, second letter), or both. A) F₂ Offspring routine metabolic rates (RMR) tested at Cool (teal; 22 °C) or Hot (red; 32 °C) thermal extremes. B) Offspring egg diameter. C) Posterior distributions of effect sizes of Parental Temperatures, Test Temperature, and their interactions on F₂ offspring RMR. Posterior means are represented by thin dark lines at the mean of each distribution; 90% and 50% uncertainty intervals are represented by increasingly light shading.*

Discussion

Although transgenerational plasticity (TGP) is an important mechanism through which organisms can modify offspring phenotypes to withstand climatic stressors, we have little empirical data describing the role of parents' early life thermal experiences on offspring responses [44]. Here, I show that biparental thermal experiences of F1 zebrafish during their first month of life strongly affected F2 offspring embryonic routine metabolic rates when exposed to stressful temperatures. Indeed, metabolic rates were over 20 percent lower at thermal extremes when parents were initially reared in challenging, thermally variable conditions compared to optimal, constant early life conditions.

Anthropogenic climate warming has increased ectotherm metabolic rates worldwide [10,36], leading to increases in energetic demands and ultimately imposing constraints on adult body sizes [8,9]. Although interpretations of plasticity-induced reductions in metabolism are context-dependent [45], lower metabolic rates are predicted to confer growth benefits under stressful thermal environments [18,36]. Although later offspring body sizes were not measured here, other studies rearing fish in warm temperatures have explicitly linked lower routine metabolic rates to improved growth rates via TGP [12,18] and within-generation plasticity [46]. Therefore, I interpret the reduction in embryonic metabolic rate as beneficial metabolic compensation, which could ameliorate negative impacts of thermal stressors on body size as offspring develop [12,18,45,46].

I also tested whether these reductions in metabolic rate were mediated via direct reductions in egg size, given that metabolic rates positively scale with organismal size [47] and later life fluctuating temperatures reduce both parental body sizes and egg provisioning in zebrafish [41]. By correcting for differences in egg size, the small decrease in offspring RMR owing to fluctuating later life parental temperatures disappeared. This may reflect a condition-transfer effect [48], in which constraints on parental energy expenditure during later life led to a reduction in egg size, and thus offspring RMR [11,41]. By contrast, the large reduction in RMR caused by fluctuating early parental temperatures occurred over and above effects on egg size. Instead, it is likely that this metabolic compensation was facilitated through alternative epigenetic mechanisms established during parental early life [25]. For example, beneficial TGP to warm temperatures has been shown to be mediated through differential gene expression,

including the upregulation of genes associated with mitochondrial activity and energy production [15,32,49]. Other potential mechanisms include methylation and histone modifications in germline cells during early parental development [6,25,44].

In this experiment, I also found a modest interaction between early parental temperatures and acute test temperatures. Contrary to previous findings, however, we found slightly less metabolic reduction at the hotter vs. the cooler offspring test temperature for offspring from early fluctuating temperature parents [12,30]. It is possible that the scope for thermal sensitivity of TGP was reduced in the acute hot test temperature because of physiological limitations, as 32 °C is the upper thermal limit for the normal development of zebrafish embryos [35].

Overall, I found that there is strong TGP in offspring routine metabolic rate in response to ecologically realistic thermal variability experienced during parental early life, but a negligible influence of later life adult thermal experiences. These data highlight the importance of studying how the timing of stressors influences plasticity, and show that early life parental environments can generate significant phenotypic variation in subsequent generations. A goal of future studies will be to understand the long-term consequences of these changes.

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Chapter 6: Conclusions

Plasticity is a key mechanism by which organisms can rapidly respond to stressors imposed by anthropogenic climate change [1–5]. The causes and consequences of plasticity are therefore of immediate interest to ecologists. Yet, we are currently uncertain about how the timing of ecological stressors impacts variation in phenotype and limited by the paucity of studies eliciting plasticity using ecologically realistic conditions. In this thesis, I address these two issues using variable thermal treatments in fish as a model system.

The goal of my thesis was to address these knowledge gaps to further our understanding of organismal responses to anthropogenic climate change. Specifically, I (i) describe and compare developmental plasticity, acclimation, and transgenerational plasticity, and (ii) contrast the effects of ecologically realistic thermal variability with constant optimal temperatures, within and across generations of zebrafish (*Danio rerio*). I accomplished this by conducting long-term factorial experiments, which allowed me to separate contributions to phenotypic variation in multiple traits arising through developmental plasticity, acclimation, and transgenerational plasticity in response to ecologically realistic thermal variability.

Within- and across-generational plasticity and climate change

Early life environments drive beneficial developmental plasticity in most physiological traits

In my within-generation experiments, adult fish displayed both developmental plasticity (during embryonic and larval life stages) and acclimation (during juvenile and adult life stages) in response to thermal variability in most traits (Table 1). Developmental plasticity had stronger overall physiological effects, leading to improvements in thermal tolerance and hypoxia tolerance, a reduction in basal metabolic demands, and enhancing the oxygen supply capacity of adult fish (Chapter 3). In contrast, acclimation to thermal variability later in life had negligible effects on the physiological traits studied, except thermal tolerance, which displayed stronger beneficial acclimation – an additive effect [6] – than beneficial developmental plasticity alone (Chapter 3).

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Table 1: *Summary of main effects of different forms of plasticity in response to thermal variability (vs. constant optimal temperatures) applied at different life-stages of zebrafish (Danio rerio), and sorted by trait class. Data summarized from experimental Chapters 3 – 5.*

Type of plasticity	Class of trait	Phenotypic Trait	Effect
Developmental plasticity *Plasticity resulting from experiencing fluctuating temperatures during embryonic and larval stages (0 – 29 days post-fertilization)*	**Morphology (Chapter 4)**	Body size (averaged between sexes)	Negligible effect
	Physiology (Chapter 3)	Thermal tolerance (CT_{max})	Increase (additive with acclimation, but weaker)
		Hypoxia tolerance (P_{crit})	Increase
		Routine metabolic rate	Decrease
		Oxygen supply capacity (α)	Increase
	Reproduction (Chapter 4)	Spawning success	Decrease (developmental plasticity alone), but with environmental matching, increase
		Fecundity (clutch size)	Increase
		Egg quality (yolk volume)	Decrease
		Male sperm quality (concentration)	Negligible effect
Acclimation *Plasticity resulting from experiencing fluctuating temperatures during juvenile and adult stages (30+ days)*	**Morphology (Chapter 4)**	Body size (averaged between sexes)	Decrease
	Physiology (Chapter 3)	Thermal tolerance (CT_{max})	Increase (additive with developmental plasticity)
		Hypoxia tolerance (P_{crit})	Negligible effect
		Routine metabolic rate	Negligible effect
		Oxygen supply capacity (α)	Negligible effect
	Reproduction (Chapter 4)	Spawning success	Decrease (acclimation alone), but with environmental matching, increase
		Fecundity (clutch size)	Decrease
		Egg quality (yolk volume)	Decrease
		Male sperm quality (concentration)	Negligible (direct effect), decrease (due to effects on body size)
Transgenerational plasticity *Plasticity resulting from parents experiencing fluctuating temperatures*	**Physiology (Chapter 5)**	Offspring metabolism	Decreased (when tested at stressful temperatures), specifically as a result of parental early life exposure to thermal variability.

Collectively, beneficial changes in these traits imply populations of wild zebrafish could experience greater survival [7–9] and metabolic efficiency [8,10–12] under increased mean temperature, heat waves, and hypoxic stressors [13,14], provided they are cued by thermal variability at appropriate time periods (*summarized in* Chapter 3). Indeed, there similar within- and across-generation plastic improvements to physiological traits associated with aerobic metabolism under heat acclimation in stickleback and killifish [15,16], suggesting this conclusion can be extended to biologically similar species, such as small-bodied fishes with similar life histories that experience environmental heterogeneity.

Acclimation induces costs, but developmental plasticity induces trade-offs in morphology and reproduction

Fish demonstrated a stronger capacity for acclimation than developmental plasticity in their morphology and reproduction, though these effects were complex, and not always beneficial (Table 1; Chapter 4). Body sizes, fecundity, yolk quality, and sperm quality were all negatively affected by adult acclimation to thermal variability relative to constant optimal temperature exposure. In comparison, developmental plasticity in response to temperature variation had negligible effects on body sizes and sperm quality. In females, however, there appeared to be a developmentally plastic trade-off between fecundity and egg quality, in which females exposed to variable temperatures during early life shifted towards producing a greater number of smaller eggs. There was also a significant, positive interaction between developmental plasticity and acclimation for spawning success. If early and late life environments matched, spawning success was greater, regardless of thermal treatment, an example of beneficial acclimation [6,17].

It is clear that there were costs associated with long-term exposure (acclimation) to thermal variability [18] – these costs manifested as overall reductions in body size, fecundity, and egg and sperm quality. These were probably driven by increased metabolic costs of coping with variable *vs.* optimal temperatures [19]. The developmental plasticity-induced trade-off between egg number and quality, however, may be beneficial, reflecting a life-history approach that optimizes egg size to thermal conditions [20,21]. Indeed, smaller zebrafish eggs have higher fertilization and hatching rates when temperatures are

hot [21]. It is interesting to consider that these changes parallel two hypotheses for the overall reduction of body size in fishes worldwide *via* plasticity: Increased warming (i) reduces body size due to metabolic increases, and (ii) causes life history trade-offs that are presumably adaptive [22,23]. In a plasticity framework, my results support both hypotheses, further emphasizing the time-dependency of plasticity.

Transgenerational plasticity of offspring metabolism was strong and influenenced by parental developmental plasticity

I detected evidence of strong transgenerational plasticity in response to the early life thermal experiences of parents. Parental exposure to thermal variability elicited an over 20% reduction in metabolic rates of their offspring tested at hot and cool thermal extremes, which is expected to benefit offspring by reducing energy expenditure at stressful temperatures [11,15]. Notably, this transgenerational effect on offspring was a result of developmental plasticity of parents, and was only influenced by their early life conditions. This finding links developmental plasticity of parents to transgenerational plasticity of offspring, which has been hypothesized to occur if there is some correlation between parent and offspring early life environments [24,25].

Summary and synthesis

Taken together, my results show evidence of plasticity to thermal variability in all traits studied, but there was variation in the type of plasticity (*i.e.*, timing of exposure) that led to these changes. Physiological changes were largely influenced by developmental plasticity, whereas morphological and reproductive metrics were largely influenced by later life acclimation, and some traits were influenced by both exposures. These results highlight that long-term experiments including all stages of ontogeny should be used if authors want to describe the true range of within- and across-generation phenotypic variation that plasticity can induce.

My experimental results also demonstrated that developmental plasticity can have strong and persistent effects in traits linked to fitness: These include surviving stressors (*e.g.*, thermal and hypoxia tolerance) and reproduction (*e.g.*, the fecundity-egg size

relationship). Recent reviews have suggested that developmental plasticity in response to temperature often has effects that are small in magnitude, throwing into question the importance of developmental plasticity in mediating the negative effects of climate change [26,27]. However, authors have also acknowledged that the overuse of constant temperatures in these experiments may bias results – for instance, if the developmental cost of coping with stressful static temperatures eliminate or obscure the signal of beneficial plasticity [26,28]. Aquatic ectotherms also display much stronger developmental plasticity than do terrestrial ectotherms [27]. Therefore, a combination of the realistic thermal regime I selected and the biological propensity towards high plasticity of zebrafish themselves may have revealed a previously cryptic capacity for developmental plasticity.

On defining forms of plasticity, irreversibility, and critical windows

There is currently a focus on the 'reversibility' of a plastic effect as a means to differentiate developmental plasticity from acclimation, even though nearly all authors acknowledge that there are many exceptions to the rule that plasticity to early life environments is irreversible (some examples of reversibility include: [29,30], *reviewed in*: [31]). A second common assertion is that developmental plasticity must occur in response to early life conditions [31], which is often taken to exclusively mean embryogenesis [32]. Yet, development is a process that occurs continuously throughout life, and organisms can retain the ability to modify many responses to environmental stimuli well past the embryo stage [33,34]. Indeed, in the seminal work *Developmental Plasticity and Evolution*, West-Eberhard explicitly makes no distinction as to the irreversibility or reversibility of plasticity, or the life-stages that differentiate types of plasticity (p. 36, [32]). Ultimately, there appears to be a range of trait- and timing-specific plastic responses in organisms, which vary from near-instantaneous responses across environmental gradients (*e.g.*, a reaction norm), to responses that cause more persistent changes (*e.g.*, shifting of a reaction norm's parameters for some longer period of time), to responses that are large in magnitude and perhaps irreversible (*e.g.*, developmental phenotypic switching); delineation of these responses is unclear [19,31,32,35].

In my own analyses of within-generation plasticity of multiple traits, I made the assumption of delineating developmental plasticity from acclimation by comparing early *vs.* late ontogenetic periods of exposure. I found a great deal of variation in the presence, magnitude, and direction of plasticity in response to these two ontogenetic thermal treatments, and the timing-specific plasticity of each trait had ecological implications for fish. The category of plasticity that each response fell into was secondary to understanding their timeframes of environmental sensitivity. Consequently, I suggest that more conceptual emphasis should be placed on the trait-specific 'critical windows' of sensitivity to exposure that cause phenotypic plasticity, and terms like *irreversible* or *developmental* are more useful as descriptors of plasticity (*sensu* West-Eberhard [32]). There are several reasons for this:

1) Individual traits differ in their ontogenetic critical windows of sensitivity to the environment, and this can have important consequences for adult phenotype [36,37]. Across my experimental chapters, some traits appeared to be sensitive only to early developmental environments: these included the traits associated with aerobic metabolism, such as routine metabolic rate and oxygen supply capacity. Other traits were only sensitive to the temperatures experienced during the juvenile and adult life stages, including body size and sperm quality. Interestingly, a few traits were sensitive to both, and the magnitude and direction of these effects differed: Thermal tolerance was additive across early and late life, and spawning success showed evidence of beneficial acclimation of prior exposure. Notably, there were opposing effects of early and late thermal experiences on fecundity. Taken together, these data suggest there is a need to understand how different time periods of exposure can affect the existence, magnitude, and direction of phenotypic plasticity; this task can be accomplished by carefully choosing critical windows.

2) Understanding critical windows lends insight into the mechanisms of plasticity [31,37]. For instance, in Chapter 3, I illustrate that early life exposure to thermal variability improved zebrafish hypoxia tolerance, but late life exposure had no effect. Consequently, this suggests mechanisms generating variation in adult hypoxia tolerance are responsive only to inputs that occur before the juvenile life-stage. And indeed, hypoxia tolerance-associated changes in gene expression pathways and cardiorespiratory organs are known to occur during early organismal differentiation [16,38–40]. In situations where mechanisms are unknown, identification of a critical window can assist authors in elucidating environmentally sensitive proximate mechanisms.

3) Critical windows during parental lives can also affect transgenerational plasticity [41–43], though parental early lives are not well-explored [1]. In Chapter 5, I showed that the embryonic and larval thermal experiences of parents led to transgenerational plasticity of offspring metabolic rates, and the subsequent eleven months of life after this period had no effect. Little attention has been given to parental critical windows for producing transgenerational plasticity, though authors have highlighted this an important future direction for transgenerational plasticity research [1,41–43].

4) Knowing critical windows for plasticity of specific traits is relevant to understanding organismal responses to climate change. Stressors associated with climate change may be more likely to occur at certain times of year; for instance, heat waves and freshwater anoxic periods are expected during summer months [44,45]. If we can accurately forecast periods of intense stressors relative to the life cycle of organisms (*e.g.*, spawning), we can make better predictions about how they will adjust phenotypes *via* plasticity in response to these stressors.

With that said, 'developmental plasticity' (during some early life-stage) and 'acclimation' (during some later life-stage) remain convenient terms with which to categorize within-generation plasticity. Despite varying definitions, they are commonly used and easily recognizable terms [31], that unite shared research interests between physiologists and ecological or evolutionary developmental biologists [46,47], and assist with straightforward experimental designs, such as the one used in my experimental chapters (*see also*: [29,30,48]). Irrespective of whether we term within-generation plasticity 'developmental plasticity' or 'acclimation,' and whether these are reversible or not, understanding the breadth and sensitivity of critical windows during ontogeny provides more useful information about how current phenotypes were generated.

Limitations

There are several limitations to my studies that suggest directions for future work. First, in relation to the section above, I only tested two critical windows of thermal sensitivity. Although my choice of breakpoint (larval metamorphosis) was biologically meaningful, this design excluded further delineation of other critical windows. For example, I was able to describe (i) the effects of the early developmental environment on adult phenotype, (ii) clearly show that these effects were persistent, and (iii) understand that they occurred as a result of the environmental experience some time between age 0 and 29 days post-fertilization. I could not, however, determine if 'acclimation' effects occurred during juvenile development (30 – 90 days) or sexual maturity (90+ days), or some other range of days during the treatment, nor could I determine how persistent these effects were.

An interesting solution to this issue comes from the temperature-dependent sex determination literature, in which temperature-switching experiments are used to determine the timing of sensitivity to temperature [49]. Here, experimenters will split clutches into treatments that compare windows of exposure, by applying a condition across multiple points during ontogeny (Figure 19). Differences between each treatment thus illuminate when critical windows exist, and the magnitudes of effects can be compared. Future studies dissecting plasticity could apply this framework to answer questions such as "when during ontogeny will climate change conditions have the

strongest, negative impacts on a given trait?" Or, as a specific example of contemporary interest in the study of cross tolerance [7,50], "What timepoints of exposure to heat generate cross-tolerance to hypoxia?"
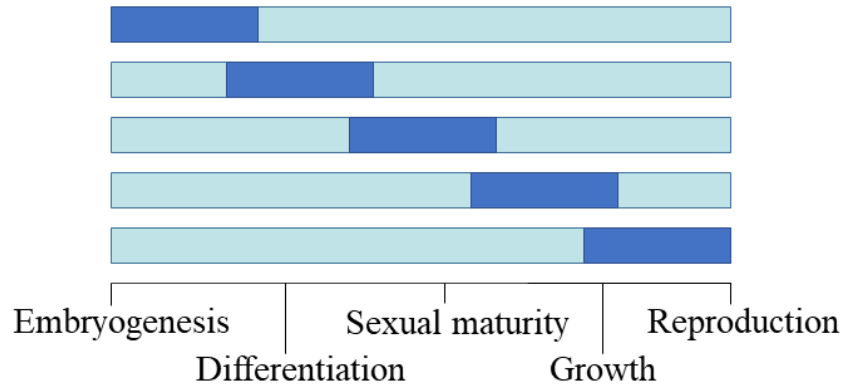
Figure 19: Example of experiment designed to investigate critical windows for plasticity, based on temperature-switching experiments originally used to determine critical periods for temperature-dependent sex determination [49]. Experimenters apply stressors (dark blue boxes) to split clutches (along light blue lines) over time at different periods of interest during ontogeny. Some periods of ontogeny are listed on a developmental timeline for illustrative purposes.

Next, for logistical reasons, I only compared two thermal regimes of exposure: an optimal temperature, and predictable diel thermal variability around that optimal temperature. I chose this approach to yield foundational insights on thermal variability's general plastic effects, but there are many alternatives. As an example, future work with zebrafish might compare current (variable) early life conditions with predicted future conditions, using thermal means and variance drawn directly from empirical data in natural habitats. It will ultimately be important to determine these treatments based on ecological knowledge of natural systems. For instance, seasonality is changing as a result of anthropogenic climate change, with longer summers and later winters in some parts of the world [51,52]. Authors might then be interested to know, for their given study population, how a longer-than-normal summer regime (*i.e.,* longer duration exposure to summer temperatures, without changing the magnitude) affects phenotypic variation in traits like winter survival or subsequent reproduction, and whether previous experience (*e.g.,* exposure to heat waves) causes persistent plasticity that modifies these effects.

Last, I chose to use a model laboratory organism to investigate my questions on plasticity to thermal variability, but this approach limited the external validity of my results. First, any laboratory reared or acclimated organism will be differently (likely, better) conditioned than their wild counterparts. This can result in an overestimation of beneficial plasticity in the wild, because organisms in better body condition can display a higher capacity for plasticity [53]. This was demonstrated by Morgan *et al*. [53], who showed that wild zebrafish had lower thermal tolerance than lab-raised zebrafish, despite the hypothesis that their thermally variable history should improve thermal tolerance through plasticity or local adaptation. Next, lab zebrafish are genetically distinct from wild zebrafish populations [54], and have undergone intense selection for fast growth in stable conditions [55]. This selection resulted in reduced physiological plasticity of laboratory strains [55], which may be reflected in my results, potentially as an underestimation of the magnitude of plasticity in some traits. Future work could improve external validity by using ecologically realistic laboratory conditions (*e.g.*, feeding fish a diet that mirrors their natural nutritional content) and using the recent descendants of wild-caught fishes, to minimize genetic divergence from focal populations.

Conclusions

In this thesis, I illustrated the distinct and interacting effects of developmental plasticity, acclimation, and transgenerational plasticity to ecologically realistic thermal variability in multiple metrics relevant to organismal fitness. Through comparing these forms of plasticity, I highlight the significance of the timing of stressors during organismal ontogeny. In particular, I show that developmental plasticity, though often ignored in the context of climate change plasticity research, can have appreciable effects on the phenotypes of both adults and their offspring *via* transgenerational plasticity. These findings empirically demonstrate that aquatic fishes may have a greater capacity than previously anticipated to beneficially respond to climatic stressors expected under climate change, and underscore the importance of ecologically realistic experimental conditions in the study of climate change responses.

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Appendix A: Supplemental Material for Chapter 2, Developmental plasticity facilitates enduring physiological tolerance under ecologically realistic temperatures

Supplemental Results

Bayesian Median Estimates, 50% and 90% Uncertainty Intervals for Fixed Effects

Appendix A Table 1: Model results for Bayesian critical thermal maximum (CT_{max}) model.

	Median	Lower 50	Upper 50	Lower 95	Upper 95
Intercept	40.06	39.97	40.15	39.78	40.34
Developmental Temperature (F)	0.29	0.18	0.40	-0.03	0.63
Acclimation Temperature (F)	1.18	1.07	1.29	0.86	1.52
Male Sex	-0.28	-0.39	-0.18	-0.58	0.02

Appendix A Table 2: Model results for Bayesian routine metabolic rate (RMR) model; posterior estimates are presented as raw model output.

	Median	Lower 50	Upper 50	Lower 95	Upper 95
Intercept	-0.62	-0.82	-0.41	-1.23	-0.03
Developmental Temperature (F)	-0.16	-0.23	-0.10	-0.38	0.04
Acclimation Temperature (F)	-0.02	-0.10	0.06	-0.26	0.25
Male Sex	0.06	-0.04	0.15	-0.21	0.33

Appendix A Table 3: Model results for Bayesian hypoxia tolerance (P_{crit}) model.

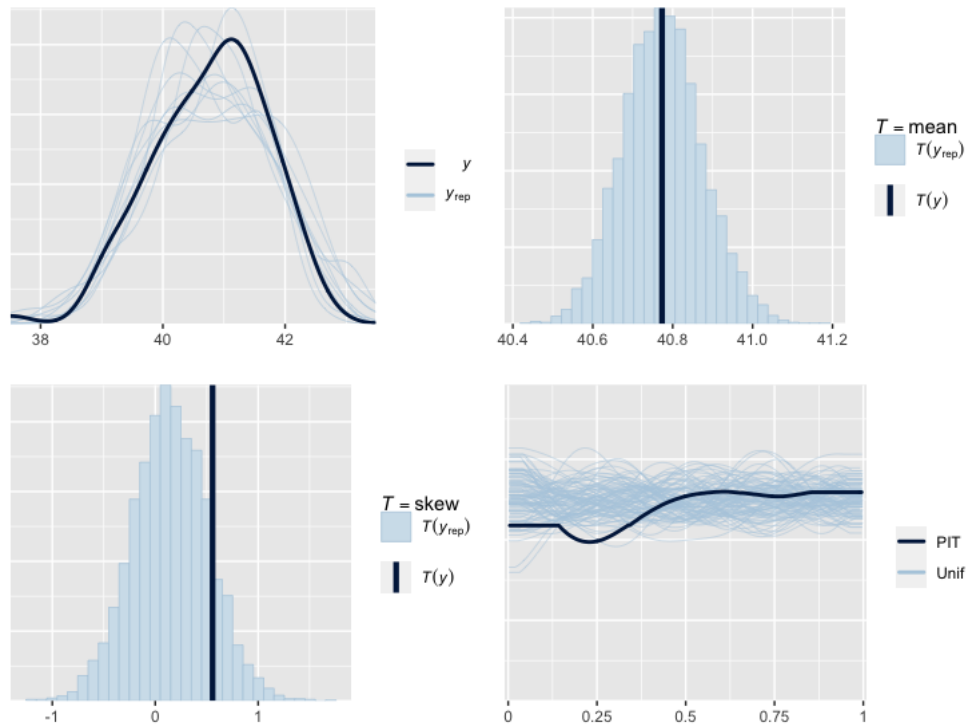|	Median	Lower 50	Upper 50	Lower 95	Upper 95
Intercept	14.83	10.68	18.86	2.65	27.03
Developmental Temperature (F)	-5.91	-7.22	-4.59	-10.04	-1.84
Acclimation Temperature (F)	0.92	-0.78	2.52	-3.82	6.15
Male Sex	0.73	-1.21	2.72	-5.14	6.51

Appendix A Table 4: Model results for Bayesian physiological oxygen supply (alpha) model.

	Median	Lower 50	Upper 50	Lower 95	Upper 95
Intercept	4.05E-02	3.59E-02	4.52E-02	2.60E-02	5.50E-02
Developmental Temperature (F)	4.43E-03	2.85E-03	5.92E-03	-2.75E-04	9.55E-03
Acclimation Temperature (F)	-1.16E-03	-3.06E-03	6.86E-04	-7.06E-03	4.76E-03
Male Sex	3.40E-03	1.14E-03	5.62E-03	-3.30E-03	9.80E-03

Visual model checks

Posterior predictive checking allows one to visually examine the fit of a model with respect to the real data. I implemented joint posterior predictive checks (on all data), as well as pointwise posterior predictive checks on statistics (median and skew of data). These checks act as a safeguard against gross misspecification. Next, I visualized Leave-One-Out Cross Validation Probability Integral Transformation (LOO-PIT) values to examine how predictive distributions are dispersed relative to conditional observations. My checks revealed adequate model specification.
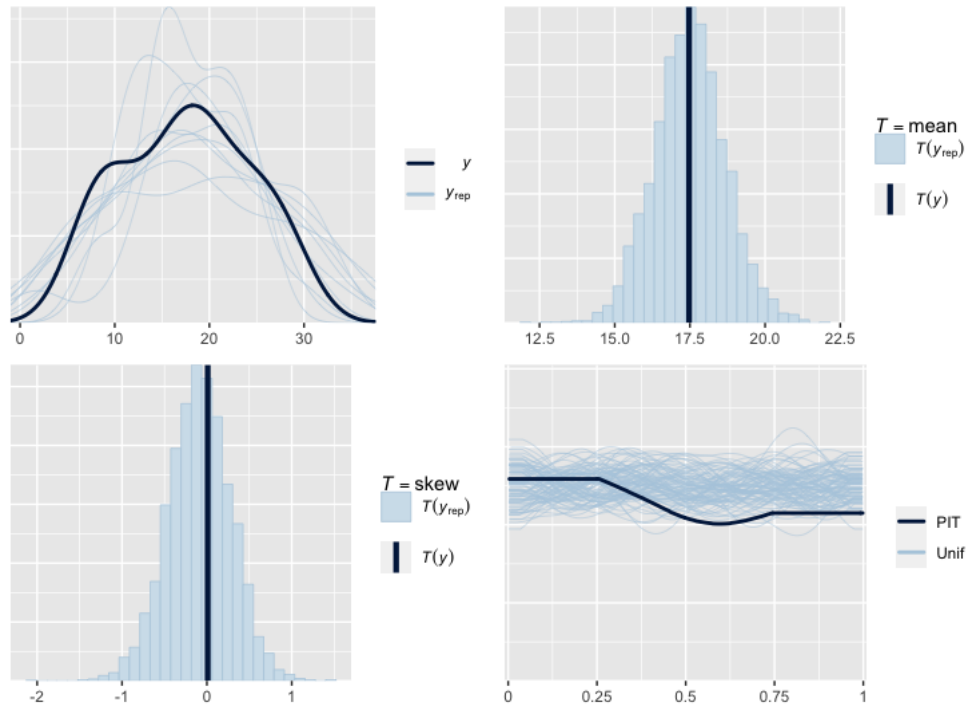
The visual model checks below are arranged as such: joint posterior predictive check (top left), median (top right), skew (bottom left), and LOO-PIT values (bottom right).
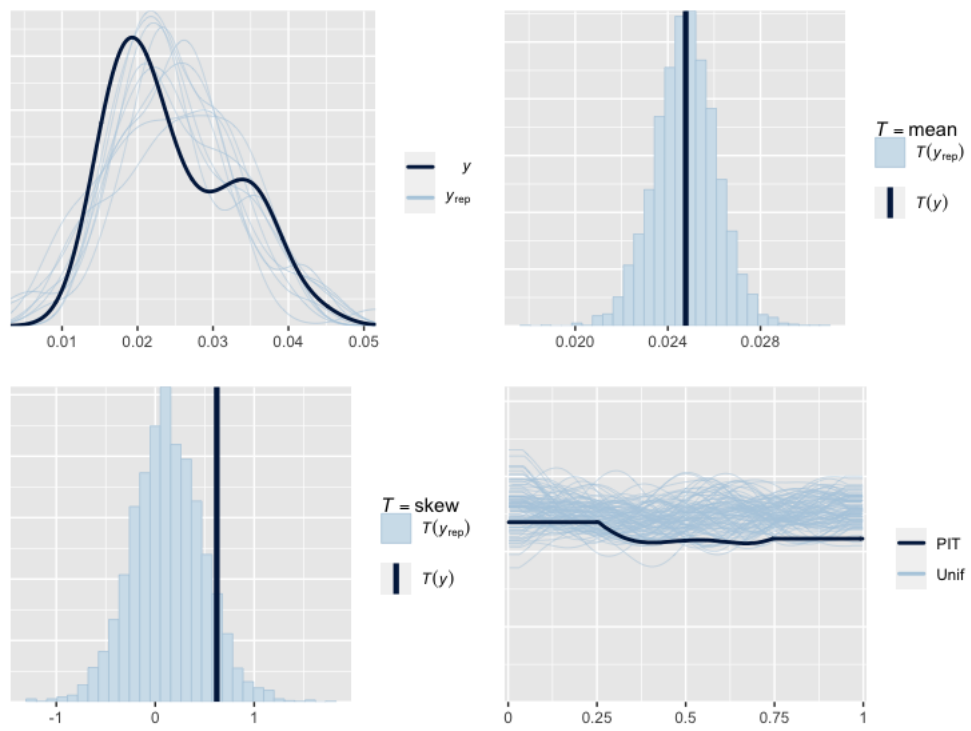

Appendix A Figure 1: Posterior predictive checks for critical thermal maximum model.
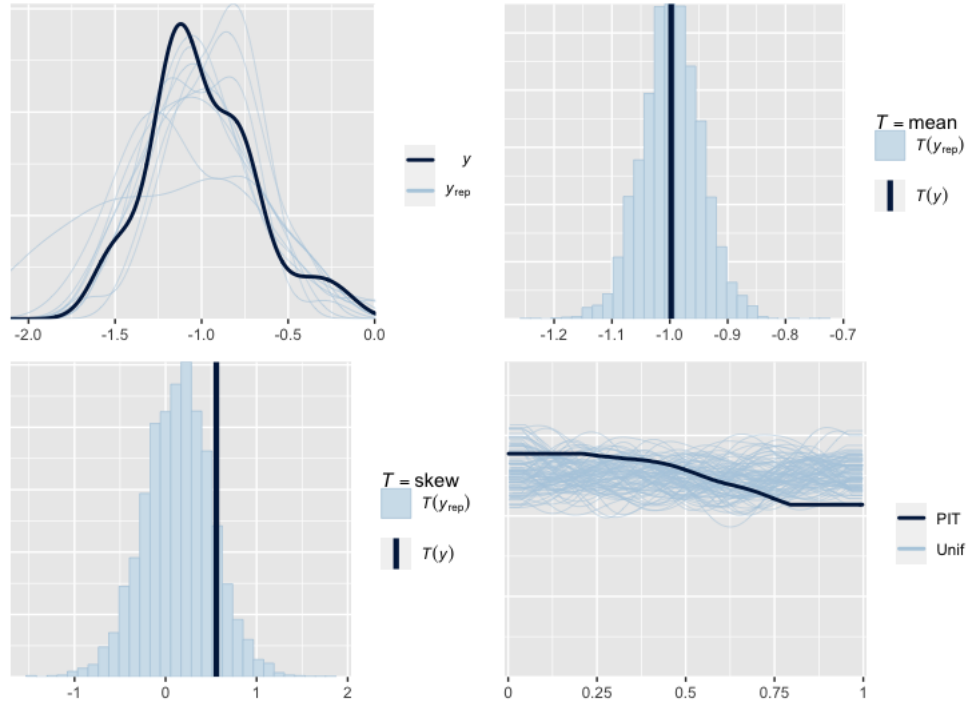
Appendix A Figure 2: *Posterior predictive checks for routine metabolic rate model.*

Appendix A Figure 3: *Posterior predictive checks for hypoxia tolerance (P_{crit}) model.*

Appendix A Figure 4: *Posterior predictive checks for physiological oxygen supply (alpha) model.*

Appendix B: Supplemental Material for Chapter 3, Differential reproductive plasticity under thermal variability in a freshwater fish (*Danio rerio*)

Rearing conditions for eggs, embryos, and fish

Ancestral stock

All fish in the ancestral stock of zebrafish from the Zebrafish Core Facility (ZCF; Dalhousie University, Halifax) were held in standard conditions (28 °C, 14 h light: 10 h dark) to control for environmentally driven parental (on F_0 fish) and grandparental effects (on F_1 eggs) in our experiment. I kept photoperiod to standard 14 h light: 10 h dark throughout our experiment.

Protocols for rearing F_0 zebrafish

Freshly laid F_0 eggs acquired from the ZCF were kept in 100 mL of embryo medium in 1 L beakers, placed in water baths set to treatment temperatures connected to the flow-through system. Eggs were checked daily to remove debris and replenish embryo medium. I set 5 days post-fertilization (dpf) as a cutoff point before transferring larvae to larger containers, as in my experience, the vast majority of viable eggs hatch by 3 dpf.

From 5 – 15 dpf, F_0 larvae were housed in approximately 0.75 L of dechlorinated City of Halifax water in 3 L zebrafish tanks placed in 10 L water baths connected to the flow-through system, such that larvae experienced treatment temperature conditions. A HOBO MX2200 temperature datalogger (Onset Corporation, Bourne, MA) was placed in one

tank per treatment that contained no larvae but had the same amount of water as treatment tanks to estimate temperatures within treatment tanks. On alternating days, 300 mL of either rotifer or algae solution were added to each 3 L larvae tank. This produced a co-culture of rotifers and algae that promoted *ad libitum* and natural feeding behaviour, while simultaneously slowly increasing water levels to promote swimming behaviour.

At 15 dpf, the fish were switched to size-appropriate commercial feed. Larvae and juveniles were fed GEMMA 150 (Skretting, Maine, USA) and adults were later fed GEMMA 300 (Skretting, Maine, USA) twice-daily. By this time, larvae were able to swim through the water column, and we connected 3 L larval tanks to the flow-through system with a gentle flow cycle. Although I did not explicitly collect data on mortality in this experiment, pilot studies in my laboratory indicated that there are no mortality differences between thermal treatment groups at 30 dpf.

At 30 dpf, when fish had reached the juvenile period, they were split randomly and evenly once more into juvenile-adult treatment groups in 3 L tanks. These final treatment tanks ultimately housed 10 – 15 adult fish, all of whom were siblings that shared the same thermal history (either CC, CF, FC, or FF).

Thermal treatment apparatus

My laboratory in the Dalhousie University Aquatron was equipped with a flow-through system connected to two zebrafish racks, one for the Constant and one for the Fluctuating treatment. Header tanks in the room supplied hot (32 °C) and cool (22 °C) water to the racks. The Constant rack used an approximately equal mixing of water from these two header tanks, resulting in ~27 °C water being input to tanks. The Fluctuating temperature rack was equipped with a custom-built 'valve apparatus', with hot and cool water entering the valve via two separate pipes, and exiting the apparatus to be fed into the rack. The valve itself was programmed to rotate 180°, alternating between the hot and cool pipes, on a 12 h basis. As the valve moved radially across 180°, it permitted mixing of water from the two separate pipes; for example, when the valve was at 90°, approximately equal amounts of hot and cool water were mixed, supplying the Fluctuating rack with 27 °C water. Likewise, when the valve was at 0°, the Fluctuating rack was supplied only with water set to 32 °C. The effect of this was such that the valve

would complete a full cycle from hot-cool-hot on a 24 h basis, resulting in approximately sinusoidal thermal curves peaking at 12:00 pm and reaching a minimum at 12:00 am to mimic natural conditions. This custom valve apparatus was engineered by Mechanical Engineer Piotr Kawalec in collaboration with Aquatron Manager Jim Eddington, both based out of Dalhousie University.

Supplemental Results

Bayesian Median Estimates, 50% and 90% Uncertainty Intervals for Fixed Effects

Appendix B Table 1: Model results for Bayesian maternal body size model.

Parameter	Median	Lower 50	Upper 50	Lower 90	Upper 90
Intercept	25.7666	25.42904	26.09367	24.90279	26.64193
Early Fluctuating	-0.5549	-0.89647	-0.19536	-1.49979	0.413275
Late Fluctuating	-1.4144	-1.76294	-1.07586	-2.33764	-0.47235
Family 2	-0.147	-0.43859	0.162229	-1.01798	0.664892
Family 3	0.8524	0.565127	1.138347	0.011775	1.624887
Week	0.1267	0.078358	0.174577	0.013604	0.240976
Early:Late Fluctuating	0.467	-0.01201	0.961048	-0.85521	1.785964
Paternal Mass					

Appendix B Table 2: Model results for Bayesian paternal body size model.

Parameter	Median	Lower 50	Upper 50	Lower 90	Upper 90
Intercept	24.1663	23.91981	24.40969444	23.49207602	24.8216681
Early Fluctuating	0.0886	-0.17755	0.35362278	-0.63537054	0.7907302
Late Fluctuating	-1.2026	-1.46678	-0.93265277	-1.90170793	-0.485071
Family 2	1.1671	0.94469	1.40301158	0.53642494	1.7978537
Family 3	1.8388	1.602745	2.07809346	1.21149338	2.4357207
Week	0.2132	0.179518	0.24605913	0.13329404	0.2891957
Early:Late Fluctuating	0.1026	-0.26928	0.49134827	-0.921945	1.1386033
Paternal Mass					

Appendix B Table 3: Model results for Bayesian breeding success model.

Parameter	Median	Lower 50	Upper 50	Lower 90	Upper 90
Intercept	-9.0832	-12.36494231	-5.61188	-17.5213	-1.04254
Early Fluctuating	-1.3881	-1.8368339	-0.9176	-2.55942	-0.26318
Late Fluctuating	-0.4166	-0.90568228	0.065739	-1.62142	0.808731
Family 2	0.2575	-0.12783816	0.658895	-0.74769	1.25407
Family 3	0.6252	0.17039988	1.12631	-0.60311	1.832097
Week	0.0811	-0.01632529	0.177319	-0.15953	0.322656
Paternal Length	0.4125	0.26823819	0.544962	0.075779	0.76327
Early:Late Fluctuating	1.7975	1.16352412	2.393003	0.178524	3.461161

Appendix B Table 4: Model results for Bayesian fecundity model.

Parameter	Median	Lower 50	Upper 50	Lower 90	Upper 90
Intercept	4.3169	3.411662	5.236444	2.081735	6.595793
Early Fluctuating	0.2738	0.090976	0.464949	-0.19318	0.771602
Late Fluctuating	-0.4569	-0.64682	-0.27101	-0.97223	0.010051
Family 2	0.1173	-0.03476	0.27349	-0.29507	0.546476
Family 3	0.4121	0.2513	0.577624	0.004798	0.849841
Week	0.1161	0.082008	0.152834	0.032249	0.203808
Maternal Length	-0.0043	-0.03871	0.031106	-0.09144	0.08085
Early:Late Fluctuating	-0.2106	-0.46424	0.048378	-0.89572	0.477613

Appendix B Table 5: Model results for Bayesian yolks volume model.

| Parameter	Median	Lower 50	Upper 50	Lower 90	Upper 90
Intercept	0.1838	1.58E-01	2.09E-01	1.22E-01	0.247246
Early Fluctuating	-0.0027	-5.77E-03	5.33E-04	-1.04E-02	0.005252
Late Fluctuating	-0.0132	-1.66E-02	-9.93E-03	-2.17E-02	-0.00506
Family 2	0.0074	4.81E-03	1.01E-02	5.52E-04	0.014342
Family 3	0.0081	5.36E-03	1.08E-02	1.10E-03	0.01491
Week	0.0039	3.25E-03	4.53E-03	2.27E-03	0.005495
Maternal Length	-0.0018	-2.78E-03	-8.44E-04	-4.25E-03	0.000583
Early:Late Fluctuating	-0.0075	-1.18E-02	-3.17E-03	-1.86E-02	0.003467

Appendix B Table 6: Model results for linear sperm concentration model.

Parameter	Median	Lower 50	Upper 50	Lower 90	Upper 90
Intercept	-6E+09	-9.7E+09	-3.2E+09	-14501784316	1551246356
Early Fluctuating	-8E+08	-2E+09	3.7E+08	-3815384349	2225437767
Late Fluctuating	-1E+09	-2.5E+09	-1.4E+08	-4411001457	1690102297
Early:Late Fluctuating	2E+09	-1.9E+08	3.3E+09	-2637161903	6019897320
Paternal Mass	3E+10	2.65E+10	4.19E+10	14735327095	53163137319

Visual model checks

Posterior predictive checking allows one to visually examine the fit of a model with respect to the real data. I implemented joint posterior predictive checks (on all data), as well as pointwise posterior predictive checks on statistics (median and skew of data). These checks act as a safeguard against gross misspecification. Next, I visualized Leave-One-Out Cross Validation Probability Integral Transformation (LOO-PIT) values to examine how predictive distributions are dispersed relative to conditional observations. My checks revealed adequate model specification.

The visual model checks below (Appendix B Figures 1 – 6) are arranged as such: joint posterior predictive check (top left), median (top right), skew (bottom left), and LOO-PIT values (bottom right). In cases where observations are discrete (*i.e.* breeding success), LOO-PIT plots are not shown as they assume continuous observations.
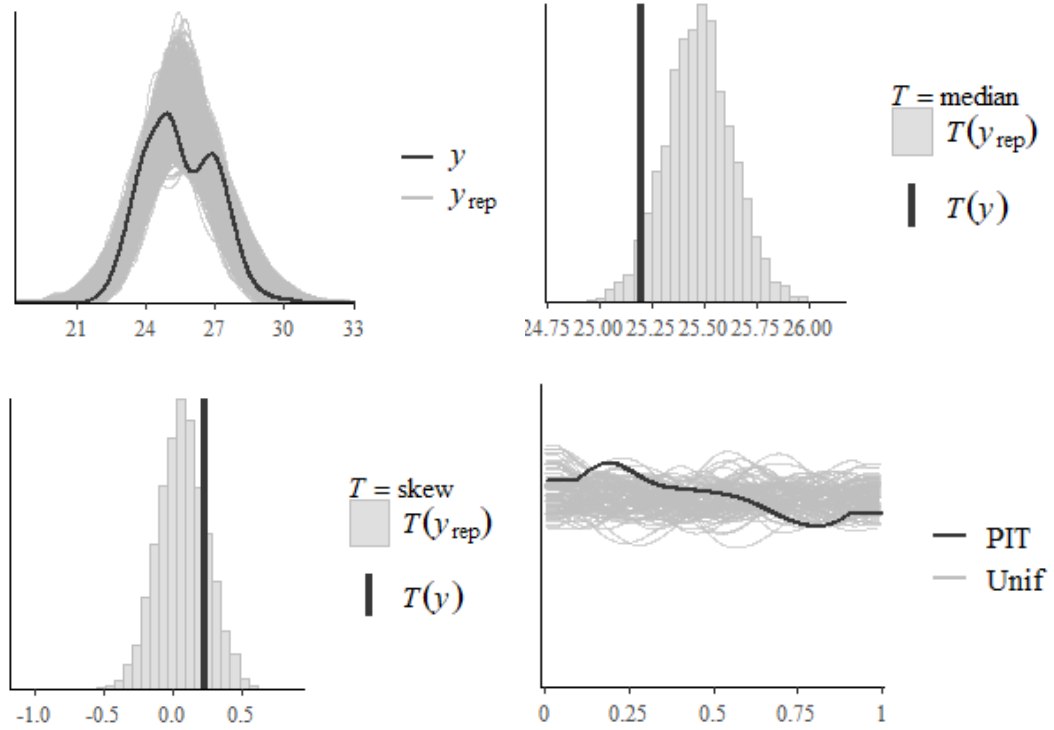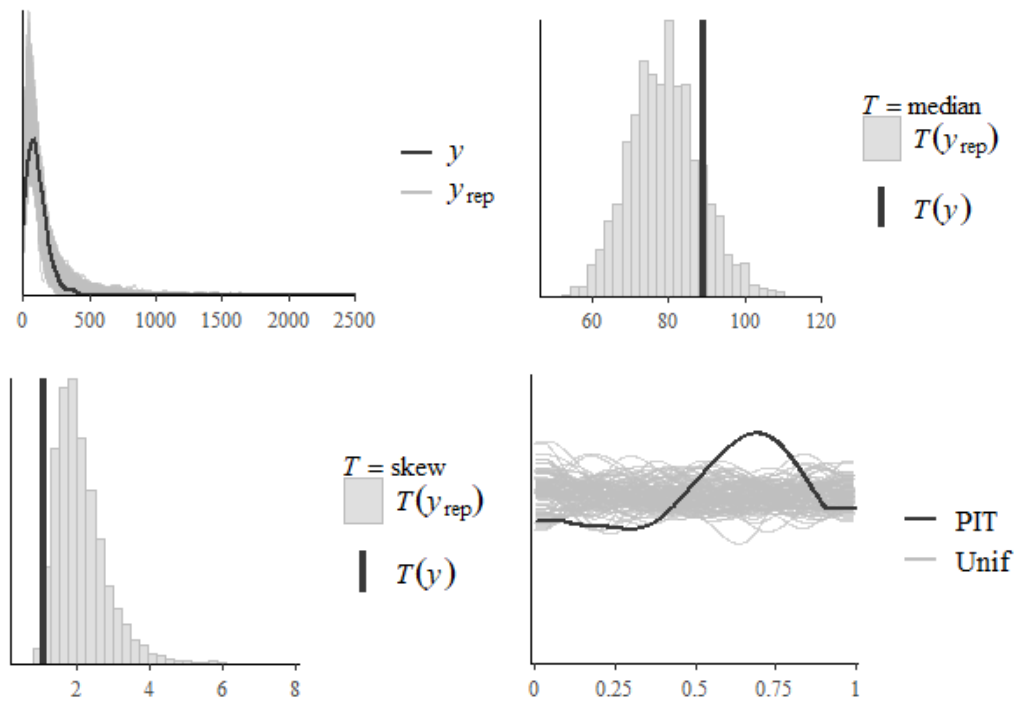
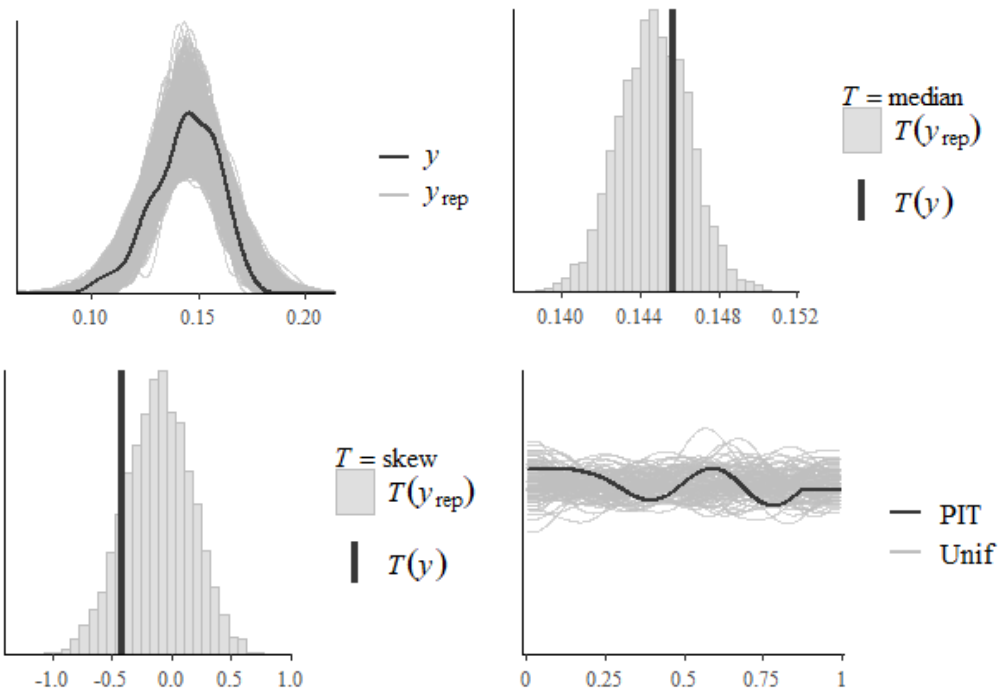***Appendix B Figure 1:*** *Posterior predictive checks for maternal body size model.*
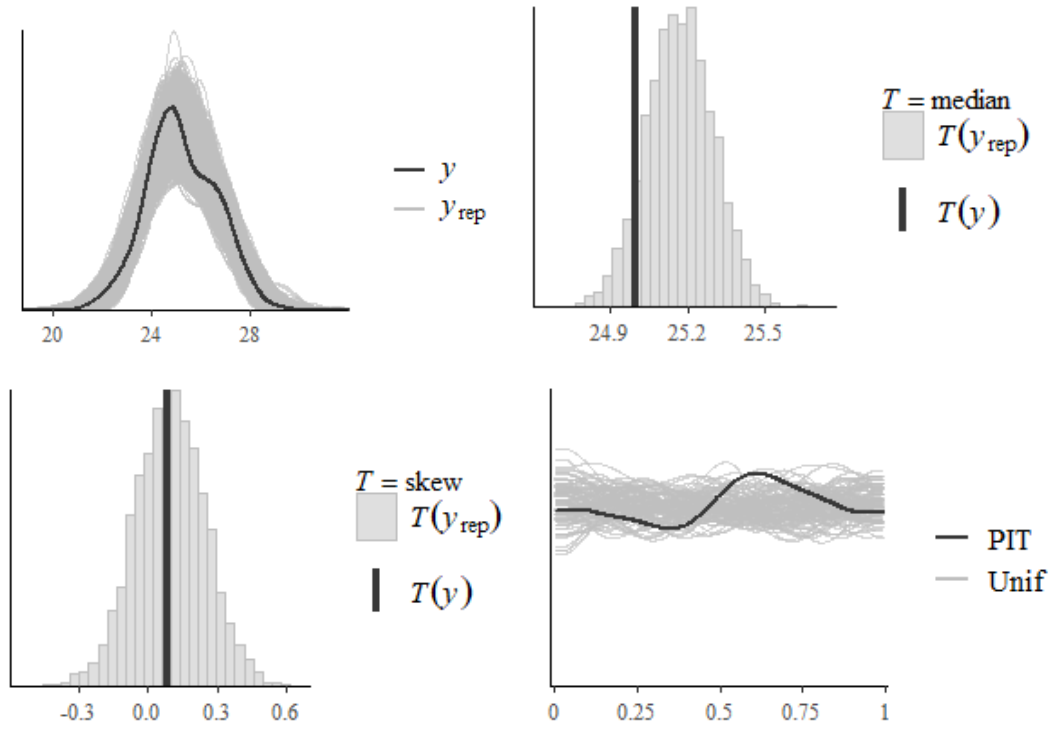
Appendix B Figure 2: *Posterior predictive checks for paternal body size model.*
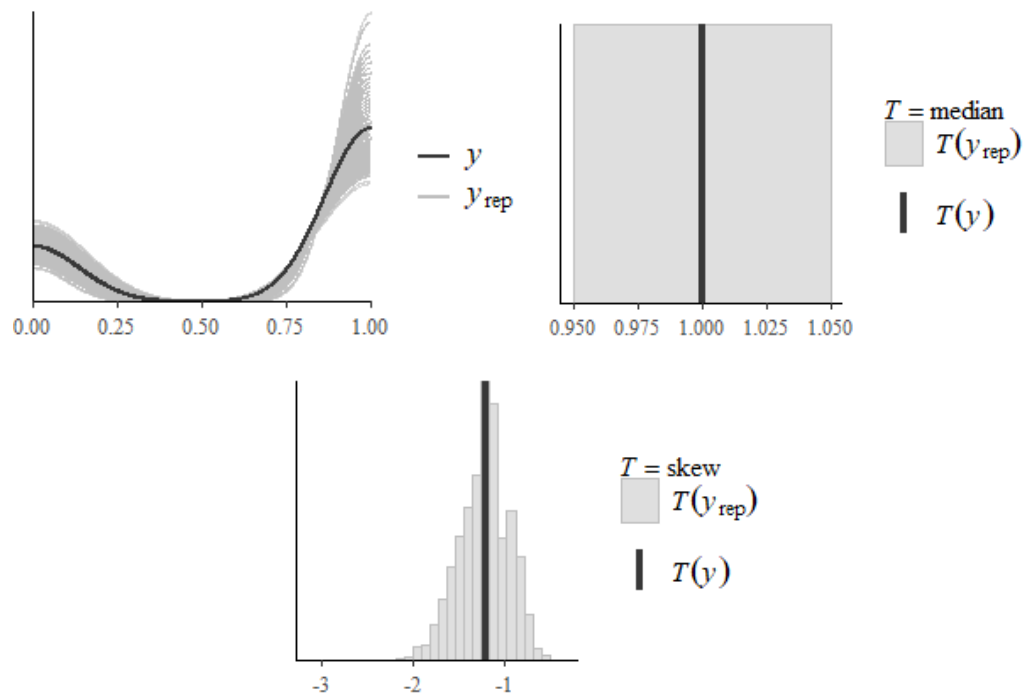
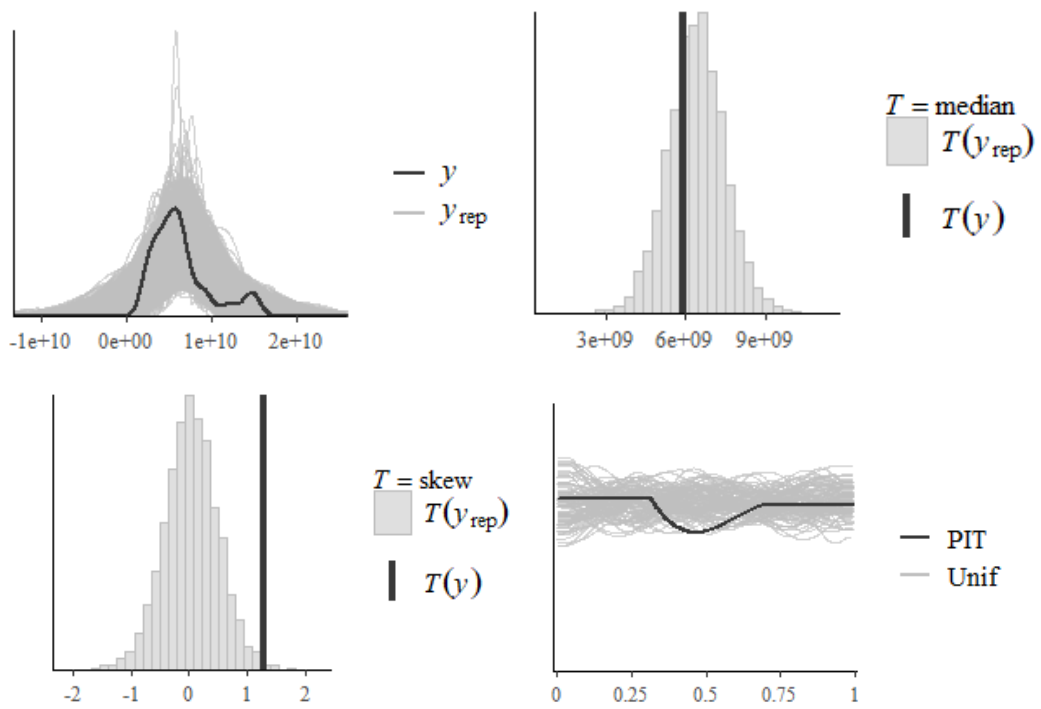***Appendix B Figure 3:*** *Posterior predictive checks for breeding success model.*

***Appendix B Figure 4:*** *Posterior predictive checks for fecundity model.*

Appendix B Figure 5: *Posterior predictive checks for yolk volume model.*

Appendix B Figure 6: *Posterior predictive checks for sperm concentration model.*

Non-viable egg data

I detected non-viable eggs in 72 spawnings. On average, fish that spawned non-viable eggs produced ~15 non-viable eggs (Appendix B Figure 7). A Bayesian mixed model with a negative binomial distribution and the same covariates used in the Fecundity model (Early*Late Treatment + Family + Maternal Size + Week + 1|Tank) suggested that acclimation to thermal variability during Late ontogeny had a positive effect on the production of non-viable eggs (posterior median estimate: 0.635; 90% UIs: [-0.224, 1.48]; 50% UIs: [0.310, 0.960]), maternal length had a positive effect (posterior median estimate: 0.120; 90% UIs: [0.034, 0.200]; 50% UIs: [0.086, 0.155]), and week had a negative effect (posterior median estimate: -0.099; 90% UIs: [-0.284, 0.086]; 50% UIs: [-0.174, 0.021]).
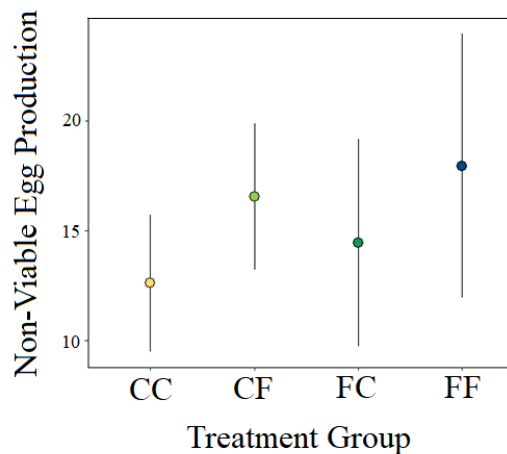
Appendix B Figure 7: *Average production of non-viable eggs between combined treatment groups.*

Therefore, female fish were positively influenced to lay non-viable eggs if they experienced thermally variable conditions during their juvenile and adult phases or were larger-bodied. In contrast, as the five weeks of breeding passed, fewer non-viable eggs were laid. The causes and consequences of non-viable eggs in zebrafish have not been well-explored and their significance in the context of thermal acclimation is unclear, so I present these data here but do not discuss these data further in the main text of Chapter 3.

Appendix C: Supplemental Material for Chapter 5, Parental early life environments drive transgenerational plasticity of offspring metabolism in a freshwater fish (*Danio rerio*)

This Appendix was submitted as Electronic Supplemental Material in:

Massey, M. D., & Dalziel, A. C. June 9th, 2023. Parental early life environments drive transgenerational plasticity of offspring metabolism in a freshwater fish (*Danio rerio*). *Biology Letters*. (RSBL-2023-0266).

Supplemental Methods

Grandparental conditions and acquisition of parental generation

F_0 grandparental wildtype-AB zebrafish were kept at the Dalhousie University Zebrafish Core Facility in control conditions. Water temperature in this facility is kept at a constant 27 °C, and photoperiod is kept to 14 hours light : 10 hours dark. I kept this photoperiod throughout our experiment.

   F_0 fish were bred by Zebrafish Core staff at 08:00 h to produce experimental F_1 parental generation fish. I collected eggs from three separate non-sibling fish pairings, which we refer to as 'families' in this study. These eggs were collected at 10:00 h and immediately brought to the Dalhousie University Aquatron Facility, then were sorted into treatment groups (Constant 27 °C or sinusoidally Fluctuating 22 – 32 °C on a diel basis) within 1 h.

Thermal treatments and fish breeding

I selected two temperature treatments to be used throughout our experiment: a 22 – 32 °C Fluctuating temperature range which reflects the range of temperatures permitting normal growth and development in zebrafish (*Danio rerio*) [1,2], and a 27 °C Constant temperature range representing the thermal optimum for growth and development, and the average temperature of the Fluctuating treatment [2] (*see* Chapter 5, Figure 17).

To separate the influences of Early and Late Parental Thermal Experience on offspring metabolism, I employed a split-clutch factorial design (e.g. [3], used previously in [4]). Here, each of the three clutches of F_1 parental embryos were immediately split equally and randomly into Constant or Fluctuating Early Parental Temperature treatments. They were reared in these treatments during their embryonic and larval periods, until the presumptive onset of the juvenile life stage at 30 days post-fertilization. Each group was then split once more into a Constant or Fluctuating Late Parental Temperature treatment. This design ultimately produced four combinations of Early and Late Parental Temperature treatments, allowing me to isolate effects on offspring that stem from either the 'Early' embryonic and larval stages of parents, or the 'Late' juvenile and adult stages.

At 1 year of age, I bred F_1 parental fish with within-treatment tankmates to produce F_2 offspring. The night before breeding, fish were placed in groups of two males: three females in zebrafish breeding boxes, with males and females being separated by a plastic divider. Overnight separation of males and females, while allowing exchange of pheromones, promotes breeding in this species; zebrafish also reproduce more effectively in breeding groups rather than in pairs [1,5] and this ratio produces high egg output (David Malloy, Dalhousie Zebrafish Facility Manager, personal communication). The breeding boxes have a sieved bottom which allow eggs to fall through later during oviposition, preventing egg cannibalism by parents. I also placed two 5 cm sanitized plastic aquatic vegetation sections in each female compartment, because this enhances reproduction by simulating natural conditions [6]. Flow-through water was provided to maintain water quality overnight.

At 08:00 the next morning, when lights came on, I disconnected the flow-through, removed dividers separating males and females, and placed tanks on a shallow incline to promote breeding (David Malloy, *personal communication*). I then allowed fish to spawn for 2 hours.

After the 2 hour spawning period, I removed F_2 eggs by passing the breeding box water through a fine mesh strainer. Eggs were rinsed with dechlorinated City of Halifax water at 27 °C and placed in petri dishes. They were further evaluated under a dissecting microscope to remove debris. 50 eggs from each breeding were randomly selected and

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placed in a 1-L glass beaker filled with 100 mL of dechlorinated City of Halifax water, and placed in a water bath set to 27 °C for 24 hours.

The next day, at 24 hours post-fertilization, I tested the acute metabolic responses of F_2 embryos. I equally and randomly split F_2 offspring from each treatment into a 22 or 32 °C acute Test Temperature, and measured their routine metabolic rates (RMR) at normoxia (>80% DO).

Model specifications

To analyze my data, I used Bayesian causal hierarchical modelling. I was interested in the effects of F_1 Early and Late Parental Treatments (Constant *vs.* Fluctuating), F_2 acute Test Temperature (22 *vs.* 32 °C), and their respective interactions on F_2 RMR. I was also interested in estimating the effect of F_2 Egg Size (continuous) on RMR, to test whether effects of Parental Treatments on egg size were the mechanistic explanation for potential changes in RMR. I specified two models based on a causal diagram [7] (Appendix C Figure 1). I posited that Early and Late Parental Treatments could affect offspring RMR. I also assumed based on metabolic scaling with temperature [8] that the acute temperature at which offspring were tested would also have an influence on RMR. Because I determined in previous work (Chapter 4, [4]) that Late (but not Early) Parental Temperature significantly influences egg size, I specified Egg Size as an intermediate between Late Parental Temperature and the RMR response, and Egg Size was included in a second model.
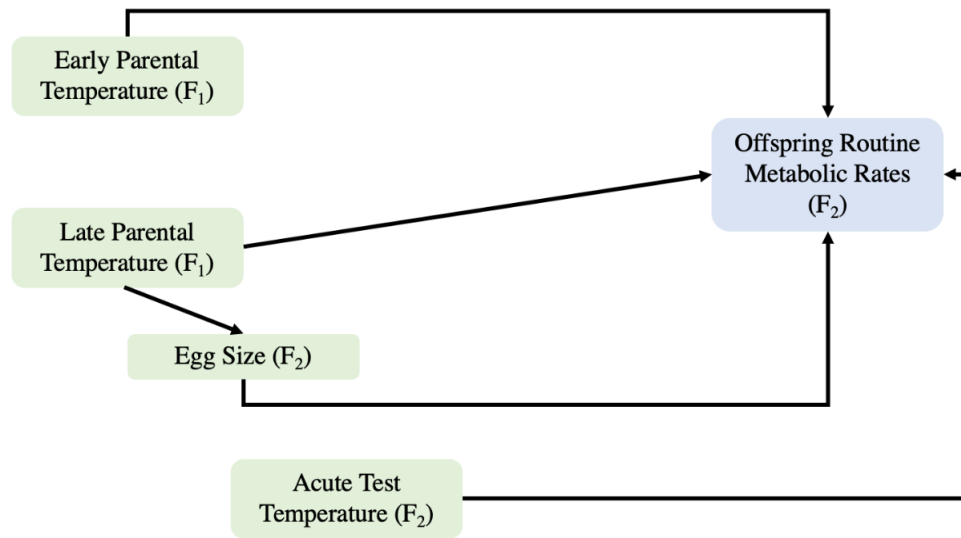
Appendix C Figure 1: Causal diagram illustrating effects of predictors (green) on offspring routine metabolic rates (RMR; blue). Based on previous work, I assumed that Late Parental Temperatures could impact Egg Size, but did not exclude Late Parental Temperatures having an effect outside of Egg Size. To avoid overcontrol bias (*e.g.,* an over- or underestimation of the effects of Late Parental Temperature, by controlling for its mechanism, Egg Size) I created two separate models, one which did not include Egg Size (to allow for proper estimation of the effects of the Late Parental Temperature) and one which did (to allow for estimation of the effects of Egg Size alone). Implicit in these diagrams is the existence of interaction terms.

I also incorporated a random intercept of 'Trial', because I calibrated the microplate daily for each Trial, and assumed that there would be slight differences in calibration between the six Trials of testing. Because only one family was tested for each Trial, this random effect also subsumes variation due to family-level effects.

I further log-transformed offspring RMR to increase normality and to aid in interpretation of results, such that effect size estimates represent proportionate changes from the reference category (*e.g*, a value of – 0.23 corresponds to a 23% decrease compared to the reference category). I specified the two models as follows:

Model 1:

$Offspring\ Metabolic\ Rate_{ij}$

$$= \beta_0 + \beta_1 EPT_i + \beta_2 LPT_i + \beta_3 TT_i + \beta_4 EPT{:}TT_i + \beta_5 LPT{:}TT_i + u_{0j}$$
$$+ \ \varepsilon_{ij}$$

Where β is the intercept, *EPT* is the Early Parental Temperature, *LPT* is the Late Parental Temperature, *TT* is the acute offspring Test Temperature, *EPT:TT* is the interaction between Early Parental Temperature and Test Temperature, and *LPT:TT* is the interaction between Late Parental Temperature and Test Tempeature (interaction terms denote the thermal sensitivity of transgenerational plasticity). *u* is the adjustment term for the random effect of Day of testing (which subsumes tank-level effects and differences caused by daily calibration of equiment), and ε is error.

This model therefore examined the total effects of Early Parental Temperature, Late Parental Temperature, Test Temperature, and their interactions on RMR.

Model 2:

$Offspring\ Metabolic\ Rate_{ij}$

$$= \beta_0 + \beta_1 EPT_i + \beta_2 LPT_i + \beta_3 TT_i + \beta_4 EPT{:}TT_i + \beta_5 LPT{:}TT_i$$
$$+ \beta_6 ES_i + u_{0j} + \ \varepsilon_{ij}$$

This model was identical to Model 1, except for the inclusion of *ES*, Egg Size. Here, I estimated the influence of Egg Size on offspring RMR. The effect of Late Parental Temperature in this model is therefore reflective of its *direct* effects on offspring RMR (arrow pointing from Late Parental Temperature directly to RMR in Appendix C Figure 1), and not its *total* effect (the sum of the arrows that point from both Late Parental Temperature and Egg Size to RMR).

Supplemental Results

Bayesian Median Estimates, 50% and 90% Uncertainty Intervals for Fixed Effects
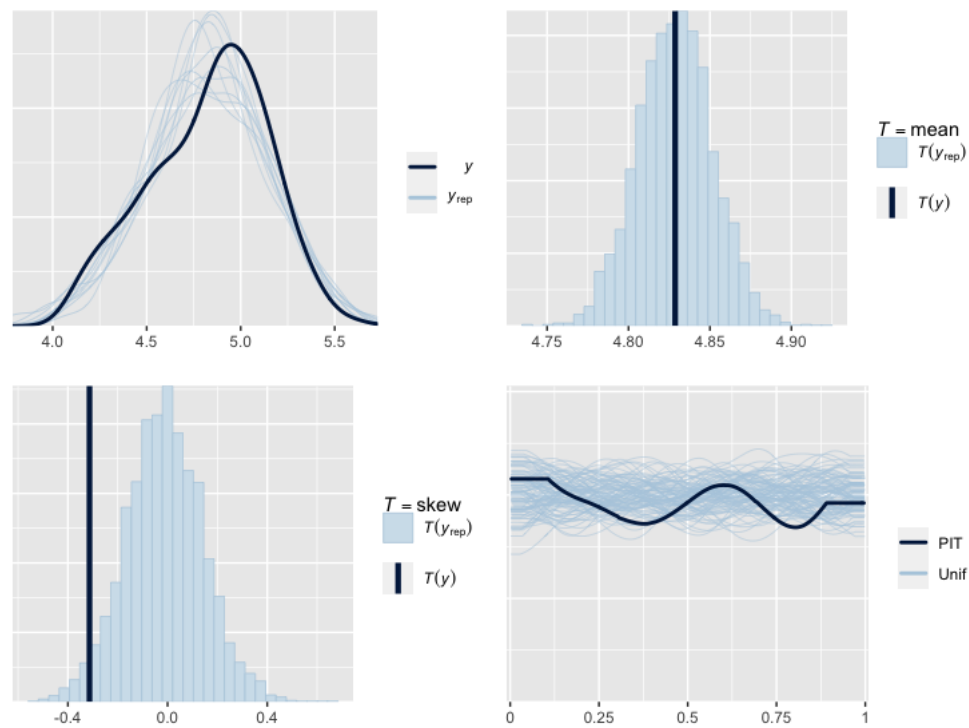
Appendix A Table 1: Bayesian Model 1 results (without egg size included); posterior estimates are presented as raw model output.

Parameter	Median	Lower 50	Upper 50	Lower 90	Upper 90
Intercept	4.84	4.78	4.90	4.63	5.04
Early Parental Temperature(F)	-0.24	-0.27	-0.21	-0.33	-0.15
Test Temperature (32 C)	0.25	0.21	0.29	0.13	0.37
Later Parental Temperature (F)	-0.03	-0.06	0.00	-0.12	0.06
Early Parental Temperature (F): Test Temperature (32 C)	0.06	0.02	0.11	-0.07	0.19
Later Parental Temperature (F): Test Temperature (32 C)	-0.01	-0.05	0.03	-0.13	0.12

Appendix A Table 2: Bayesian Model 2 results (with egg size included); posterior estimates are presented as raw model output.

| Parameter	Median	Lower 50	Upper 50	Lower 90	Upper 90
Intercept	4.22	4.02	4.44	3.61	4.82
Early Parental Temperature(F)	-0.23	-0.26	-0.20	-0.32	-0.14
Test Temperature (32 C)	0.25	0.21	0.29	0.13	0.37
Later Parental Temperature (F)	0.01	-0.02	0.04	-0.09	0.11
Egg Size	0.53	0.35	0.69	0.05	1.03
Early Parental Temperature (F): Test Temperature (32 C)	0.06	0.02	0.10	-0.07	0.19
Later Parental Temperature (F): Test Temperature (32 C)	-0.03	-0.07	0.02	-0.15	0.11

Visual model checks

Posterior predictive checking allows one to visually examine the fit of a model with respect to the real data. I implemented joint posterior predictive checks (on all data), as well as pointwise posterior predictive checks on statistics (median and skew of data). These checks act as a safeguard against gross misspecification. Next, I visualized Leave-One-Out Cross Validation Probability Integral Transformation (LOO-PIT) values to examine how predictive distributions are dispersed relative to conditional observations. My checks revealed adequate model specification.

The visual model checks below are arranged as such: joint posterior predictive check (top left), median (top right), skew (bottom left), and LOO-PIT values (bottom right).

Appendix C Figure 2: *Posterior predictive checks for Model 1.*
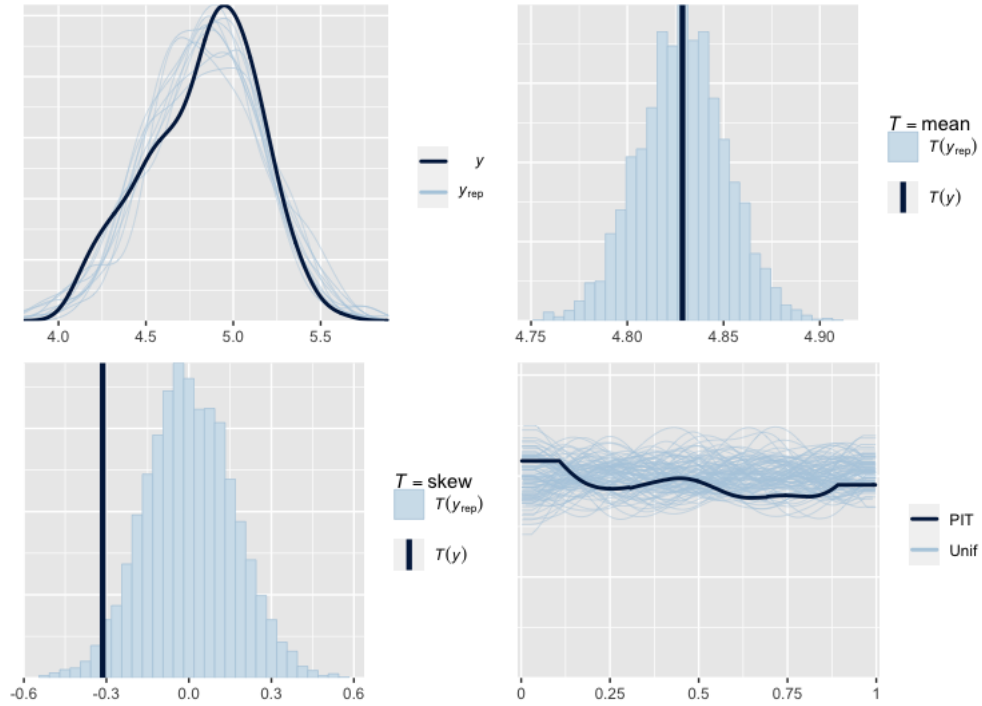
Appendix C Figure 3: *Posterior predictive checks for critical thermal maximum model.*

Egg size data

I detected changes in Egg Size, as measured in diameter (mm), which occurred as a result of Late Parental Temperature Treatment (Appendix C Figures 4, 5). F_1 parental fish who were exposed to Fluctuating temperatures during their late life (juvenile and adult stages) laid smaller eggs, on average (~0.1 mm in diameter difference; Appendix B Figure 4), as expected from a previous experiment in Chapter 3 sharing the same experimental setup with a much larger sample size (N = 1194 *vs.* N = 218 in the present experiment) for egg measurements [4].
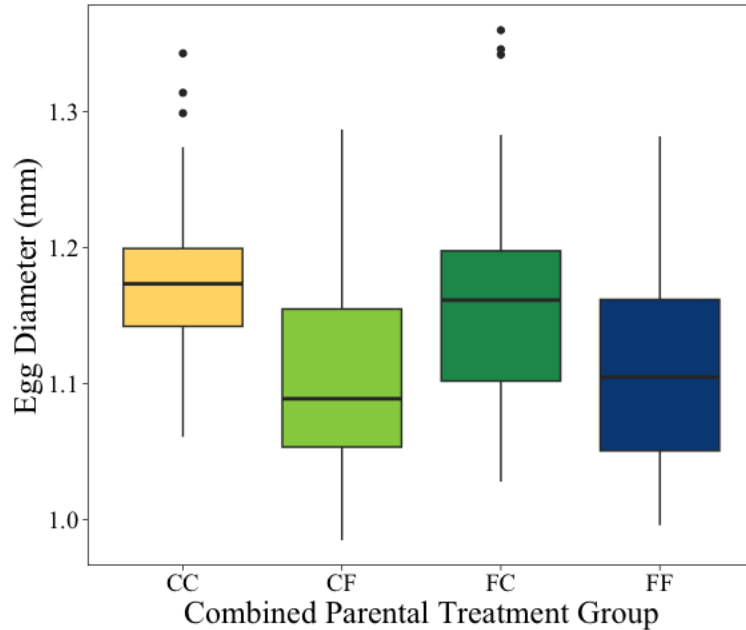
Appendix C Figure 4: *Egg sizes (diameter in mm) of F₂ offspring that underwent metabolic testing, based on combined parental thermal treatment group in a transgenerational plasticity experiment in zebrafish (*Danio rerio*). Each group represents a combination of Parental Temperatures, where C denotes a Constant (static 27 °C) thermal treatment and F denotes a Fluctuating (22 – 32 °C on a diel basis) thermal treatment. The first letter represents the Early Parental Temperature (from 0 – 29 days post-fertilization) and the second letter represents the Late Parental Temperature (30 days post-fertilization – 1 year post-fertilization).*

The results of Model 2 show the inclusion of Egg Size causes the modest effect of Late Parental Temperature to disappear (from a 2.7% decrease in Model 1, to a 1.4% increase in Model 2, highlighted in Appendix C Figure 5), suggesting Egg Size mediates Late Parental Temperature's effect on offspring RMR. Increases in Egg Size ultimately drive positive changes in offspring RMR, such that a 1 mm increase in egg diameter causes a mean 70.4% increase in RMR (Appendix C Figure 5). Because the estimate for Early Parental Temperature did not meaningfully change (-21.3% to -20.1%), we assume that there was minimal influence of Early Parental Temperatures on egg size (as found in previous work [4]), and effects of Early Parental Temperatures occurred over-and-above changes to Egg Size through unknown epigenetic mechanisms.
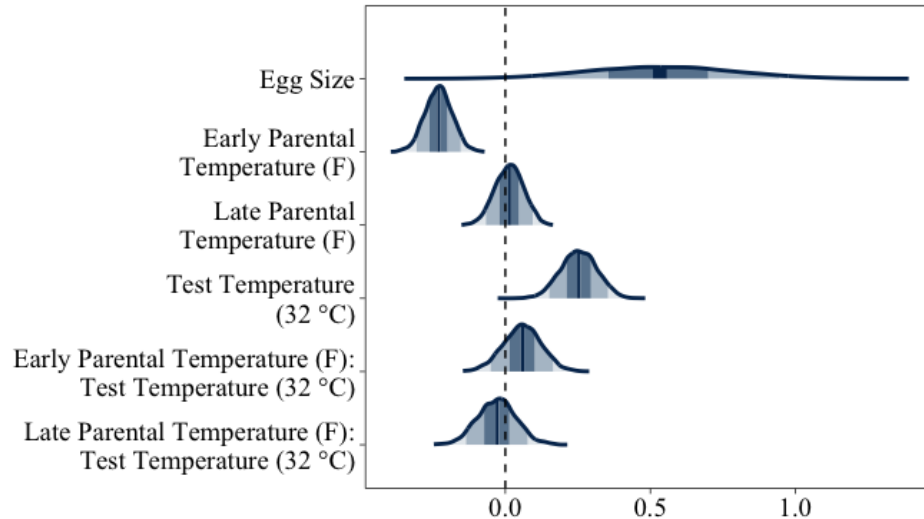
Appendix C Figure 5: *Visual model results of a Bayesian hierarchical model of a transgenerational plasticity experiment in zebrafish (*Danio rerio*), showing distributions of effect size estimates of Early Parental Temperature (Fluctuating, 22 – 32 °C), Late Parental Temperature (Fluctuating), Test Temperature (32 °C), their interactions, and Egg Size on back-transformed offspring routine metabolic rate. Reference categories are Constant (27 °C) for Early and Late Parental Temperatures, and 22 °C for Test Temperature. Note that routine metabolic rate was log-transformed so effect size estimates should be interpreted as proportionate changes (*e.g., for Egg Size, 0.59 would correspond to a 59% increase in offspring RMR for a given 1 unit (mm) increase in Egg Size). Posterior means are represented by thin dark lines at the mean of each distribution; 90% and 50% uncertainty intervals are represented by increasingly light shading.*

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The citation for Chapter 2 is as follows:

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