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Environmental Sensitivity of Maternal and Offspring Phenotype in the Green Anole (Anolis carolinensis) Lizard

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Abstract

Animals dynamically invest their acquired energetic resources into fitness-related traits, and life-history trade-offs occur when limited resources are invested in a given trait at the expense of another. The phenotypic effects of life history trade-offs are well documented, but the mechanisms facilitating these trade-offs are poorly understood. One such mechanism is the insulin/insulin-like signaling (IIS) network, and specifically its two primary hormones: insulin-like growth factor 1 (IGF1) and insulin-like growth factor 2 (IGF2). IGF1 is well-characterized but IGF2 is severely understudied, though it is present in nearly all amniotes and sometimes expressed at higher levels than IGF1 in adulthood. I tested how different environmental pressures affect expression of these hormones in adult female green anoles (*Anolis carolinensis*). Because maternal effects, which are transgenerational effects whereby the mother's environment influences offspring phenotype, can also promote life-history trade-offs, I also tested how these same environmental manipulations affect egg and offspring phenotypes. IGFs are affected by diet restriction and sprint training in females, albeit in different ways that are also dependent on mass and energetic history of the individual in question. IGF1 and IGF2 are therefore implicated in the response to variation in environment and the manner in which energetic environment is manipulated matters. Similarly, manipulating diet and locomotor investment also had distinct effects on both egg and offspring phenotypes, again in a manner that depended on the mass of the mother. These results implicate both the IIS and related pathways in life-history trade-offs involving the maternal and offspring phenotypes in green anoles.

Key words: maternal effects, diet manipulation, sprint training, *Anolis carolinensis*

Introduction

Environmental variation can affect phenotypic expression both within generations via phenotypic plasticity, and between generations via maternal and paternal effects. The most important environmentally sensitive pathway mediating plasticity is the insulin and insulin like signaling network (IIS) (Regan et al. 2020). This pathway is a biochemical link between environment and phenotype, yet it has traditionally been considered as only a nutrient sensing pathway, and little is known about its response to various (and varying) environmental conditions. Similarly, although a large literature exists documenting the effects of maternal resource allocation on offspring phenotype, the effects of changes in the maternal IIS to such effects are poorly understood.

My dissertation **tests how maternal energetic environment and factors that affect it (namely, dietary restriction and sprint training) affect phenotypes of both mothers and offspring in green anole (*Anolis carolinensis*) lizards.** Specifically, I propose to test how environmental pressures affect insulin-like growth factor (*IGF*) expression in mothers and how those same pressures alter maternal investment and consequently the phenotype of the offspring. In doing so, I aim to achieve a greater understanding of how components of the IIS in particular respond to environmental variation, as well as of the consequences of that variation for offspring phenotypic expression in a model organism for the study of ecology and evolution.

I. Background and Significance

The environment exerts a variety of pressures on organisms in nature. Over evolutionary time, organisms respond to these pressures via the mechanism of

differential survival and reproduction known as natural selection. However, environmental variation can also affect aspects of the integrated organismal phenotype over far shorter time scales. Although a growing literature exists documenting the causes and consequences of both adaptive and non-adaptive plasticity, the dynamic nature of phenotypic expression within an organism's lifetime has been studied for far longer under auspices of life-history theory. The disposable soma theory, for example, posits that organisms acquire limited resources from the environment and prioritize investment of those resources in either reproduction or survival in such a way as to optimize lifetime reproductive success (van den Heuvel et al. 2016). These investment decisions are made repeatedly over an individual's lifetime, and manifest ultimately as patterns of aging and age-related trait expression (Kirkwood, 1977).

Although life-history theory offers a powerful conceptual framework for understanding patterns of resource acquisition and allocation, it offers little insight into the mechanisms underlying these investment "decisions". Reproductive females are faced with the challenge of dynamically allocating resources to optimize their fitness, as well ensuring survivability of offspring. To understand how, when, and where females allocate their resources, different environmental stressors can be experimentally implemented to manipulate an animal's energetic environment. Consequently, manipulating energetic environment promotes life history trade-offs, exposing how an animal is affected by its changing environment. Different levels of trade-offs exist: for example, intermediate level trade-offs are at the physiological level such as endocrine function, whereas trade-offs at the phenotypic level involve whole-organism performance and morphology (as defined by Stearns, 1989).

Whole organism performance refers to “ecologically relevant tasks” that enhance fitness, such as sprinting ability to evade predators (Lailvaux and Husak, et al. 2014). Sprinting has significant functional requirements, and increasing investment in sprint performance should elicit changes in the underlying physiological and morphological pathways supporting that function (Husak et al. 2015). Changes in nutrient availability, specifically diet restriction, is a ubiquitous conserved mechanism that promotes life history trade-offs within an animal (Shanley and Kirkwood, 2000; Chiba et al. 2007). Investment into performance enhancing traits can also promote trade-offs (Lailvaux et al. 2012; Husak et al. 2016). The specific underlying physiological pathways that can potentially facilitate changes in the phenotypes of both sprint trained and diet restricted animals, is the insulin/insulin-like signaling (IIS) network. The IIS network is a ubiquitous system functioning in nearly all animals (Barbieri et al. 2003; Mathew et al. 2017). Its primary role is to promote growth, cellular reproduction, and metabolic functions (van Heemst, 2010; Schwartz and Bronikowski, 2016). The IIS comprises two primary hormones besides insulin itself: insulin-like growth factor (IGF) 1 and IGF2. The former is well characterized and is necessary for growth and reproduction (Swanson & Dantzer 2014). IGF2 is generally considered in the context of embryonic growth because most information comes from lab rodents which do not express IGF2 postnatally (Werner et al. 2008). However, many animals, including humans, express IGF2 throughout adulthood and at higher levels than IGF1 (Bentov and Werner, 2004; Werner et al. 2008; Beatty and Schwartz, 2020, Beatty et al. 2022), which is why it is important to study the responsiveness of IGF2 to environmental pressures.

Changes in the environment can be passed on to offspring via nongenetic maternal effects which encompass the response offspring have to maternal environment, such as trade-offs among components of the integrated offspring phenotype (Wells, 2018). The thrifty phenotype hypothesis posits that suboptimal prenatal conditions alter juvenile metabolism to better cope with the physiological challenges of limited food for the remainder of their lives; however, this is advantageous only if offspring face the same poor resource availability as the mother (Wells, 2003). Oviparous organisms are unique in that a mother must provision an egg with all necessary nutrients to complete development. Developmental biology primarily focuses on how a mother can manipulate embryonic development via variations in yolk nutrients. However, when a mother experiences varying environmental conditions, she might also manipulate the egg itself by altering the egg size and composition of the eggshell, too. Ultimately, these maternal effects are expected to be adaptive providing the maternal and offspring environment are the same.

Insulin/Insulin-like signaling network

The insulin/insulin-like signaling network (IIS) is a highly conserved pathway present in nearly all animals (Barbieri et al. 2003; Mathew et al. 2017). The IIS is sensitive to changes in nutrients and many studies exist to characterize its response to variation in caloric intake and nutritional geometry (Chiba et al. 2007; Taguchi and White, 2008; Duncan et al. 2015; Rahmani et al. 2015; Regan et al. 2020). The two primary hormones of the IIS are insulin-like growth factor (IGF) 1 and IGF2. IGF1 is an important regulator of cell proliferation and reproduction (Mathew et al. 2017). IGF1

receives a significant amount of attention because it is negatively correlated with longevity and has supposed anti-cancer properties (Mathew et al. 2017). IGF2 is typically discussed within the context of embryonic development because most studies of the IIS occur in lab rodents that do not express IGF2 postnatally (Carter et al. 2002; Werner et al. 2008), even though humans, non-human primates, birds, and reptiles exhibit postnatal IGF2 expression (Werner et al. 2008; Beatty and Schwartz, 2020). In many of these animals, IGF1 decreases in response to DR which could increase longevity (Weindruch and Sohal, 1997; Heilbronn and Ravussin, 2003). The underlying mechanisms as to how caloric restriction impacts the IIS remain to be elucidated.

Recently, the IIS was noted as not only being a nutrient-sensing pathway but also a crucial link between an organism's environment and its phenotype (Regan et al 2019). Environmental cues drive changes in an animal's underlying physiology depending on the intensity and nature of those cues (Regan et al. 2019). Just as diet restriction affects energetic environment, experimentally implemented exercise training forces an animal to invest limited resources to traits that will maximize survival (Husak et al. 2016). Sprint training forces animals to invest in protein synthesis, specifically fast twitch skeletal muscle growth (Jansson et al. 1990) which is energetically costly (Husak et al. 2016). In humans, exercise training induces variable endpoint measurements of IGF1 and IGF2 that are contingent upon baseline IGF (Devin et al. 2016), age, sex, diet etc. (Sellami et al. 2017). Sprint training typically leads to increases in IGF1 and IGF2 while endurance training does not exhibit these effects (Sellami et al. 2017), but to reiterate, these results are restricted by age and baseline athletic ability. However, the sensitivity of the IIS to exercise training in reptiles is unknown.

Maternal effects

A mother can plastically affect the phenotype of her offspring based on what she experiences in her lifetime in ways that are potentially adaptive (Uller et al. 2013). Oviparous mothers can anticipate what their offspring will need based on the current maternal environment, but in reptile species that lack maternal care their influence on offspring phenotype ceases at oviposition (Mitchell et al. 2015). Therefore, oviparous animals are a great model organism to test how offspring phenotype is influenced by the environment the mother is experiencing. The phenomenon of mothers manipulating offspring for the environment they will be born into is called maternal effects (Wolf and Wade, 2009). Maternal effects can affect offspring phenotype in a variety of ways such as via hormone provisioning to the eggs (Ensminger et al. 2018), sex ratios (Mousseau and Fox, 1998; Martins, 2004), or manipulating offspring size (Stearns, 1989; Sinervo and Huey, 1990; Brown and Shine, 2009). One mechanism known to promote maternal effects is diet restriction (Chapman and Partridge, 1996; Mair and Dillin, 2008; Moatt et al. 2016; Regan et al. 2020). It affects nearly all animals and is known to decrease reproduction but can also cause maternal effects such promoting a slower growth rate and smaller final body mass in female offspring compared to male offspring of zebra finch (*Taenopygia guttata*) (Martins, 2004). Although the effects of diet restriction are well characterized, ecologically relevant tasks such as performance traits are less studied within the context of maternal effects. The effect of performance traits such as sprinting deserves attention because it too forces an animal, such as green anoles, to allocate limited energy away from fitness enhancing traits towards the underlying machinery supporting locomotion. Examining how maternal environment influences

offspring phenotype could help garner a more complete picture about the impact of maternal effects.

I propose to conduct three experiments that will test the following explicit hypotheses regarding maternal energetic environment and offspring phenotype in the green anole lizard *Anolis carolinensis*:

H1) *IGF1* and *IGF2* expression will respond differently to dietary restriction;

H2) *IGF1* and *IGF2* expression will be affected by sprint training;

H3) Maternal dietary restriction and maternal sprint training will affect offspring phenotype differently.

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Chapter 1 – Diet Restriction (H1)

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Expression of insulin-like growth factors depends on both mass and resource availability in female green anoles (*Anolis carolinensis*)

Abstract

The insulin and insulin-like signaling (IIS) network is an important mediator of cellular growth and metabolism in animals, and is sensitive to environmental conditions such as temperature and resource availability. The two main hormones of the IIS network, insulin-like growth factor 1 (IGF1) and insulin-like growth factor 2 (IGF2), are present in all vertebrates, yet little is known regarding the responsiveness of IGF2 in particular to external stimuli in non-mammalian animals. We manipulated diet (low or high quantity of food: low and high diet group, respectively) in adult green anole (*Anolis carolinensis*) females to test the effect of energetic state on hepatic gene expression of *IGF1* and *IGF2*. The absolute expression of *IGF2* in female green anoles was 100 times higher than that of *IGF1* regardless of diet treatment, and *IGF1* and *IGF2* expression interacted with post-treatment body mass and treatment, as did the expression of the purported housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and eukaryotic elongation factor 2 (*EEF2*). The low diet group showed a negative relationship between body mass and gene expression for all genes, whereas the relationships between body mass and gene expression in the high diet group were either absent (in the case of *IGF1*) or positive (for all other genes). After accounting for total change in mass, the low diet group expressed *IGF2*, *GAPDH* and *EEF2* at higher

levels compared with individuals in the high diet group of a similar change in mass. These results illustrate that expression of *IGF1* and *IGF2*, and of the housekeeping genes is affected by energetic status in reptiles.

Introduction

Life histories are shaped by trade-offs in trait expression (Stearns, 1989; Roff, 2002). A central and ubiquitous trade-off is that between survival and reproduction, and animals inhabiting environments where resources are limited will allocate acquired resources in such a way as to maximize residual reproductive value (Williams, 1966). This trade-off is enabled by the insulin and insulin-like signaling (IIS) network (Dantzer and Swanson, 2012; Smykal and Raikhel, 2015), a highly conserved pathway that is present in animals ranging from fungi to primates (Barbieri et al., 2003) and whose primary function is to facilitate cell growth and metabolism as well as control physiological responses to changes in nutrient and environmental status (Regan et al., 2020). Consequently, activity in the IIS network has been implicated as a key factor mediating the vertebrate slow–fast life-history continuum (Dantzer and Swanson, 2012). For example, the circulating levels of insulin-like growth factor 1 (IGF1), a primary endocrine signal to upregulate the IIS network, is positively correlated with growth and reproduction yet negatively related to lifespan across 41 species of mammals (Swanson and Dantzer, 2014).

The sensitivity of IGF1 production in response to environmental conditions is well documented. For example, it is this sensitivity that facilitates the inverse relationship between caloric intake and lifespan such that dietary restriction tends to enhance

longevity in a wide variety of animal species (Weindruch and Sohal, 1997; Heilbronn and Ravussin, 2003). However, other endocrine regulators of the IIS network, though potentially no less important than IGF1, are poorly understood. The action of IGF1 is well characterized in adult animals, but the other primary hormone of the IIS network, insulin-like growth factor 2 (IGF2), has received less attention (Schwartz and Bronikowski, 2018; Beatty and Schwartz, 2020). IGF2 is produced at high levels in early developmental stages and is thought to be crucial for embryonic development (Harvey and Kaye, 1992; Yue et al., 2014). However, most of what is known about IGF2 derives from work on laboratory rodents, which do not express the *IGF2* gene postnatally (Carter et al., 2002) and exhibit monoallelic *IGF2* expression as a result of paternal imprinting (Chao and D'Amore, 2008). Given the ubiquity, complexity and importance of the IIS network, understanding the relative sensitivity of IGF1 and IGF2 production in response to environmental variation should be a priority if we are to fully comprehend the ecological relevance of the IIS network.

Reptiles are of interest for testing the environmental sensitivity of IGF2 because some evidence suggests that they exhibit postnatal *IGF2* gene expression without paternal imprinting (Reding et al., 2016; Schwartz and Bronikowski, 2016). Indeed, there is also evidence that IGF2 might significantly affect postnatal growth and development in reptiles (Reding et al., 2016), and it has been proposed that IGF2 could be more environmentally sensitive than IGF1 in non-placental vertebrates (McGaugh et al., 2015). IGF1 levels tend to decline with dietary restriction and lower temperatures in ectotherms (Beckman, 2011; Reindl and Sheridan, 2012), although a previous study on the lizard *Sceloporus undulatus* reported decreases in hepatic *IGF1* gene expression

only in animals under negative energy balance (Duncan et al., 2015). Although responsiveness of the IIS network to a manipulated energetic environment has been tested in a handful of reptile species, IGF2 has never been characterized in this regard.

To further understand the potential relationship between IIS network regulation and energetic state in reptiles, we altered the diet of adult female green anoles (*Anolis carolinensis*) and measured hepatic gene expression of both *IGF1* and *IGF2*. Green anoles are a useful organism for testing hypotheses regarding energetic state and IIS activity in reptiles because their genome is well annotated and previous studies have shown that a decrease in nutrient availability suppresses reproduction (Lovern and Adams, 2008), growth (Lailvaux et al., 2012) and immune function (Husak et al., 2016) in this species. We tested the hypothesis that *IGF1* and *IGF2* expression respond differently to the energetic state of the animal, whether they are in a positive energetic state and gaining mass; maintaining a steady body mass; or losing mass, indicating a negative energetic state. Specifically, we predicted that *IGF1* expression would be downregulated in animals in an intended negative energetic state (hereinafter, referred to as a low diet, LD) relative to animals that are in an intended positive energetic state (hereinafter, referred to as a high diet, HD), as is the case in mammals (Breese et al., 1991; Fontana et al., 2008; Rahmani et al., 2019) and other reptiles (Duncan et al., 2015). However, given the paucity of information regarding IGF2 and nutrient availability in reptiles specifically, we made the null prediction that *IGF2* expression would be unaffected by energetic state.

Materials and Methods

Husbandry

All procedures were approved by the UNO Institutional Animal Use and Care Committee protocol #19-003. We captured adult (snout–vent length, SVL >40 mm) *Anolis carolinensis* Voigt 1832 females (*N*=100) from urban populations in Orleans parish in Louisiana in June 2019, during the green anoles breeding season (Jenssen et al., 1995). We used reproductively mature females because this experiment was a part of a larger project testing for effects of environmental variation on maternal condition and maternal effects. Adult, reproductively mature females are continuous reproducers, so under good conditions they would be in a state of follicular development (Sparkman et al., 2010) during this time. We recorded SVL to the nearest 0.05 mm and body mass to the nearest 0.01 g on the day of capture. The mass range of the lizards was 1.91–4.25 g and their SVL range was 44.68–56.03 mm. The lizards were held in a climate-controlled room set at 28°C and 70% humidity. They were misted daily with water and singly housed in 36.6 cm×21.6 cm×24.9 cm plastic terrariums with ∼1.25 cm layer of mulch as substrate along with a wooden rod to perch on, and kept on a light:dark cycle of 13 h:11 h. Animals were haphazardly assigned a location in the room and we circulated the location of the lizards throughout the room weekly to minimize local position effects. All animals were given 1 week to acclimate before beginning treatment.

Diet Treatments

To alter the energetic environments, the experimental animals were randomly assigned to LD and HD groups. Although the initial mass of the treatment groups was significantly

different, with the LD group starting out slightly larger than the HD group (Table S2). All lizards were given ~1.25 cm crickets (*Acheta domesticus*). The LD group was fed one cricket coated in ZooMed ReptiCalcium powder, 3 times weekly and the HD group was fed an *ad libitum* diet of three crickets, 3 times per week supplemented with ZooMed ReptiCalcium powder (as in Lailvaux et al., 2012; Husak et al., 2016). Reproduction did drop within the LD group, as expected (Husak et al., 2016). Having animals from a size continuum of about 2–4 g on set HD and LD is expected to create an energetic state continuum in which small animals on the HD would increase in mass (positive energy balance) whereas bigger animals on the HD would either not change or slightly lose mass. Small animals on the LD would either maintain their mass or have minimal mass loss, and bigger animals on the LD would be in negative energy balance and lose mass (Fig S3). In this way, we could test for the effect of the categorical energetic environment (treatment group), and the continuous variable of energetic state (represented by either final body mass at the end of the experiment or change in mass over the time of the experiment).

Mass of females was recorded weekly for 8 weeks and females that lost >33% of initial body mass were temporarily removed from the experiment and put on an *ad libitum* diet. This only occurred in two lizards, one of which was included in the gene expression analysis. They were put back on the treatment if they reached the accepted threshold the following week. Any individual that had fallen below the body mass threshold more than once was excluded from gene expression analysis.

Post Treatment

At week 8 of the experiment, the green anoles were rapidly euthanized. Twenty-five individuals from the LD group and 25 individuals from the HD group were immediately dissected post-mortem. Liver tissue was removed, minced, and stored in RNAlater at 4°C for 3 weeks prior to gene expression analysis.

IGF gene expression analysis

Liver samples (*n*=19 for LD; *n*=22 for HD) for each treatment were randomized before RNA isolation. Liver samples were vortexed in DEPC-treated sterile water to rinse off the RNAlater. RNA was extracted with an Illustra RNAspin Mini kit according to the manufacturer's protocol (GE, 25-0500-70). Briefly, samples were lysed in RNAspin Lysis Buffer (GE, 25-0500-70) with 5 mm stainless steel beads (Qiagen 69989) using a Tissuelyser II (Qiagen) at 30 Hz for a period of 3 min. Proteinase K (Qiagen, 19131) was added post-homogenization to degrade proteins during cell lysis. During RNA isolation, a DNAse digestion was included according to the manufacturer's protocol. Total RNA was quantified on an Agilent 2200 TapeStation. All samples were standardized by making a 100 ng μl^{-1} dilution. Following the manufacturer's protocols, total RNA (100 ng) was used in cDNA synthesis reactions using qScript XLT cDNA SuperMix (QuantaBio, 95161-500). cDNA for all samples was made in the same 96-well plate.

Primers were designed for four target genes: *IGF1*, *IGF2*, *GAPDH*, and *EEF2* (Table 1). Primer and probe pairs for these genes were designed with Geneious Prime (Kearse et al., 2012; version 2019.0.4) using the publicly available green anole genome

from the NCBI gene database (version AnoCar2.0). An absolute standard curve for each gene was produced using a minigene synthesized by Integrated DNA Technologies (see Supplementary Materials and Methods). Amplicon regions of the four target gene regions with a 10 bp flanking region at each end were strung together and produced as a single synthetic plasmid (pUCIDT-KAN+Vector, Ref. 220963291). The circular plasmid was reconstituted to a concentration of 40 ng μl^{-1} and 1 µg of plasmid was digested using BgIII (NEB, R0144) to a final concentration of 20 ng μl^{-1}. Total copy number was calculated from concentration and plasmid length (Staroscik, 2004; https://cels.uri.edu/gsc/cndna.html). The plasmid was diluted to a concentration of 1×10^8 copies μl^{-1} and used to produce a serial dilution ranging from 1×10^7 to 1×10^2 copies μl^{-1}. In order to standardize the total amount of nucleic acid in each standard, Lambda DNA (NEB, N3011S) was prepared at a concentration of 310 ng μl^{-1} and used to balance each standard solution.

Gene	Amplicon Length (bp)	Primer Name	Primer Sequence (5'-3')
IGF1	115	GA_IGF1_440F	GGA GGC AAT CGA CGT TCA GT
		GA_IGF1_555R	ACG GAT CGT GCG GTT TTA TCT
		GA_IGF1_Probe516	/56-FAM/TGACCTGAC/ZEN/ACGACTGGAG/3IABkFQ/
IGF2	116	GA_IGF2_581F	CTG TGG GCA GAA ACA GAG GA
		GA_IGF2_697R	TGA TTT TGC ACA GTA GGT TTC CAA
		IGF2_Asag_Probe_HexZen	/5HEX/TGT GGA GGA /ZEN/GTG CTG CTT CCG GA/3IABkFQ/
EEF2	124	GA_EEF2_549F	GAA CCA GAA GAC ATA CCT ACC G
		GA_EEF2_673R	AAG TGG CGG ATT TCT CTT GG
		GA_EEF2_Probe585	/5Cy5/TTGCTGAGC/TAO/GTATCAAGCCA/3IAbRQSp/
GAPDH	110	GA_GAPDH_510F	AGT GAA TGG CCA ACG AGG
		GA_GAPDH_620R	AGA TGG CAT TCA GGA TCT CC
		GA_GAPDH_Probe77	/5TexRd-XN/CTGCTGGCATTGCTCTCAAC/3BHQ_2/

Table 1. Primers for each gene were developed using Geneious Prime (F) software and were created at IDT DNA Technologies.

Real-time quantitative PCR (qPCR) was conducted as described in Beatty and Schwartz (2020) to quantify *IGF1*, *IGF2*, *GAPDH* and *EEF2* in a multiplex qPCR reaction containing 1× PrimeTime Gene Expression Mastermix (Integrated DNA Technologies DNA, 1055772), 0.3 µmol l^{-1} of each primer, 0.2 µmol l^{-1} of each probe, 3 µl of cDNA at a 1:100 dilution in a final volume of 20 µl volume. Samples were randomized to 2 plates and ran in triplicate reactions on a BioRad CFX96 qPCR thermal cycler: 3 min 95°C initial activation, 2-step amplification cycle of 15 s at 95°C and 1 min at 60°C, repeated for 45 cycles. Imaging occurred immediately following each extension using the FAM, HEX, Tex615 and Cy5 fluorophore channels.

qPCR quality filtering

CFX Maestro Software (BioRad) was used to calculate PCR efficiency, CQ (quantification cycle) values, standard deviations, and absolute copy number of each gene. PCR efficiency was as follows: *IGF1* 101.8% (r^2=0.992), *IGF2* 106.4% (r^2=0.987), *GAPDH* 102.8% (r^2=0.988) and *EEF2* 101.5% (r^2=0.993). All data filtering was based on the output CQ values. Final data analyses were based on absolute copy number determined within the software from standard curve and CQ values, accounting for PCR efficiency. However, additional care was taken to randomize samples during RNA isolation, cDNA synthesis and qPCR stages, and to normalize samples before cDNA synthesis.

All analyses were run in R version 3.6.0 (http://www.R-project.org/). We used a two-tailed *t*-test to determine confidence intervals for genes and made subsets of data by gene. We removed replicates of samples and housekeeping genes that deviated by more than 0.2 cycles from the mean of the triplicate. We excluded samples from analysis that required the removal of more than one replicate.

Statistical analysis

Because of the documented relationships between components of the IIS network and growth, and because growth is affected by the energetic state of an organism that will certainly be altered by our diet treatment, we conditioned all of our analyses on one of two measures of body mass. The energetic environment, as defined by the HD and LD treatments, is expected to affect the energetic state of the animals, as indicated by the change in body mass by the end of the experiment. Additionally, the

energetic environment can have an independent effect beyond a change in mass. Thus, to test our hypothesis that *IGF1* and *IGF2* expression may respond differently to the energetic state of the animal, as well as the energetic environment, we analyzed absolute copy number for each gene in two different ways: (1) using models with treatment as a factor and final body mass at the end of the experiment as a covariate; and (2) using models with treatment as a factor and total change in body mass (Δmass, calculated as the difference between post- and pre-treatment mass) over the 8 weeks of the experiment as a covariate, representing energetic state at the time of sampling for measuring gene expression. We used mass instead of SVL because in adult animals, changes in mass are more sensitive to diet than changes in SVL might be over the time scale of this experiment. We used the *nlme* package (https://CRAN.R-project.org/package=nlme) to fit all general linear mixed models, and Box–Cox transformed dependent variables as required to meet model assumptions of normality. In cases where mixed models still exhibited heteroscedasticity following transformations, we dealt with this by fitting an exponential variance structure (Zuur et al., 2009). Because a penalty factor is applied to random effects during calculation of the likelihood function, *P*-values associated with individual factors are approximate. Consequently, we did not rely on Wald *P*-values for interpretation of mixed-model factor significance, nor do we report them; rather, we based our interpretations on model simplification achieved via log-likelihood deletion tests (see Silk et al., 2020, for a recent review). Once minimum adequate model structure was determined, we refitted final models using restricted estimate maximum likelihood (REML). We used the *visreg* package (Breheny and Burchett, 2017) to plot partial residuals of absolute copy number

from the final minimum adequate models for each gene. Partial residuals describe the relationship of interest (in this case, between treatment and copy number) while holding all other factors in the final models constant (Breheny and Burchett, 2017). To test the effect of treatment on final body mass, we ran two different linear models. Both models had treatment set as the independent variable and post-experimental mass as the dependent variable. Lastly, to facilitate comparison with previous studies that made interpretations based on absolute gene expression, we provide those models in the Supplementary Materials and Methods (see Figs S1, S2 and Table S1); however, in the discussed results we analyze mass-dependent relationships throughout.

Final mass analysis

We fitted general linear models to copy number for each gene measured, with treatment, final body mass and their interaction as fixed factors and individual as a random factor to account for the repeated measures of gene expression. We fitted exponential variance structure to models for *IGF1*, *IGF2* and *GAPDH* to deal with heteroscedasticity.

Change in mass analysis

To understand how energetic state affects *IGF1* and *IGF2* gene expression, we fitted general linear models to absolute copy number for each gene measured with treatment, Δmass and their interaction as fixed factors and individual as a random factor, as above. We dealt with heteroscedasticity in the *IGF1* model by fitting an exponential variance structure.

Results

Final body mass analysis

Following simplification via log-likelihood ratio tests, the minimum adequate model for *IGF1* retained an interaction between the main effects of treatment and final body mass (Table 2), such that LD lizards exhibited a negative relationship between *IGF1* expression and final body mass, whereas HD animals showed no such relationship (Fig. 1A). The final model for *IGF2* also retained a significant interaction between final body mass and treatment (Table 2) such that LD led to a negative relationship between body mass and gene expression, while the two factors were positively related in HD individuals. This same interaction was also retained in the models for housekeeping genes *GAPDH* and *EEF2* (Fig. 1C, D, Table 2). Within the housekeeping genes, the LD group had a negative correlation with expression levels and final body mass while the HD group had a positive direct correlation between expression levels and final body mass.

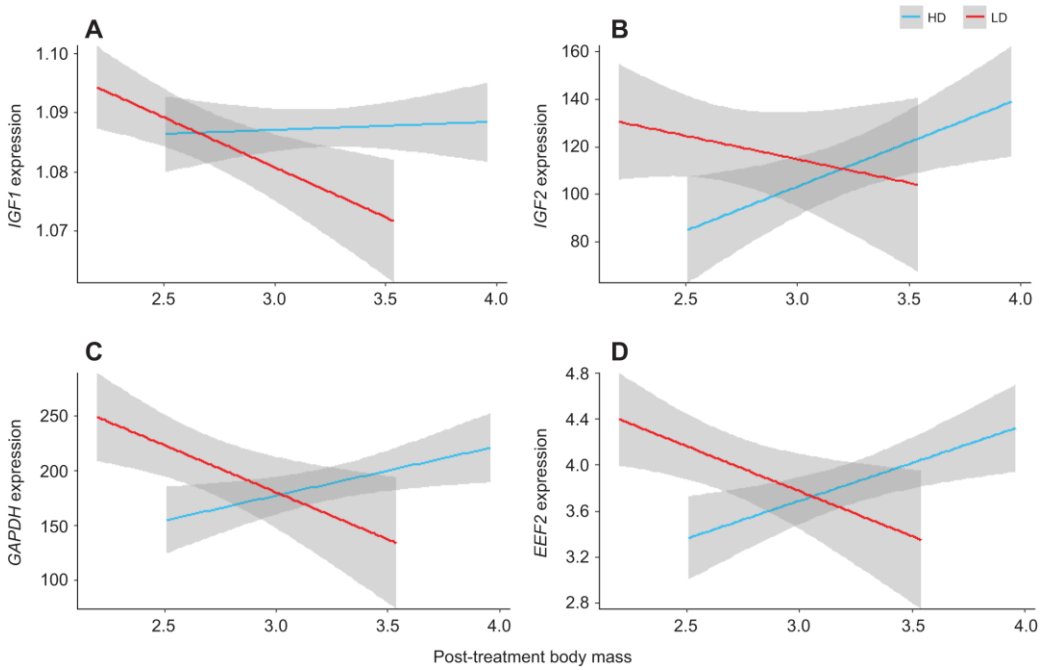

Fig. 1. Relationship between insulin-like growth factor 1 and 2 (*IGF1* and *IGF2*) gene expression and final body mass in female green anoles in the two diet groups. Partial residuals illustrating expression (copy number) of (A) *IGF1*, (B) *IGF2* and the housekeeping genes (C) glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and (D) eukaryotic elongation factor 2 (*EEF2*) in individuals from the high diet (HD; *n*=22) and low diet (LD; *n*=19) groups after accounting for the effects of final body mass. The optimal transformation given by the Box–Cox transformations resulted in a negative exponent for *IGF1*. To be consistent with interpretations, we show it here with a positive exponent – it still fitted well with our models.

(A) (IGF1)	Model term	Coefficient	SE
	Intercept	1.088	0.026
	Treat (LD)	0.045	0.035
	Final Body Mass	-0.0001	0.008
	Treat (LD):Final Body Mass	0.018	0.012
(B) (IGF2)			
	Intercept	7.72	90.92
	Treat (LD)	185.98	123.30
	Final Body Mass	32.11	28.058
	Treat (LD):Final Body Mass	-61.72	41.43
(C) (GAPDH)			
	Intercept	47.40	137.60
	Treat (LD)	411.87	186.64
	Final Body Mass	43.06	42.46
	Treat (LD):Final Body Mass	-141.49	62.70
(D) (EEF2)			
	Intercept	1.77	1.48
	Treat (LD)	4.60	2.00
	Final Body Mass	0.64	0.46
	Treat (LD):Final Body Mass	-1.55	0.67

Table 2: Best-fitting models describing the variation in copy number of (A) (*IGF1*), (B) (*IGF2*), (C) (*GAPDH*), and (D) (*EEF2*) with final body mass as a covariate. The reported coefficients give estimated change in the dependent variable between the baseline category and the category named in the table. Baseline category was the High Diet group.

Change in mass analysis

The change in mass is indicative of the energetic state of the animals at the time gene expression was analyzed. The final model for *IGF1* retained only an effect of Δmass, indicating no treatment effects on gene expression (Table 3). We included an interaction between treatment and Δmass to account for both simultaneously. While the interaction was non-significant for *IGF1*, there was a positive relationship between change in body mass and expression of *IGF1* over the course of the experiment (Fig. 2A).

However, *IGF2*, *GAPDH* and *EEF2* all included treatment in the final model conditioned on Δmass, indicating that LD lizards expressed *IGF2*, *GAPDH* and *EEF2* at higher levels compared with those individuals in the HD group of similar Δmass (Table 3). Furthermore, the positive relationship between Δmass and gene expression observed for *IGF1* was also seen in *IGF2*, *GAPDH* and *EEF2*.

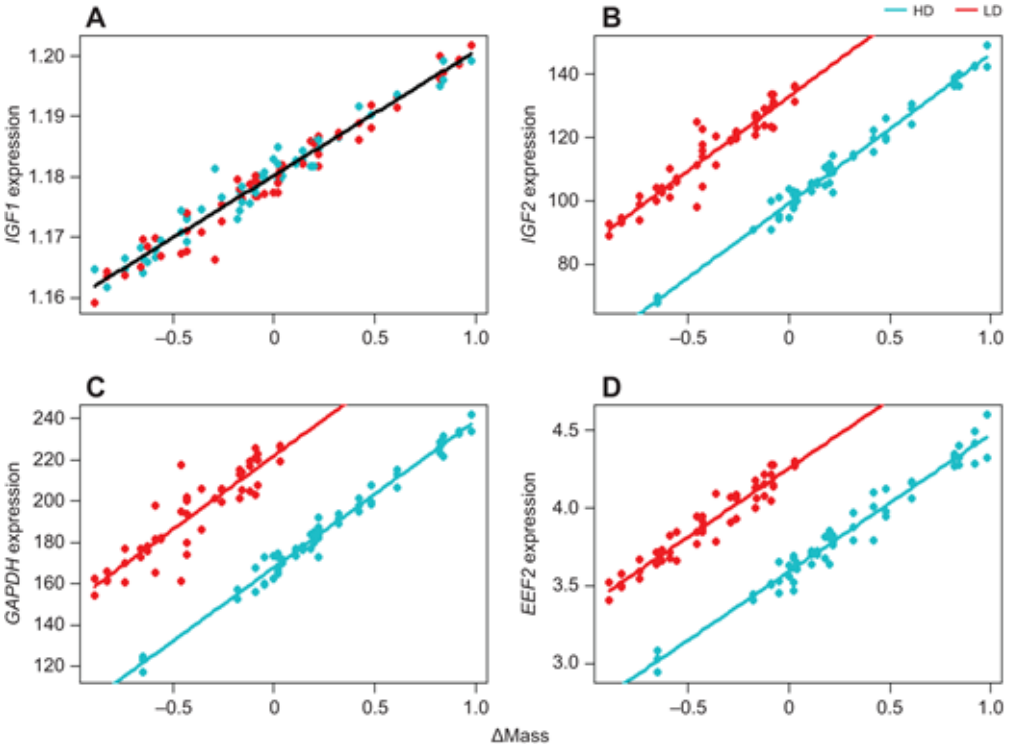

Fig. 2. Relationship between *IGF1* and *IGF2* gene expression and change in body mass in female green anoles in the two diet groups. Estimated marginal means for gene expression when accounting for the change in body mass (Δmass). Treatment was included in the models for *IGF2* (B), *GAPDH* (C) and *EEF2* (D) when conditioned with Δmass. Treatment was not included in the final model for *IGF1* (A). HD, *n*=22; LD, *n*=19.

(A) (*IGF1*)	Model term	Coefficient	SE
	Intercept	1.180	0.005
	Change in Body Mass	0.021	0.016
(B) (*IGF2*)			
	Intercept	99.10	12.41
	Treat (LD)	33.58	22.31
	Change in Body Mass	46.90	23.63
(C) (*GAPDH*)			
	Intercept	167.90	19.62
	Treat (LD)	54.95	35.29
	Change in Body Mass	71.07	37.36
(D) (*EEF2*)			
	Intercept	1.57	1.25
	Treat (LD)	0.54	0.37
	Change in Body Mass	2.04	1.13

Table 3: Best-fitting models describing the variation in copy number of (A) (*IGF1*), (B) (*IGF2*), (C) (*GAPDH*), and (D) (*EEF2*) with change in body mass as a covariate. The reported coefficients give estimated change in the dependent variable between the baseline category and the category named in the table. Baseline category was the High Diet group.

Although HD lizards were fed in such a way as to increase the energetic environment, four individuals lost mass over the course of the experiment (Lovern et al., 2004). Because we do not know why those lizards lost mass, we included them in our main analyses here and interpret the results of modeling all of the data, but we also analyzed the data with those four lizards removed (see Table S3). When those lizards were removed, the change in mass was no longer retained as a significant factor in the final minimum adequate models for *IGF2*, *GAPDH* and *EEF2*.

Discussion

The responsiveness of IGF1 to the energetic environment is well characterized (Breese et al., 1991; Fontana et al., 2008; Duncan et al., 2015; Rahmani et al., 2019), but the factors affecting IGF2 levels are poorly understood. In this study, we manipulated diet and compared gene expression of *IGF1* and *IGF2* with the goal of testing the hypothesis that *IGF1* and *IGF2* respond differently to the energetic environment and the energetic state.

Our prediction that *IGF1* would be downregulated in animals with a negative energy status (LD) was supported. Our minimum adequate model retained a significant interaction between treatment and final mass, such that LD animals exhibited a clear negative effect of final body mass on *IGF1* expression that was not seen in the HD animals (Fig. 1A, Table 2). Larger animals in resource-limiting environments (LD treatment) may express *IGF1* to a lesser extent than smaller animals, in that same environment, because of the level of resources available relative to size. Further, our data indicate that larger females that are losing mass have reduced *IGF1* expression relative to smaller females that are maintaining mass, when resources are scarce. Our data also show that lizards that gained mass over the course of the experiment, regardless of treatment, had higher expression of *IGF1* relative to those animals that maintained or lost mass (Fig. 2A), recapitulating an important general result that IGF1 expression and energetic state are directly correlated. Our data therefore support the effect of resource environment and energetic state on *IGF1* expression in reptiles.

Our second prediction, that *IGF2* expression would be unaffected by energetic environment, was not supported. Our minimum adequate model retained final mass as

a covariate (Fig. 1, Table 2), and also retained a significant interaction between treatment and final mass, such that HD lizards that were maintaining or gaining body mass showed a positive relationship between *IGF2* expression and final body mass, whereas LD lizards that were maintaining or losing mass showed a negative relationship (Fig. 1B, Table 2). Further, when the change in body mass over the experiment was accounted for, we found that animals in a low energetic environment (LD) exhibited higher expression of *IGF2* relative to animals in a high energetic environment (HD) (Fig. 2B). Our data therefore indicate that *IGF2* is responsive to the energy environment beyond the effect of energetic state. These results highlight a key difference in the action of components of the IIS under resource-limited conditions on reptiles compared with rodent models, where *IGF2* is not expressed in adulthood. The very novelty of this result limits our ability to interpret it within a properly comparative context, although we hope it will serve as a foundation for future studies.

Housekeeping genes are commonly used to normalize data in studies of gene expression (Mane et al., 2008). In theory, expression of housekeeping genes should be consistent between individuals, regardless of treatment, because they are required for normal cellular function. We used two of the most common housekeeping genes, *GAPDH* and *EEF2*, in this study; however, because there is evidence from mice that *GAPDH* in particular is not a stable reference gene under caloric restriction (Gong et al., 2016), we controlled for the eventuality that neither gene might be appropriate for normalizing our expression data by randomizing samples at RNA isolation, cDNA synthesis and qPCR steps to disperse technical error amongst treatments, as well as normalizing RNA amounts when making cDNA (Beatty and Schwartz, 2020). Indeed,

we found that expression levels of *GAPDH* and *EEF2* differed between treatments, both when accounting for final body mass (Fig. 1C, D, Table 2) and when accounting for change in body mass (Fig. 2C, D, Table 3). This suggests that both *GAPDH* and *EEF2* are affected by the animal's energetic state. Given that *GAPDH* is essential to break down glucose for ATP (Nicholls et al., 2012), it is possible that the females receiving lower levels of nutrients needed to upregulate *GAPDH* production for increased efficiency in metabolism (Vaquero and Reinberg, 2009; but note Mozdziak et al., 2003). In this respect, our results are consistent with results from mammals, illustrating that *GAPDH* is unsuitable for reference in energetics studies in reptiles as well, and may in fact be implicated in the key life-history trade-off between survival and reproduction. The role of *EEF2* is to conduct the elongation step in protein translation, and it is naturally expressed at low levels within both mammalian and reptilian cells (Kaul et al., 2011; Taha et al., 2013), consistent with our results here. In mammals, low nutrition status leads to inhibition of *EEF2* and ultimately protein synthesis (Proud, 2002; Kaul et al., 2011). The increase in *EEF2* expression seen in the LD animals (Figs 1D and 2D, Tables 2 and 3) appears to further indicate increased metabolic efficiency, although more research is needed to elucidate the effects of changes in energetic environment on *EEF2* in reptiles.

Taken together, we can conclude that energetic environment affects the responsiveness of *IGF1*, *IGF2*, *GAPDH* and *EEF2* within green anoles. This is evidenced by the fact that treatment (representing the energetic environment) was still a significant factor even after change in mass (representing energetic state) was accounted for, which suggests that some other mechanism is also driving changes in

gene expression beyond the change in mass of the animals. Although the nature of this mechanism is not apparent from our dataset, the effects of both treatment and change in mass on the expression of housekeeping genes nonetheless highlight a fundamental issue in molecular biology: that common housekeeping genes suitable and well characterized in mammalian models may not always be adequate for non-model species. Despite the status of anoles as model organisms for evolutionary studies (Camargo et al., 2010), little effort has been devoted to finding effective reference genes for species outside of a biomedical context (such as these organisms), and thus for reptiles in general.

A final constraint to using oviparous organisms, such as green anoles, as a model organism is that green anoles lay eggs every 7–14 days, so the initial mass may be reflective of egg retention while the final mass may be reflective of mass following oviposition (Lovern et al., 2004). This could be why four HD lizards lost mass over the course of the experiment. This could also be due to either the artificial laboratory environment or the fact that the intended *ad libitum* feeding regime did not provide enough energy to maintain their starting mass. When these lizards were removed from the dataset, Δmass was no longer included as a significant factor (see Table S3). Although our results give insight into the function of the IIS in reptiles, an important caveat is that increases in *IGF2* or the housekeeping genes in the LD lizards could be due to the nature of endpoint measures of gene expression, showing only a momentary snapshot of transcription. Additionally, we did not measure circulating levels of IGF1 or IGF2 proteins because no such assay has been validated for green anoles. We also did not assay insulin-like growth factor binding proteins (IGFBPs), which can positively or

negatively manipulate the effects of circulating IGFs (Denley et al., 2005), although we have limited information on their binding relationships to these hormones in reptiles (McGaugh et al., 2015; Schwartz and Bronikowski, 2018); nor did we test IGF1 receptor density, which would moderate the downstream effects of the hormone expression. Furthermore, although we sampled liver tissue because the vast majority of IGF production is of hepatic origin, especially for endocrine regulation, paracrine production of IGFs occurs in other tissue types such as skeletal muscle and the brain (Chao and D'Amore, 2008; Reding et al., 2016). It is therefore possible that the diet treatments led to the differential regulation of *IGF1* and *IGF2* expression in these tissues that we did not measure. The complexity of the IIS network means that considering all of these aspects of IGF expression and regulation within a single study is enormously challenging, and logistical constraints precluded us from doing so here. Future research in the field should focus on the development of these additional assays listed in green anoles and subsequent testing of these other components of the IIS to better understand its reactivity to environmental pressures.

The IIS network is highly conserved, and is responsible for nutrient signaling of the energetic environment to regulate cell proliferation and differentiation in nearly all animal species. In this paper, we demonstrated that gene expression of both *IGF1* and *IGF2* is subject to modification by the energetic environment as well as the energetic state in female green anoles. These results are crucial to filling in the knowledge gap regarding the actions of *IGF1* and *IGF2* in reptiles, and provide a foundation for future understanding of the mechanisms effecting *IGF* expression. Continuing research on the IIS network in response to external physiological stressors is essential to understand

how reptiles can adapt to subpar conditions, including those caused by climate change (Böhm et al., 2016), and ultimately to comprehend the mechanisms by which the IIS network mediates life-history trade-offs.

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Chapter 2 – Sprint training (H2)

(Accepted in the journal of General and Comparative Endocrinology, 2022)

Sprint training interacts with body mass to affect hepatic insulin-like growth factor expression in female green anoles (*Anolis carolinensis*)

Abstract

Locomotor performance is a key predictor of fitness in many animal species. As such, locomotion integrates the output of a number of morphological, physiological, and molecular levels of organization, yet relatively little is known regarding the major molecular pathways that bolster locomotor performance. One potentially relevant pathway is the insulin and insulin-like signaling (IIS) network, a significant regulator of physiological processes such as reproduction, growth, and metabolism. Two primary hormones of this network, insulin-like growth factor 1 (IGF1) and insulin-like growth factor 2 (IGF2) are important mediators of these processes and, consequently, of life-history strategies. We sprint-trained green anole (*Anolis carolinensis)* females to test the responsiveness of *IGF1* and *IGF2* hepatic gene expression to exercise training. We also tested how sprint training would affect glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and eukaryotic elongation factor 2 (*EEF2*). The former is a crucial enzyme for glycolytic function in a cell, and the latter is necessary for protein synthesis. Resistance exercise forces animals to increase investment of resources towards skeletal muscle growth. Because *IGF1* and *IGF2* are important hormones for growth, and *GAPDH* and *EEF2* are crucial for proper cellular function, we hypothesized that these four genes would be affected by sprint training. We found that sprint training affects *IGF* and *EEF2*

expression, such that larger sprint-trained lizards express hepatic *IGF1*, *IGF2,* and *EEF2* to a lesser extent than similarly sized untrained lizards. These results demonstrate that the IIS, and pathways connected to it, can react in a size-dependent manner and are implicated in the exercise response in reptiles.

Introduction

Each day, animals are required to conduct a variety of dynamic, ecologically relevant tasks that can directly affect survival and reproductive success (Bennett & Huey, 1990; Irschick & Garland, 2001). Locomotor performance is a key target of selection (Arnold, 1983) and is linked to fitness in selective contexts ranging from dispersal (Phillips et al., 2006) to male combat (Husak & Fox, 2008; Hall et al., 2010) and predation (Domenici et al., 2008; Bro-Jørgensen, 2013). Although individual locomotor traits such as sprint speed or endurance capacity have clear effects on Darwinian fitness (Irschick et al., 2008), such traits do not exist in isolation and exhibit functional, genetic, and physical links with other performance traits and other aspects of the integrated whole-organismal phenotype (Ghalambor et al., 2003, 2004; Pasi & Carrier, 2013; Lailvaux & Husak, 2014; Husak & Lailvaux, 2022).

Resource-based life-history trade-offs are the result of allocating limited acquired energetic resources from one fitness enhancing trait to another (De Jong & Van Noordwijk, 1992; Roff & Fairbairn, 2006). Changes in the environment can therefore prompt differential resource allocation between specific traits, depending on the ecological and selective context, and whole-organism performance traits are no exception to this phenomenon (Ghalambor et al., 2004; Reznick et al. 2004; Lailvaux and Husak, 2014). The resulting phenotypic performance trade-offs can be revealed by: 1) manipulating or limiting available resources, and thus resource acquisition (Lailvaux et al. 2012, 2020); 2) manipulating traits that are linked to performance, such as immune function (Kelly, 2014; Zamora-Camacho et al., 2015; Husak et al., 2021); or 3) by directly manipulating performance itself such as via exercise training (Husak et al., 2015, 2016;

Careau & Wilson, 2017). The resulting direction and nature of trade-offs involving performance will depend on the type of performance trait in question. For example, aerobic performance traits such as endurance capacity are bolstered by efficient cardiac function and oxygen delivery, whereas burst traits such as sprint speed are anaerobic and require investment in the development and growth of skeletal muscle comprising appropriate muscle fiber types. These different performance traits incur distinct costs (Husak & Lailvaux, 2017) and likely also elicit activity in disparate metabolic and biochemical pathways (Chung et al., 2021; Husak & Lailvaux, 2022). Despite the attention paid to the physiological and genetic factors underlying locomotor performance (Sorci et al., 1995; Bouchard, 2012; Sharman & Wilson, 2015; Chung et al., 2021), it remains unclear how increased investment in specific types of performance mechanistically affects other aspects of the integrated phenotype. This poor understanding in turn impedes our ability to comprehend both the proximate trade-offs involved in performance expression, as well as the effects of such trade-offs on developmental and evolutionary trajectories (Lailvaux & Husak, 2014, Husak & Lailvaux, 2022; Garland et al., 2022).

The insulin/insulin-like signaling (IIS) network is a highly conserved environmental sensing network that mediates growth and metabolism and is thus a likely regulator of muscle growth and metabolism in response to increased anaerobic activity such as sprinting. Two of the primary hormones of this network are insulin-like growth factor 1 (IGF1) and insulin-like growth factor 2 (IGF2). IGF1 is an important catalyst for cellular growth and has been studied extensively throughout the lifespan of rodents and humans (Junnila et al., 2013; Vitale et al., 2019). Work on IGF2 is limited, since rodents as the

primary biomedical models, do not express IGF2 post-natally, and nearly all available studies on IGF2 are within the context of the mammalian placenta and embryonic growth (Sun et al. 1997; Fagerberg et al., 2014; Yue et al., 2014; White et al. 2018). Although there is a growing body of literature regarding the role of IGF1 in human exercise training (Carro et al., 2000; Llorens-MartÍn et al., 2010; Cui et al., 2015), these studies are typically not conducted within a comparative context (but see Raichlen and Gordon, 2011) and yield mixed results regarding the directional effect of sprint training and IGF expression. Furthermore, the role of IGF2 in growth of adult organisms is vastly understudied, although a recent survey of post-natal IGF expression across 82 species of amniotes has shown that hepatic *IGF2* expression was nearly ubiquitous, and hepatic *IGF2* was often expressed at a higher level than *IGF1* (Beatty et al. 2022). This work in combination with earlier studies have repeatedly shown that reptiles express both *IGF1* and *IGF2* post-natally (McGaugh et al., 2015; Reding et al., 2016; Schwartz and Bronikowski, 2016; Beatty & Schwartz, 2020). Marks et al. (2021) found that both hepatic *IGF1* and *IGF2* gene expression are affected by decreased energetic intake in adult female green anoles, indicating that *IGF2* likely has important post-natal function in reptiles. Since IGF1 and IGF2 compete for binding to the IGF1 cellular receptors (IGF1R) (Denley et al., 2005), it is plausible that both hormones play a role in cellular growth, specifically muscle growth, and may affect sprint speed in lizards.

In addition to IGF1 and IGF2, we examined the response of two additional important metabolic genes involved in growth that are also affected by resource limitation (Marks et al., 2021), and that may also respond to exercise. *GAPDH* is a central component of glucose metabolism, and at the cellular level is connected to the mTOR

complex 1 (mTORc1) pathway (Lee et al., 2009; Nicholls et al., 2012). Similar to IIS, this pathway is involved in cell growth and is environmentally sensitive to external stimuli such as resource availability (Sarbossov et al., 2005; Lee et al., 2009; Regan et al., 2020). As such, if exercise-induced changes in *GAPDH* simulate those of a low-glucose environment, this could inhibit actions of the mTORc1 (Lee et al., 2009) which would constitute a potential mechanistic link between the effects of exercise and muscle growth. The second gene of interest, *EEF2*, is important for the elongation step of protein formation (Kaul et al., 2011), and thus could be implicated in muscle growth (Atherton & Smith, 2012). We know that a highly conserved kinase in mammals, EEF2K, acts as an inhibitor to EEF2 and this kinase is upregulated by environmental factors such as low nutrient availability within a cell (Kenney et al., 2014). EEF2K activity is inversely related to the activity of mTORc1 (Kenney et al. 2014), recapitulating an important point that the combined effects of these genes, along with *IGF1* and *IGF2*, emphasize the integrated response of an organism to external stimuli.

Over the last several years, green anole lizards (*Anolis carolinensis*) have emerged as a useful model system for understanding the effects of exercise training on both performance capacities and the expression of traits linked to performance. Previous studies have shown that green anoles show physiological changes in response to sprint training, including differences in muscle fiber size (Husak et al., 2015), metabolic rate (Lailvaux et al., 2018) and immune function (Wang and Husak, 2020) compared to untrained controls. In this experiment, we sprint-trained adult female green anole lizards for six weeks, thereby forcing them to increase allocation of energy resources to muscle growth (Husak et al., 2015). We tested the hypothesis that hepatic expression of *IGF1*

and *IGF2* is affected by sprint training because IGFs are important regulators of cellular reproduction and ultimately skeletal muscle growth. Specifically, we predicted that *IGF1* and *IGF2* would be upregulated in sprint-trained lizards compared to untrained lizards. We also tested the additional hypothesis that both *GAPDH* and *EEF2* would be affected by sprint training, as well, given the previously demonstrated effects of the energetic environment on the expression of these genes.

Materials and Methods

Husbandry

The UNO Institutional Animal Use and Care Committee protocol #19-003 permitted all procedures outlined below. All housing conditions are consistent with those of Marks et al. (2021). In June 2020, we caught adult (snout-vent length (SVL) > 40mm) *A. carolinensis* females (N=96) from urban populations in Orleans parish in Louisiana. We concentrate specifically on adult reproductively-active female lizards in this study both to facilitate comparison to Marks et al. (2021), which also exclusively used reproductively-active adult females, and because the present study is part of a larger experiment aimed at understanding maternal effects in green anoles. A Mitituyo digital caliper was used to measure SVL to the nearest 0.05 mm and a digital scale was used to measure body mass to the nearest 0.01 g on the day of capture. The climate of the lizard room was maintained at 28 °C and 70% humidity, with a light:dark cycle of 13:11 hours. Lizards were individually held in 36.6cm x 21.6cm x 24.9cm plastic terrariums that had a wooden dowel to perch. The lizards received water daily by misting the terraria, and they were fed a high diet (Marks et al., 2021) of three ~1.25cm crickets (*Acheta*

domesticus) dusted with mineral supplements three times per week (also referred to as *ad libitum* in Lailvaux et al. 2012; Husak et al. 2016). This diet aimed to inhibit trade-offs associated with low nutrition status and therefore any variation in gene expression would be due to sprint training. Local position effects were reduced by haphazardly relocating the lizards around the room once per week. All animals were acclimated for a period of one week prior to the treatment implementation.

Sprint Training

Lizards were trained on a 2.0-m long, 5-cm cork dowel set at a 45° incline three times each week for six weeks with each trial consisting of 3 runs separated by 1 hr. After two and four weeks, training intensity was increased by hanging off the lizard's weight (centrifuge tubes filled with clay) equivalent to ~ 25% and 50% respectively of the weekly lizard body mass (Husak & Lailvaux, 2019; Wang & Husak, 2020). In each trial, lizards were taken out of their cage and immediately encouraged to run down the dowel of the racetrack by lightly tapping their tail. As the lizards ran up the track, they broke infrared beams generated by photocells situated every 25cm. As each beam broke, the time was recorded in the computer software TrackMate (Trackmate Racing, Surrey, BC, Canada). This training regime was previously shown to be effective and not too strenuous for green anoles (Husak & Lailvaux, 2019; Wang & Husak, 2020). Untrained (UT) lizards were removed from their cages once per training day and briefly handled to simulate handling effects experienced by sprint-trained (ST) animals (Husak et al., 2015).

Three sprint times were recorded for each lizard on both the first day of the experiment and on the last day of the experiment, consistent with both standard maximum performance protocols (Losos et al., 2000; Adolph & Pickering, 2008) and similar training experiments (Husak et al., 2015; Lailvaux et al., 2020; Wang & Husak, 2020). For each lizard, starting and final sprint times were analyzed by filtering out data points (each 20 cm recorded) that were more than two standard deviations away from the mean for each of the three trials. The fastest 20 cm for each lizard from the starting sprint time and final sprint time was used in the sprint times analysis (Losos et al., 2000). When green anoles are sprint-trained, there is often no significant difference in final sprint time because the experimental group becomes habituated to the treatment (Husak et al., 2015; Lailvaux et al., 2020). Sprint training nonetheless has significant physiological effects on the animal, increasing skeletal muscle growth (Husak et al., 2015); suppressing immune function (Wang and Husak, 2020); as well as altering resting metabolism (Lailvaux et al., 2018) and impacting survival (Husak and Lailvaux, 2019).

Post-Treatment

The green anoles were rapidly euthanized via decapitation 24 hours after the final sprint training trial (week -6). All lizards were euthanized within an eight-hour period. Twenty-eight individuals from the sprint-trained group and 27 individuals from the untrained group were randomly selected to be dissected post-mortem. Liver tissue was

immediately removed, minced, and placed in 2.0mL screw top microcentrifuge tubes that contained ~250µl of RNAlater. These were then stored at 4°C for 4 weeks prior to gene expression analysis.

Insulin-Like Growth Factor Gene Expression Analysis

We randomized liver samples (n= 28 for ST; n = 27 for UT) for each treatment prior to RNA isolation. To rinse off the RNAlater, we washed the minced liver tissue by rinsing in DEPC treated sterile water and briefly vortexing the sample to remove the water. RNA extraction and gene expression analysis were performed as described in Marks et al. (2021). In brief, we used an Illustra RNAspin Mini kit according to manufacturer protocol (GE, Cat. No: 25-0500-70) to extract RNA. Samples were lysed in RNAspin Lysis Buffer (GE, Cat. No. 25-0500-70) with two 5mm stainless steel beads (Qiagen Cat. No. 69989) using the Tissuelyser II (Qiagen) at 30Hz for a period of 3 minutes. A proteinase K digestion (Qiagen, Cat. No. 19131) was performed post-homogenization along with a DNAse digestion during extraction. Total RNA was quantified on an Agilent 2200 TapeStation. For each sample, RNA concentration was standardized to 100 ng/µL. Total RNA (100 ng) was used in cDNA synthesis reactions using qScript XLT cDNA SuperMix (QuantaBio, Cat. No. 95161-500).

We used previously validated primers for *IGF1*, *IGF2*, *EEF2*, and *GAPDH*, and an absolute standard curve, in quantitative PCR (qPCR) amplification (Marks et al., 2021). The absolute standard curve was prepared as previously described (Beatty et al., 2020; Marks et al., 2021) using a custom-made plasmid containing the four targets

across seven serial dilutions ranging from $1x10^7$ to $1x10^2$ copies per µL, and balanced using Lambda DNA as a carrier (NEB, Cat. No. N3011S). Samples were randomized at each stage (*i.e.,* RNA isolation, cDNA synthesis, and qPCR stages).

We conducted real time qPCR as described in Beatty and Schwartz (2020) to quantify *IGF1*, *IGF2*, *GAPDH* and *EEF2,* utilizing the green anole primer and fluorescently-labeled probe sequences published in Marks et al. (2021). The multiplex qPCR reaction contained 1X PrimeTime Gene Expression Mastermix (IDT DNA, Cat. No. 1055772), 0.3µM of each primer, 0.2µM of each probe, 3µl of 1:100 dilution of cDNA (or standard) in a final reaction volume of 20µl volume. Samples were randomized on two 96-well plates and were run in triplicate reactions on the BioRad CFX96 qPCR thermal cycler: 3-minute 95°C initial activation, 2-step amplification cycle of 15 seconds at 95°C and 1 minute at 60°C, repeated for 45 cycles. Imaging occurred immediately following each extension using the FAM, HEX, Tex615, and Cy5 fluorophore channels.

qPCR Quality Filtering

We used CFX Maestro Software (BioRad) to calculate PCR efficiency, CQ (quantification cycle) values, standard deviation, and absolute copy number of each gene using standards 1 through 6 (30,000,000 – 300 copies when using 3 µl per reaction). The last (7^{th}) standard was removed from each run due to copy numbers below the detection limit (30 copies when using 3 µl per reaction), which greatly

improved the calculated PCR efficiency. PCR efficiency for *IGF1* was 98.93% (r^2= 0.992); *IGF2* was 99.3% (r^2= 0.993); *GAPDH* was 98.3% (r^2=0.994); and *EEF2* was 98.4% (r^2= 0.995). Reported efficiency and r^2 values are calculated as multi-plate averages across.

We assessed data quality per sample triplicate. If the mean CQ value deviated by more than 0.2 cycles from the mean, one of two approaches was taken: (1) if there was a clear outlier in the triplicate set (*i.e.*, a failed reaction), the outlier was removed to decrease the deviation to less than 0.2 cycles, and if this was not possible (2) the sample (all three reactions) was excluded from analysis. We based final data analyses on absolute copy number determined within the software from standard curve and CQ values, adjusted for PCR efficiency.

Statistical Analysis

We ran all analyses in R version 3.6.0 (R Core Team 2019). We used a two-tailed t-test to determine confidence intervals for genes and made subsets of data by gene. Because we had three replicate measures of gene expression (copy number) for each individual, we used mixed-models with individual lizard as a random factor for all gene expression analyses to use all of the available data rather than taking an average (as in Marks et al. 2021).

Although we randomly allocated the lizards to different treatments, there was nonetheless a significant difference in body mass (N=55, $F_{1,586}$ = 28.74, p<0.0001) and SVL (N=55, $F_{1,658}$=26.22, p<0.0001) between the two groups at the beginning of the

experiment, with the sprint-trained lizards being larger for both measures. These lizards were larger in mass (N=55, $F_{1,658}$=76.52, p<0.0001) and SVL (N=55, $F_{1,622}$= 32.41, p<0.0001) than the untrained group to an even greater extent by the end of the experiment. Group differences despite randomization will occur during the course of proper experimental design at a rate of ~5%, but are under-reported in the literature, possibly due in part to reverse *P*-hacking (Chuard et al., 2019). To deal with the group difference here, and to account for the known influence of mass on *IGF* expression in female green anoles (Marks et al., 2021) we conditioned all our statistical models on one of two morphometric measurements. First, we analyzed absolute copy number with treatment as a fixed factor; final body mass at the end of the experiment (when the liver sample was taken) as a covariate; and individual as a random factor to account for triplicate measures at the qPCR stage. Second, we analyzed absolute copy number with treatment as a fixed factor; percent change in body mass over the course of the experiment as a covariate (%*Δ* mass, calculated as the difference between post- and pre-treatment mass, to account for the size difference between treatments); and individual as a random factor.

Exploratory analyses revealed nonlinear relationships between gene expression and mass measures; consequently, we also included nonlinear terms for both final mass and percent change in body mass in the respective models. Finally, we also included interaction terms between those linear mass effects and treatment in each model to allow for the possibility that different treatments exhibited different nonlinear gene expression with regard to mass. The addition of random slopes for treatment (Schielzeth & Forstmeier, 2009) did not affect parameter composition of any of the

minimum adequate mixed models, but did cause convergence issues with the *IGF2* model. Consequently, we present the results of our mixed models here without random slopes.

We used the *nlme* package (Pinheiro and Bates, 2013) to fit all mixed effect models. We used Box-Cox transformed dependent variables as required to meet model assumptions of normality. We dealt with heteroscedasticity where it occurred by fitting an exponential variance structure (Zuur et al., 2009; Marks et al., 2021). We used log-likelihood deletion tests to determine final models (Silk et al., 2020). To accurately visualize the nonlinear relationships between gene expression and the model factors, we then fit generalized additive models from the package *psych* (Revelle, 2021).

Results

1) Final Body Mass analysis

The final model for *IGF1* (Fig 1A and 1B; Table 1A) and *IGF2* (Fig 2A and 2B; Table 1A) retained a nonlinear interaction between the main effect of treatment and final body mass. The larger animals in the sprint-trained group expressed *IGF1* (Fig.1B) and *IGF2* (Fig. 1D) to a lesser extent than similarly-sized untrained animals (Fig. 1A and Fig. 1C, respectively). Lastly, regardless of treatment, hepatic *IGF2* gene expression was expressed higher than *IGF1,* which is consistent with previous studies examining *IGF* gene expression in anoles (Beatty et al., 2020; Marks et al., 2021).

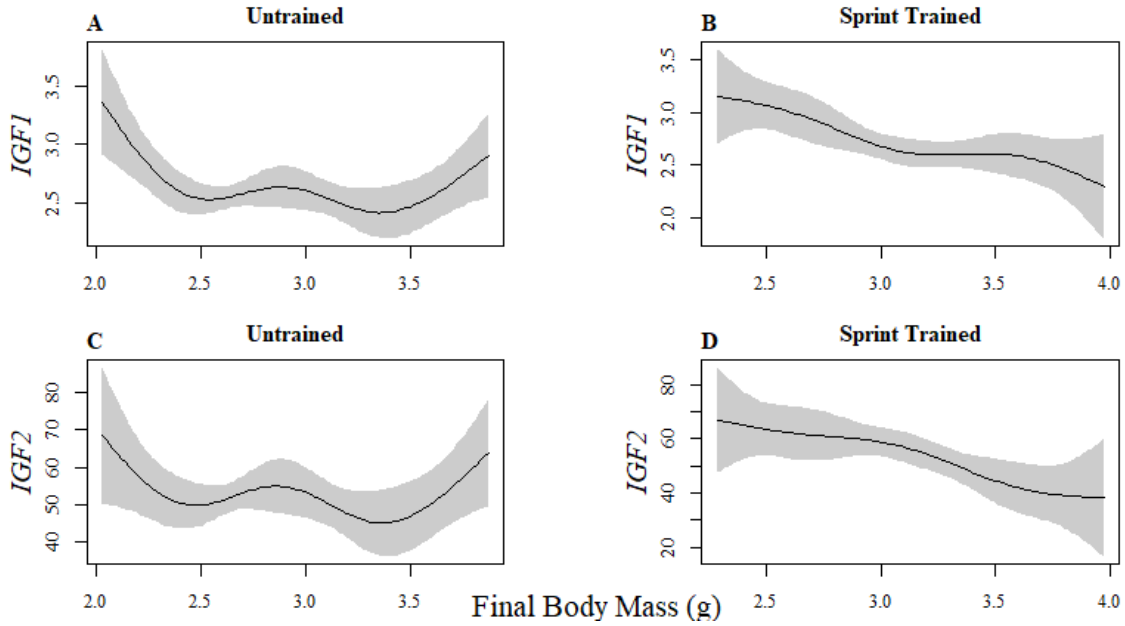

Figure 1. Absolute values of gene expression conditioned with final body mass (in grams) showing expression of (A) *IGF1* in the untrained lizards; (B) *IGF1* in the sprint-trained lizards; (C) *IGF2* in the untrained lizards; (D) *IGF2* in the sprint-trained lizards. Nonlinear interactions between treatment and final body mass are seen in *IGF1* and *IGF2*.

(A) (IGF1)	Model term	Coefficient	SE
	Intercept	7.34	2.49
	Treat (ST)	0.94	0.48
	Final Body Mass	-3.18	1.69
	I(Nonlinear Final Body Mass^2)	0.52	0.28
	Treat (ST): Final Body Mass	2.17	3.45
	Treat (ST): I(Final Body Mass^2)	-0.08	0.05
(B) (IGF2)			
	Intercept	54.29	24.83
	Treat (ST)	60.45	39.19
	Final Body Mass	-0.67	8.62
	I(Final Body Mass^2)	8.75	11.93
	Treat (ST): Final Body Mass	117.28	145.64
	Treat (ST): I(Final Body Mass^2)	-3.50	2.10

Table 1: Best-fitting models describing the variation in copy number of (A) (*IGF1*) and (B) (*IGF2*) with final body mass as a covariate. The reported coefficients give estimated change in the dependent variable between the baseline category and the category named in the table (ST = sprint-trained). Baseline category was the untrained group.

2) Percent change in body mass analysis

The final models for *IGF1* (Fig. 2A; Table 2A), *IGF2* (Fig 2B; Table 2B) and *GAPDH* (Fig. 2C; Table 2C) retained an effect of percent change in body mass on gene expression. Although these models did not retain a treatment effect, animals that gained the most mass over the course of the experiment expressed *IGF1*, *IGF2* and *GAPDH* to a greater extent than animals who maintained or lost body mass. The final model for *EEF2* retained a significant interaction between treatment and percent change in body mass (Fig. 3A and 3B; Table 2D). Sprint-trained animals (Fig. 3B) that gained body mass over the course of the experiment expressed *EEF2* to a lesser extent than similarly sized untrained lizards (Fig 3A).

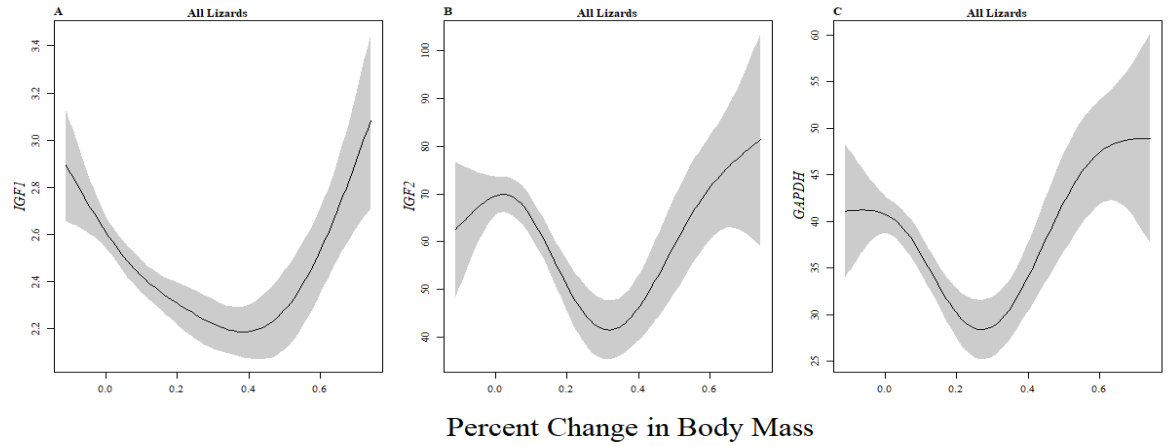

Figure 2. Absolute values of gene expression conditioned with % change in body mass of (A) *IGF1* in the untrained and sprint-trained lizards; (B) *IGF2* in the untrained and sprint-trained lizards; (C) *GAPDH* in the untrained and sprint-trained lizards. There was no effect of treatment on *IGF1*, *IGF2*, nor *GAPDH* when models were conditioned with percent change in body mass, yet a nonlinear effect of percent change in body mass was included in these models.

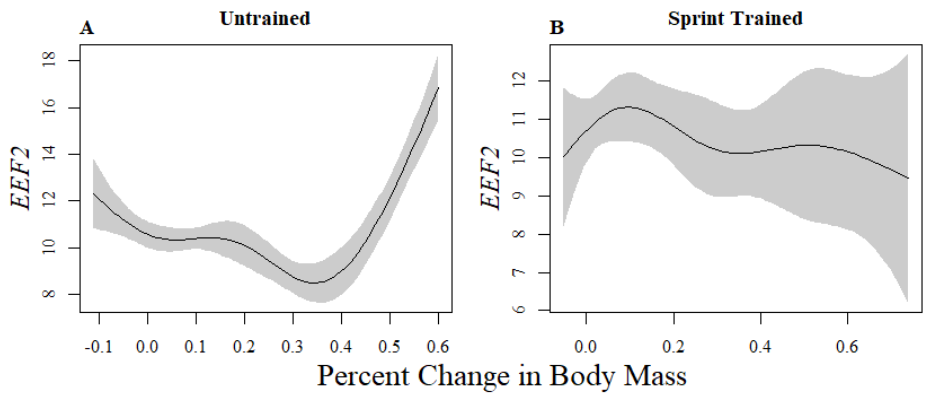

Figure 3. Absolute values of *EEF2* gene expression conditioned with % change in body mass for (A) untrained lizards and (B) sprint-trained lizards. There is a nonlinear interaction between treatment and percent change in body mass for *EEF2*.

(A) (IGF1)	**Model term**	**Coefficient**	**SE**
	Intercept	2.58	0.094
	%Δ mass	-2.59	0.66
	I(%Δ mass^2)	4.28	1.38
(B) (IGF2)			
	Intercept	65.74	5.87
	%Δ mass	-120.63	41.38
	I(%Δ mass)	217.34	86.20
(C) (GAPDH)			
	Intercept	97.28	8.81
	%Δ mass	-189.82	62.77
	I(%Δ mass^2)	417.08	130.41
(D) (EEF2)			
	Intercept	10.32	0.70
	Treat (ST)	0.99	0.87
	%Δ mass	-6.86	4.91
	I(%Δ mass^2)	23.88	10.21
	Treat (ST): I(%Δ mass^2)	-16.22	7.0

Table 2. Best-fitting models describing the variation in copy number of (A) (*IGF1*), (B) (*IGF2*), (C) (*GAPDH*), and (D) (*EEF2*) with percent change in body mass as a covariate. The reported coefficients give estimated change in the dependent variable between the baseline category and the category named in the table (ST = sprint-trained). Baseline category was the untrained group.

Discussion

Investment in sprinting ability via exercise training involves increased resource allocation towards skeletal muscle growth (Atherton and Smith, 2012; Husak et al., 2015), yet the molecular mechanisms and pathways involved are poorly understood for non-model organisms, which impedes our understanding of how sprinting is incorporated into the multivariate organismal phenotype. In this experiment, we sprint-trained female green anoles to test the hypotheses that hepatic *IGF1*, *IGF2*, *GAPDH* and *EEF2* expression respond to anaerobic exercise training.

Our hypothesis that hepatic *IGF1* expression would be affected by sprint training was supported (Fig. 1A and 1B; Table 1A), albeit not in the expected direction. Although we predicted that sprint training would upregulate *IGF1* expression, our results show that this phenomenon was size-dependent, such that larger lizards expressed *IGF1* to a lesser extent within the sprint-trained lizards than untrained lizards. Sprint-trained lizards at the lower end of the mass spectrum did express *IGF1* to a greater extent than their larger counterparts, but not more than similarly sized untrained lizards after accounting for effects of body size. In humans, *IGF1* expression in skeletal muscle tissue can increase during exercise and the recovery period, but these elevated levels are typically maintained no more than an hour (Kraemer et al., 2017). However, Marks et al. (2021) found that a limited calorie diet also decreased *IGF1* within larger female green anoles over a comparable time period. It could be that larger females are suppressing growth and reproduction via decreased *IGF1* production when resources are limited, or when they are forced to be diverted elsewhere, as in our manipulation here. Alternatively, the larger lizards may have upregulated *IGF1* within the muscle

tissue (rather than hepatic measured here) or altered cellular receptor availability, the latter of which occurs in elderly humans (Urso et al., 2005). Future studies that consider tissue-specific expression and regulation of IGF in response to sprint training would be extremely valuable for understanding the contributions of both hormones to the exercise response.

When the models testing *IGF1* expression were conditioned on percent change in body mass, there was no treatment effect (Fig. 2A; Table 2A). Percent change in body mass was included in the final model, though, which means that body size is a crucial component to *IGF1* gene expression within the context of sprint training, consistent with Marks et al. (2021) who also found mass to be a determining factor of *IGF1* expression in green anole lizards. The nonlinear effect of body size shows that lizards exhibiting the greatest changes in body mass (positive or negative) express *IGF1* to a greater extent. It is possible that lizards that lost mass increased *IGF1* transcription via an upregulated somatotropic axis to increase energy availability via growth hormone effects. It is also possible that younger lizards are growing faster than older lizards regardless of training effects, but these lizards were wild caught, and we have no information on their ages other than they were above the size threshold for being sexually mature females (Vanhooydonck et al., 2005). In a previous study, endurance training enhanced growth of adult female green anoles, but did not affect juveniles, suggesting that age can impact performance-growth trade-offs (Husak et al., 2017).

Our prediction that *IGF2* expression would be upregulated in response to sprint training was not supported when models were conditioned with final body mass (Fig 1C

and 1D; Table 2B). Although smaller lizards within the sprint-trained group expressed *IGF2* to a larger extent than their untrained counterparts, this pattern was inverted at the larger end of the size continuum. When the data were conditioned with percent change in body mass, treatment was again no longer included in the final model (Fig 2B; Table 2B), but lizards that gained mass expressed *IGF2* to a greater extent. This relationship shows the likely importance of *IGF2* for growth in green anoles. Although treatment was not included in the final model with percent change in body mass for *IGF1* and *IGF2*, it is clear that sprint training affects the growth of the animal and *IGF1* and *IGF2* are involved in physiological changes, albeit via possible indirect effects (Swanson and Dantzer, 2014). Alternatively, these findings may be a result of when the tissue was sampled in comparison to when the final sprint trial was performed. Larger lizards may have been suppressing hepatic *IGF2* expression and upregulating skeletal muscle *IGF2*. *IGF2* might have been affected by the treatment but is undetected when using percent change in body mass because only hepatic transcription of *IGF2* was measured, rather than paracrine and autocrine activity at the receptor level, or circulating hormone levels (Marks et al. 2021). There is currently no assay available to measure circulating levels of IGF1 and IGF2 in green anoles, but validating the relationship between gene expression and circulating hormone levels at the whole-organism level is an important future goal. Furthermore, because no studies in other species exist that specifically test *IGF2* expression in response to sprint training, it is difficult to place our results here within an appropriate comparative context.

GAPDH and *EEF2* are traditionally used as housekeeping genes. Housekeeping genes are those expressed in all cells for normal physiological function and used to

normalize data in qPCR because they should be expressed similarly across all treatments in a study (Thellin et al., 1999). Contrary to this, Marks et al. (2021) found that *GAPDH* and *EEF2* genes are in fact significantly altered by the energetic environment. Although this effect renders them impractical as housekeeping genes, they nonetheless give us further insight into whole-organism genetic effects of environmental variation.

GAPDH is a critical enzyme for glucose metabolism during glycolysis (Nicholls et al., 2012), while *EEF2* is important in protein elongation by assisting with ribosomal movement across mRNA to build proteins (Kaul et al., 2011). Our hypothesis that sprint-training would affect *GAPDH* expression was not supported by either model. *GAPDH* was not affected when the model was conditioned with final body mass. When the model was conditioned with percent change in body mass (Fig. 2C; Table 2C), there was a nonlinear effect of percent change in body mass on *GAPDH* expression, such that animals that grew more, regardless of treatment, expressed *GAPDH* to a greater extent than animals that grew less. Interestingly, animals at the lower end of the percent change in body mass spectrum expressed *GAPDH* to a greater extent than animals in the middle of the spectrum. This could be representative of the pleiotropic effects of *GAPDH.* The lizards at the smaller end of the percent change in body mass spectrum may have had low glucose levels, which could increase expression of *GAPDH* and binding to Rheb, a GTPase (Lee et al., 2009). Increased GAPDH-Rheb interactions would inhibit the mTORc1 pathway which is a central component of growth (Lee et al., 2009; Nicholls et al., 2012).

Final body mass was not included in the final model for *EEF2*, but percent change in body mass was (Fig 3A and 3B; Table 2D), which supports our hypothesis that sprint training would affect *EEF2*. There was a nonlinear interaction between treatment and percent change in body mass, with this interaction especially obvious on the larger end of the change in body mass continuum. Untrained animals that grew more also had greater expression of hepatic *EEF2* than the corresponding sprint-trained lizards. This is consistent with Marks et al. (2021), where green anole females in a negative energetic environment expressed both *GAPDH* and *EEF2* to a greater extent than their control counterparts (Marks et al., 2021). The sprint-trained group expressed *EEF2* to a lesser extent than the untrained group. Protein elongation is an energetically costly task, which could explain why the sprint-trained lizards expressed this gene to a lesser extent than the untrained lizards within the liver. However, if sprint training increases muscle mass, there should be more protein production. It could be that hepatic protein production was downregulated with reduced *EEF2* expression (and perhaps increased EEF2K activity), whereas *EEF2* expression in the muscle (which would have been undetected by our method), where necessary to respond to training, was upregulated. Most of these studies (Rose et al., 2005; Van Proeyen et al., 2011) test *EEF2* from skeletal muscle tissue, so future studies should examine if they are consistent with those from hepatic origin.

From mammalian studies, IGFs are known to play key roles in muscle growth and cell proliferation (Duan et al., 2010; but see Atherton & Smith, 2012), but are also important for responding to environmental challenges related to resource availability and activity levels (Fontana et al., 2008; Rahmani et al., 2019). Our results provide one

more piece to the puzzle of how this pathway functions in a reptile: when green anoles invest energy into movement, the insulin and insulin like signaling network is implicated in the response. We found that small females had higher hepatic *IGF1* and *IGF2* expression than larger females when they are forced to sprint more. Large sprint-trained females may be suppressing hepatic IGFs for metabolic reasons, but increasing skeletal muscle IGFs to enhance muscle mass. On the other hand, untrained, small females may upregulate IGFs for growth, whereas large ones may increase it for reproductive purposes. The results of this experiment, taken together with those of Marks et al. (2021), show that future studies of this hormonal network should consider sex differences, as well as body size in analyses and should focus experiments on skeletal muscle expression of IGFs and the receptors, to further understand the contribution of the insulin and insulin-like signaling pathway to muscle growth in reptiles. Although our results raise many new questions, they are an important step in our understanding of how IIS functions in non-mammalian systems. In short, although the IIS network is highly complex, we have provided evidence that multiple aspects of this network are involved in response to exercise in reptiles.

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Chapter 3 – Offspring phenotype (H3)

The maternal energetic environment affects both egg and offspring phenotypes in green anole lizards (Anolis carolinensis)

Abstract

Animals exist in dynamic environments that may affect both their own fitness and that of their offspring. Maternal effects allow mothers to prepare their offspring for the environment in which they will be born via a number of mechanisms, not all of which are well understood. Resource scarcity and forced resource allocation are two scenarios that could affect maternal investment by altering the amount and type of resources available for investment in offspring, albeit in potentially different ways. We tested the hypothesis that maternal dietary restriction and sprint training have different consequences for the offspring phenotype in an oviparous lizard (*Anolis carolinensis*). To do this, we collected and reared eggs from adult diet restricted females (Low Diet [LD] or High Diet [HD]) or sprint trained females (Sprint Trained [ST] or Untrained [UT]) and measured both egg characteristics and hatchling morphology. ST and LD mothers laid both the fewest and heaviest eggs overall, and ST, UT and LD eggs also had significantly longer incubation periods than the HD group. Hatchlings from the diet experiment (LD and HD offspring) were the heaviest overall. Furthermore, both body mass of the mother at oviposition and change in maternal body mass over the course of the experiment had significant and sometimes different effects on egg and offspring phenotypes, highlighting the importance of maternal energetic state to the allocation of maternal resources.

Introduction

An animal's environment is constantly changing, with many taxa facing variable temperatures, changes in food availability, or changes in predator presence over relatively short timescales. Phenotypic plasticity might ameliorate the fit between individual and environment (Ghalambor et al. 2007), and females can influence offspring phenotypes and fitness via maternal effects, defined as the phenomenon where the offspring phenotype is affected by the environment that the mother experiences (Wolf & Wade, 2009). Maternal effects can manifest directly as alterations in sex ratios (Mousseau and Fox, 1998), brood size (Stearns, 1989; Brown and Shine, 2009), or hatchling size (Stearns, 1989; Sinervo and Huey, 1990; Brown and Shine, 2009), amongst other effects, or indirectly via manipulation and allocation of hormones in eggs (Ensminger et al. 2018). Although female plasticity is well documented, we lack an understanding of how specific female plastic responses to environmental variation affect offspring resource allocation, and ultimately offspring phenotype. Causal factors driving these maternal effects are typically labeled broadly as 'stress' or 'environmental quality' which gives little insight into the underlying mechanistic cause for the differences manifested (Glavin, 1984; Boots and Roberts, 2012; Peixoto et al., 2020). Understanding these mechanisms is imperative for uncovering the functional links between life-history trade-offs (Sterns, 1989), transgenerational effects, and phenotypic variation (Bonduriansky and Day, 2018).

One such mechanism that is known to drive trade-offs in nearly all animals, specifically the trade-off between survival and reproduction, is diet restriction (Chapman and Partridge, 1996; Mair and Dillin, 2008; Moatt et al. 2016; Regan et al. 2020).

Limiting resource acquisition can affect maternal provisioning, and thus drive maternal effects. Oviparous females in particular provide researchers with a glimpse into the maternal strategies employed in the face of different environmental pressures because mothers must proactively provision their eggs for the current environment (Giron & Casa 2003; Saino et al. 2006; Romano et al. 2008). In addition to the phenotype of the offspring themselves, maternal effects can also affect characteristics of the eggs, including their size, shape, and incubation periods (Dzialowski & Sotherland 2004). For example, Madagascar ground geckos (*Paroedura picta*) under limited resource conditions not only exhibit longer periods between laying eggs, but those eggs are also smaller than those of well-fed lizards (Kubička and Kratochvíl, 2009). Egg size also correlates with hatchling size, such that the resource-limited females produced smaller juveniles (Kubička and Kratochvíl, 2009). Although the trade-off between a maternal low-diet and offspring phenotype is well-documented, there is a huge gap in literature that compares the effects of different maternal environmental conditions on offspring phenotype.

In addition to resource limitation, changes in environmental conditions can also drive crucial allocation trade-offs in females, which could in turn affect the amount and type of resources available for mothers to allocate towards offspring. For instance, energetic investment into performance related traits such as predator evasion, foraging, and sprinting can also lead to changes in maternal phenotype which can in turn affect offspring phenotypes (Sheriff and Love, 2012; Bro-Jørgenson, 2013; St-Cyr et al. 2017). Increased activity or use of locomotor capacities, such as sprinting, can force an animal to invest energy into the underlying morphological and physiological mechanisms

supporting that function, which can in turn promote trade-offs (Lailvaux & Husak 2014; Irschick et al. 2008; Husak and Lailvaux, 2019). In green anoles, sprint training was shown to increase both overall muscle size and investment in slow oxidative muscle fibres (Husak et al. 2015). Investment in muscle is especially costly, and likely incurs significant production and maintenance costs (Husak & Lailvaux, 2017). Because investment in locomotion can be easily manipulated in the laboratory through the implementation of specialized training regimes, this presents a useful opportunity to understand the effects of forced maternal allocation to an ecologically relevant trait.

In this experiment, we used green anole females (*Anolis carolinensis*) which are continuous reproducers (Love and Williams, 2008; Sparkman et al. 2010). Continuous reproducers have incessant ovarian cycles and can store sperm to produce eggs throughout the breeding season (Awruch, 2015). The goal of this experiment was to compare how female green anoles prioritize egg investment when resources are severely limited versus when they are forced to directly invest energy into a specific trait – in this case, locomotor capacity. We tested the hypothesis that maternal dietary restriction and maternal investment into sprint training would differently affect offspring phenotype. We made five specific predictions to test this hypothesis: (P1) the low diet (LD) and sprint trained (ST) animals would lay significantly fewer eggs than the high diet (HD) and untrained (UT) lizards; (P2) eggs and (P3) offspring from the LD and ST lizards would weigh less than those from the UT and HD moms; (P4) treatment would not affect SVL; (P5) the incubation period for the treatment groups would be longer than that of their control counterparts.

Materials and Methods

The eggs and offspring used in this experiment were derived from prior experiments aimed at understanding how environmental variation, namely decreased resource acquisition (Marks et al. 2021) and increased investment in locomotion (Marks et al. 2022) affects the maternal phenotype. For continuity purposes we chose to label our control groups based on their titles within the two previous manuscripts. The control group within the diet experiment is labeled High Diet (HD) and the control group from the sprint experiment is labeled Untrained (UT).

The UNO Institutional Animal Use and Care Committee protocol #19-003 permitted all procedures outlined below. We captured adult, reproductively mature (snout-vent length (SVL) > 40mm) *A. carolinensis* females from urban populations in Orleans parish in Louisiana in June of 2019 (N=100) and June of 2020 (N=100), during the green anole breeding season (Jenssen et al. 1995). We recorded SVL to the nearest 0.05 mm and body mass to the nearest 0.01 g on day of capture. The adult lizards were acclimated for one week prior to either treatment.

Diet Treatments

In June 2019, we tested the effects of energetic environment of insulin-like growth factor expression in wild-caught female green anoles by randomly allocating them to either a High Diet (HD) or Low Diet (LD) group. Following the treatment protocol in Marks et al. (2021), all lizards were given ~1.25 cm crickets (*Acheta domesticus*). The LD group was fed one cricket coated in ZooMed ReptiCalcium powder, three times weekly, which is an

established diet known to promote trade-offs, whereas the HD group females were fed an *ad libitum* diet of three crickets, three times per week supplemented with ZooMed ReptiCalcium powder (as in Lailvaux et al., 2012; Husak et al., 2016). The HD "treatment" is therefore equivalent to the control situation, although we refer to these groups here as LD and HD to be consistent with Marks et al. (2021). The LD group was effective in decreasing reproductive output, consistent with Husak et al., (2016).

Sprint Training

In June of 2020, wild-caught adult female lizards were randomly allocated to the Untrained (UT) group or the sprint-trained (ST) group. Both treatments were fed the same as the HD group in the previous experiment, which again corresponds to a "normal" or control diet. The ST group was trained following previously established protocol (Husak & Lailvaux, 2019; Wang & Husak, 2020; and Marks et al. 2022.) The ST lizards were sprint trained three times a week for six weeks. The ST lizards were encouraged to run up the dowel of a racetrack four times each day they were trained and each trial was separated by at least one. The UT lizards were handled for 30 seconds three times a week to mitigate any effects due to the increase in handling time experienced by the ST animals. As for the diet treatment, we use the UT and ST labels for consistency with the earlier study (here Marks et al. 2022), but we note that the UT treatment corresponds to the control situation in sprint training studies (Husak et al. 2015; Husak & Lailvaux 2019; Lailvaux et al. 2020)

Egg and Hatchling Husbandry

The following protocol applies for female lizards caught in 2019 and 2020. Terraria were checked three times weekly for eggs by lightly sifting through the soil substrate on the bottom of the lizard terrarium. Dead and/or unfertilized eggs were recorded (i.e. date laid and maternal identification) and discarded. When an egg was found, it was placed on a digital scale and weighed to the nearest 0.01g. Its length and width were also recorded with Mitutuyo digital calipers to the nearest 0.05mm. Once morphometric measurements were taken, it was placed in a petri dish with moist vermiculate. Eggs were individually held in petri dishes and were labeled with the date they were found as well as maternal ID and were given a unique egg ID. This was then placed in an incubator set to 28.6 °C (Lovern and Wade, 2003; Lovern et al. 2004). Eggs in the incubator were watered gently with a spray bottle every other day and were rotated weekly to avoid position effects within the incubator. Eggs were checked daily for hatchlings.

When an egg hatched, the petri dish was removed from the incubator and the hatchling was immediately weighed to the nearest 0.01g then housed in a terrarium under the same conditions as the adult females for future experiments. Offspring born in the 2020 sprint training experiment had their SVL measured to the nearest 0.05mm with a Mitutuyo digital caliper on the same day they were removed from the incubator.

In short, we recorded the total number of eggs laid from each individual female and the total number of incubation days from oviposition to hatching. We also measured mass of the egg, initial mass at hatching, as well as the snout-vent length of the hatchlings from the sprint training experiment. Hatchling SVL was not recorded for the

diet experiment due to unforeseen logistical challenges, and so we are unable to present or analyze those data here.

Statistical analyses

We used R version 3.6.0 (R Core Team 2019) for all analyses. All models used maternal treatment (diet or sprint training) as a fixed factor. We included maternal body mass as a covariate because maternal body mass affects aspects of maternal physiology (see Marks et al. 2021 and 2022) and is known to influence offspring phenotype (Shine and Downs, 1999; Warner and Lovern, 2014). We also included percent change in maternal mass over the course of the experiment (denoted as %Δm.mass) as an additional covariate as in Marks et al. (2022). This was calculated from the initial mass and final body mass measured. For mixed models, all saturated models contained maternal identification nested within year of the experiment as a random factor to control for non-independence of eggs from the same mother, and for year-to-year variation that might otherwise confound our results. We performed log-likelihood deletion tests using the *MASS* package (Silk et al., 2020), to find minimum adequate models (i.e. the simplest models that explained the most amount of variation (Crawley, 1993).

1) *Total Number of eggs laid*

We used the *glmer* command from the *lme4* package to fit a generalized linear mixed effects model with a Poisson distribution to test our first prediction (P1) that the number

of eggs laid across treatments will be different. To visualize the model, we used packages *emmeans* and *ggplot2* to plot the treatment residuals after accounting for effects of model covariates (as in Marks et al. 2021; 2022).

2) *Egg Mass*

To test P2, we used the *nlme* package to fit linear mixed effect models with our most saturated models contained %Δm.mass or maternal mass at oviposition as covariates. Maternal identification was again nested within year as a random effect. Maternal mass at oviposition told us the energetic state of the mother when the egg was laid while %Δm.mass told us the change in energetic state over the course of the experiment. To visualize, we used packages *emmeans*, *ggplot2* and *gridExtra*.

3) Hatchling Mass

We used the *nlme* package to test our third prediction (P3) that maternal treatment affects mass of offspring at time of hatching. The saturated model contained the following covariates: egg mass, number of days in incubator, mass of the mother at oviposition, and %Δm.mass. To visualize the model, we generated a boxplot from *ggplot2* and used the *rstatix* package to overlay p-values from a pairwise t-test using a false discovery rate.

4) SVL of Hatchlings

We did not obtain SVL measurements at hatching from the 2019 diet experiment. However, we present the results from the 2020 sprint experiment to highlight the fact that the sprint training affected SVL of the offspring. To test our fourth prediction (P4)

that sprint training affects offspring phenotype, we ran a linear mixed effects model with maternal identification as a random factor and included the following covariates to test if they affected SVL of the hatchlings: number of incubation days, %Δm.mass, egg mass, and maternal mass at oviposition. We visualized the data using *ggplot2* and used the package *rstatix* to overlay p-values from a pairwise t-test using a false discovery rate (Garcia 2004).

5) Total Incubation Time

We used the *lme4* package in R (Pinheiro and Bates, 2013) to fit an initial generalized linear mixed model with Poisson errors and maternal identity (because mothers produced multiple eggs) nested within year (i.e. 2019 or 2020) as random factors to test our fifth prediction (P5) that maternal energetic environment affects total incubation time of offspring and to deal with any year-to-year variation in these data. However, the model fit was not improved by the inclusion of any random factors; consequently, we fit a generalized linear model with only fixed factors to the incubation time data. To deal with underdispersion in the resulting model, we fit a quasipoisson distribution to the final minimum adequate model, which included an effect of %Δm.mass on incubation time. We used packages *emmeans* and *ggplot2* to visualize the final model by plotting the partial residuals. A partial residual is the distance between the predicted value and our data point when additional covariates are controlled for in the model (Cook, 1993).

Results

1) *Total Number of Eggs Laid*

Although our model had a poor overall fit, fitting a negative binomial distribution returned qualitatively the same results, suggesting that our results are robust to distributional assumptions (Schielzeth et al., 2020; see also Warton et al., 2016 for discussion of distributional assumptions in count data). An effect of percent change in body mass of the mother was retained within the final model. All treatments displayed a negative correlation between total number of eggs laid and %Δm.mass (Figure 4; Table 4). Both the LD and ST group laid significantly fewer eggs compared to the HD lizards, although egg number did not differ significantly eggs between the HD and UT control groups. Maternal identification was included in the final model as a significant random effect.

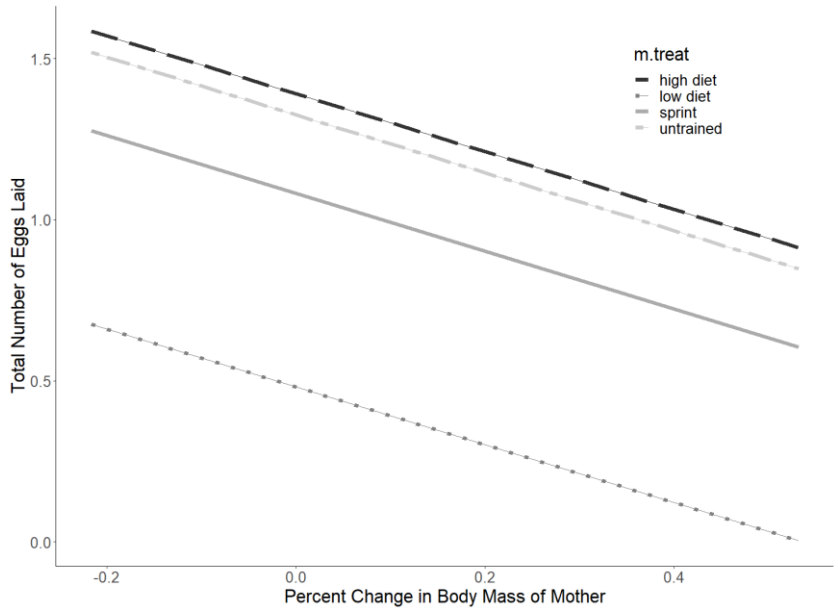

Figure 1. Graphs of the estimated marginal means for total number of eggs laid when percent change in body mass of the mother is accounted for.

Total Number of Eggs Laid	Model term	Coefficient	SE
	Intercept	1.39	0.11
	Treat (LD)	-0.91	0.18
	Treat (ST)	-0.31	0.12
	Treat (UT)	-0.064	0.10
	%Δm.mass	-0.90	0.37

Table 1: Best-fitting models describing the variation in total number of eggs laid with %Δm.mass as a covariate. The reported coefficients give estimated change in the dependent variable between the baseline category and the categories named in the table (ST = sprint-trained, UT = untrained, LD = low diet). Baseline category was the high diet group.

2) *Egg Mass*

Percent change in body mass of the mother interacted with treatment such that ST and LD lizards that gained mass over the course of the experiment laid lighter eggs than similarly sized UT and HD lizards (Figure 2A; Table 2A). ST and LD lizards that lost mass over the course of the experiment laid heavier eggs than their control counterparts. When looking at the model with mass of the mother at oviposition (Figure 2B; Table 2B), the final model retained an effect of maternal mass at oviposition, which was positively correlated to egg mas, regardless of treatment.

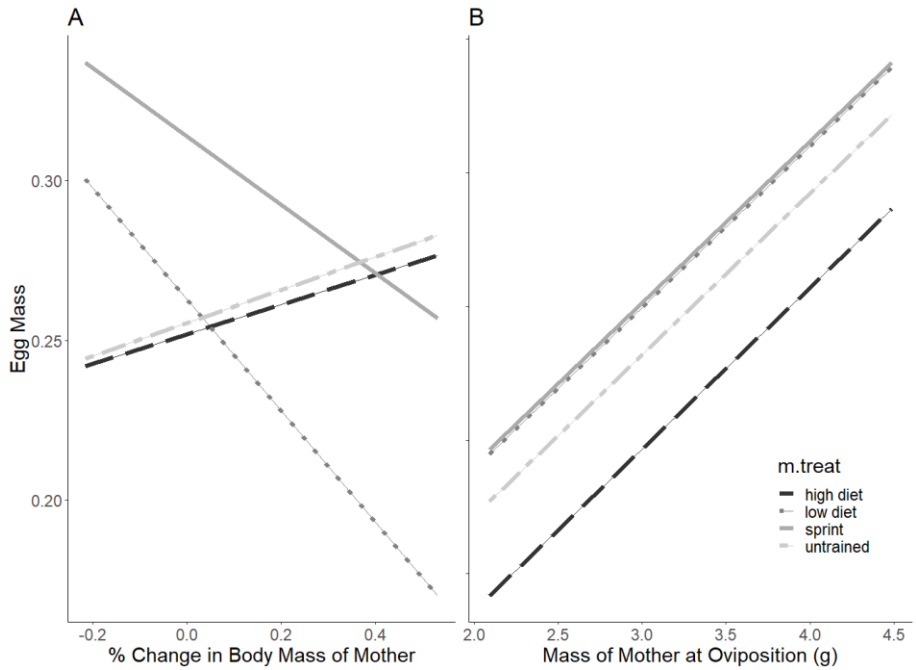

Figure 2. Graphs of the estimated marginal means for offspring egg mass when (A) percent change in body mass of the mother and (B) mass of the mother at oviposition are accounted for.

Egg Mass	Model term	Coefficient	SE
	Intercept	0.25	0.015
	Treat (LD)	0.0042	0.019
	Treat (ST)	0.065	0.034
	Treat (UT)	0.0029	0.023
	%Δ mass	0.052	0.045
	Treat (LD) : %Δm.mass	-0.31	0.16
	Treat (ST) : %Δm.mass	-0.17	0.10
	Treat (UT) : %Δm.mass	0.0073	0.063

Table 2A: Best-fitting models describing the variation in egg mass with percent change in final body mass of the mom as a covariate. The reported coefficients give estimated change in the dependent variable between the baseline category and the categories named in the table (ST = sprint-trained, UT = untrained, LD = low diet). Baseline category was the high diet group.

Egg Mass	Model term	Coefficient	SE
	Intercept	0.19	0.026
	Treat (LD)	0.021	0.011
	Treat (ST)	0.022	0.018
	Treat (UT)	0.014	0.018
	m.assovi	0.024	0.007

Table 2B: Best-fitting models describing the variation in egg mass with mass at oviposition of the mom (m.massovi) as a covariate. The reported coefficients give estimated change in the dependent variable between the baseline category and the categories named in the table (ST = sprint-trained, UT = untrained, LD = low diet). Baseline category was the high diet group.

3) *Hatchling Mass*

Our simplest model, which we found by comparing AIC values, did not contain any covariates, but maternal treatment did affect the mass at hatching. Maternal identification was nested within year as a random effect. The mass of the hatchlings from the UT and ST lizards averaged significantly less than the HD and LD lizards (Figure 3; Table 3). There was no significant difference in hatch mass between the UT and ST or between the HD and LD lizards (note that year was included as a random factor in this analysis and thus accounted for).

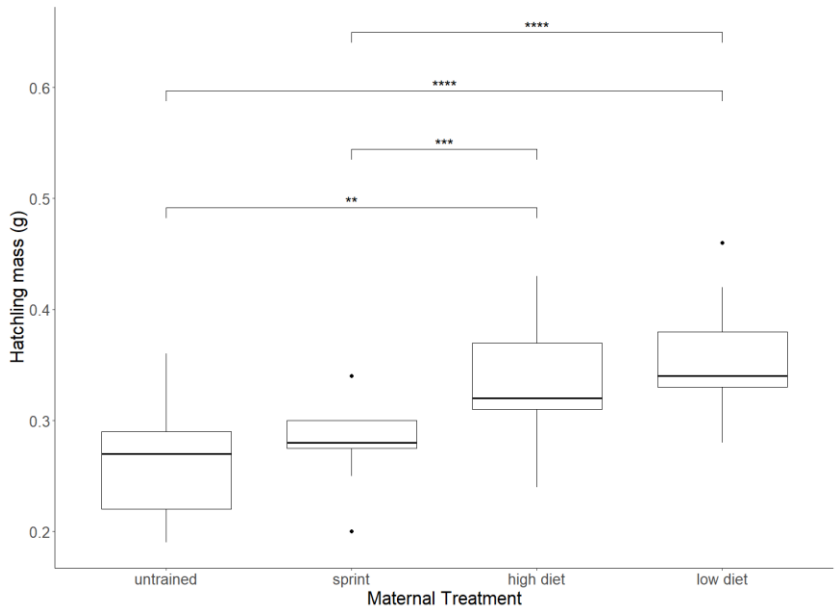

Figure 3. Boxplot showing the average mass of offspring at hatching. P-vales generated from a false discovery rate pairwise t-test are shown above plots (0.001 > ***; 0.004 > **).

Hatchling Mass	Model term	Coefficient	SE
	Intercept	0.33	0.007
	Treat (LD)	0.017	0.014
	Treat (ST)	-0.050	0.016
	Treat (UT)	-0.066	0.016

Table 3: Best-fitting models describing the variation in hatch mass among the four treatments. The reported coefficients give estimated change in the dependent variable between the baseline category and the categories named in the table (ST = sprint-trained, UT = untrained, LD = low diet). Baseline category was the high diet group.

4) *SVL of Hatchlings*

Our final model did not include any covariates. Offspring from ST mothers were significantly longer than clutches from UT individuals, exhibiting significantly larger SVLs at hatching (Figure 5; Table 5).

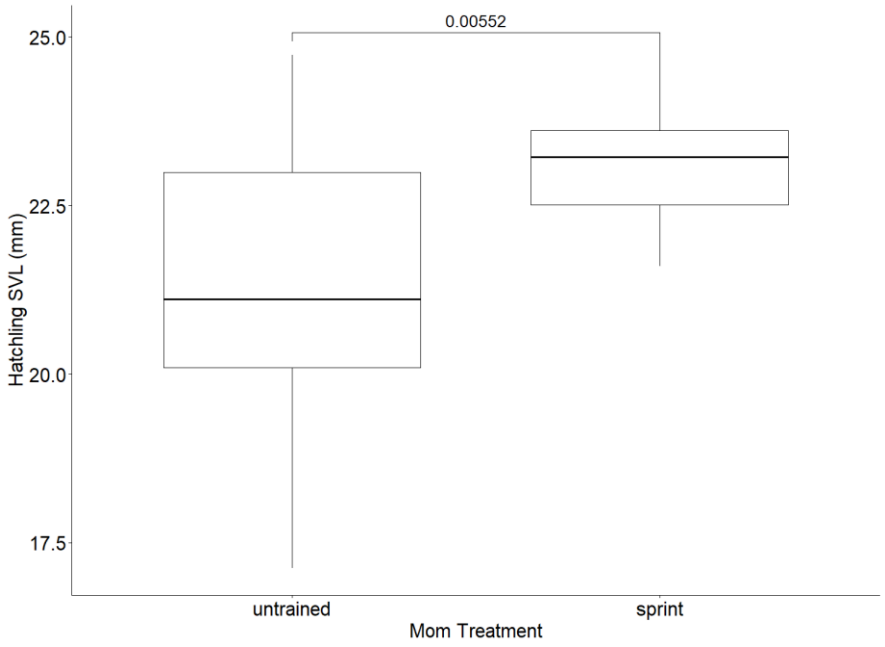

Figure 4. Boxplot showing the average SVL of offspring at hatching. P-vales generated from a false discovery rate pairwise t-test are shown above plots. Data for SVL at hatching from the 2019 diet experiment was not obtained.

Hatchling SVL	Model term	Coefficient	SE
	Intercept	23.13	0.30
	Treat (UT)	-1.84	0.50

Table 4: Best-fitting model describing the variation in hatchling SVL. The reported coefficients give estimated change in the dependent variable between the baseline category and the category named in the table (UT = untrained). Baseline category was sprint trained group.

5) Total Incubation Time

The final model for incubation time retained an effect of percent change in body mass of the mother (Table 1). The total incubation time for the offspring from the Sprint Trained (ST) and Untrained (UT) groups was significantly longer than that of the High Diet (HD) group (Figure 1). Incubation times of offspring from the Low Diet (LD) group did not differ significantly from the HD group.

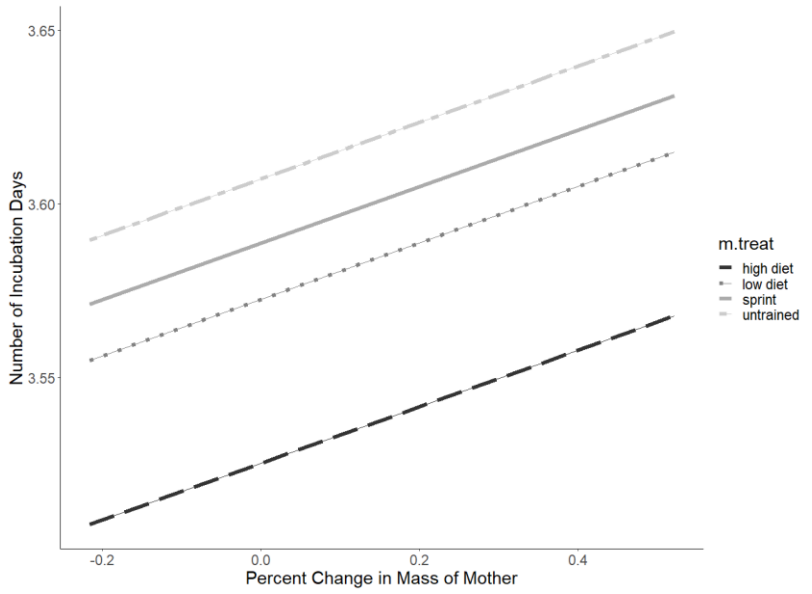

Figure 5. Estimated marginal means for total incubation time when accounting for percent change in body mass.

Number of Incubation Days	Model term	Coefficient	SE
	Intercept	3.53	0.017
	Treat (LD)	0.047	0.028
	Treat (ST)	0.063	0.024
	Treat (UT)	0.082	0.018
	%Δm.mass	0.081	0.075

Table 5: Best-fitting models describing the variation in incubation days with percent change in final body mass of the mom as a covariate. The reported coefficients give estimated change in the dependent variable between the baseline category and the categories named in the table (ST = sprint-trained, UT = untrained, LD = low diet). Baseline category was the high diet group.

Discussion

Understanding maternal effects and the factors that drive them is vital to learn about how mothers can best prepare their offspring for current environmental conditions. In this experiment, we compared egg and offspring characteristics from female green anoles that were either diet restricted or sprint trained. We tested specific predictions to determine how these different environmental pressures affected their offspring. Body size is particularly important to incorporate into our analyses because of the known allometric effects of maternal body mass on offspring size (Sakai and Harada, 2001; Kindsvater et al. 2012). Additionally, we refer to “energetic state” or “energetic environment” of the mother throughout as this terminology acknowledges that the changes implemented by the treatments affect the amount of available and allocable energy (Marks et al. 2021; Marks et al. 2022).

Our first prediction, that the LD and ST animals would lay significantly fewer eggs than the HD group was supported (Figure 1; Table 1). Percent change in mass of the mother was included in the final model and there was a negative correlation between egg number and percent change in mass. The quintessential life-history trade-off is that between survival and reproduction, which diet restriction is known to promote (Chapman and Partridge, 1996; Mair and Dillin, 2008; Moatt et al. 2016; Regan et al. 2020). Females will forgo current reproduction and extend lifespan to wait for an environment with a more suitable source of resources (Stearns, 1989; Thompson, 2012; Regan et al. 2020; Sultanova et al. 2021). Our results here are consistent with these earlier results, in that limiting available resources also resulted in decreased reproductive rate in female green anoles. We also found that the ST group laid

significantly more eggs than the LD group. It could be that the physiological changes wrought by sprint training are less energetically taxing than diet restriction is to green anoles; indeed Lailvaux et al. (2018) found that sprint training reduces resting metabolic rates in green anoles, which may result in more energetic resources being available for allocation to reproduction in sprint trained mothers. Comparison among other studies testing maternal effects of sprint training in reptiles is difficult though as this is severely understudied.

Our second prediction, that egg mass would be lower within the ST and UT group, was not supported when the maternal mass at oviposition was included in the model (Figure 2B; Table 2B). Within this model, there is a strong positive correlation between egg mass and mass of the mother at oviposition, and the HD group laid the lightest eggs compared to the other treatments. When looking at the model with %Δm.mass (Figure 2A; Table 2A), there is an interaction between body mass of the mother and treatment where the LD and ST lizards have a negative relationship between %Δm.mass and egg mass, while the HD and UT retain the positive relationship. This tells us that the treatment lizards in a negative energetic environment may have invested energy into laying larger eggs rather than more eggs. These results follow the principles of the bet-hedging model, where females will lay fewer eggs in order to invest more energy into individual offspring (Nussbaum, 1981; Seger and Brockman, 1987; Reznick and Yang, 1993, Mitchell et al., 2018). An example of this phenomenon is seen in brown anoles (*Anolis sagrei*). Mitchell et al. captured brown anoles at multiple time points throughout a breeding season and found that groups

caught later in the season laid fewer eggs but invested more resources into each egg to produce larger offspring (Mitchell et al. 2018).

The mass of the offspring at time of hatching was not affected by maternal treatment; consequently, our third prediction, that offspring from the LD and ST lizards would weigh less than those from the UT and HD moms was unsupported. Although year was controlled for as a random factor in our model there is a significant difference in hatchling mass between the diet experiment and the sprint experiment (Figure 3; Table 3), where the offspring from the diet experiment were significantly heavier at hatching. Because of the known allometric effect of maternal body size on offspring body size (Sakai and Harada, 2001; Kindsvater et al. 2012), we included percent change in mass of the mothers as a covariate to control for any differences in maternal body mass between the experiments. This metric was not significant here, and was therefore omitted from the final model.

A potential mechanism underlying the differences in offspring phenotype between the treatments could be the insulin/insulin-like signaling network (IIS). This is a highly conserved pathway and its main roles are to facilitate cell growth and division and aspects related to reproduction and metabolism (Duan et al. 2010; Schwartz and Bronikowski, 2016; Regan et al. 2020). Altering maternal environment manipulates hormones within the insulin/insulin-like signaling (IIS) network, specifically hepatic expression of insulin-like growth factor 1 (IGF1) and insulin-like growth factor 2 (IGF2) (Marks et al. 2021; Marks et al. 2022; Regan et al. 2020). The offspring tested within this experiment are derived from two larger, prior experiments where we measured *IGF* expression and showed that diet restriction affects *IGF1* and *IGF2* expression (Marks et

al. 2021), and that sprint training also affects *IGF1* and *IGF2* but in a different manner than diet restriction (Marks et al. 2022). It could be that the difference in hatchling mass, and ultimately incubation period, is due to the increase in experimenter handling time experienced by the mothers in the sprint training experiment. This handling time may have affected maternal IGF expression within these mothers which could impact IGF, and ultimately phenotype, of the offspring. Although this experiment was not designed to explicitly test the link between maternal IGF expression and offspring phenotype, it would be a logical next step to test this relationship.

We made the null prediction (P4) that SVL would not differ between the ST and UT lizards. Offspring from the ST lizards had significantly longer SVLs than those from the UT moms (Figure 5; Table 5), yet the average mass between treatments was not different (Figure 3; Table 3), suggesting that ST offspring potentially allocated energy to bone growth. Differentiation of mesenchymal stem cells occurs during development, producing cells which facilitate bone, muscle, and fat growth (Lanham et al. 2010; Du et al. 2011; Sanger et al. 2012). Variation in maternal environmental conditions in pigs and cattle, such as low nutrient availability, lead to differences in mesenchymal cell differentiation in their offspring (Du et al. 2010). Maternal sprint training may also induce differences in mesenchymal cell differentiation and these differences may manifest themselves by supporting skeletal growth in offspring. Future work should test this theory, though, to better understand the scope of maternal effects in reptiles.

The significant difference between experiments seen in hatchling mass was also seen when testing incubation period. Our fifth prediction that incubation period within the LD and ST group would be higher than the controls, (Figure 5; Table 5), was not

supported. Outside of questions focusing on incubation temperature, egg phenotype is rarely studied within the context of maternal effects in vertebrates. Development time is commonly measured in entomological research because of its clear effects on offspring phenotype. For example, development time can be affected by maternal diet in large milkweed bugs (*Oncopeltus fasciatus)*. Offspring reared on different diets than their mothers had longer developmental times than siblings reared on the same host plant as their mother (Newcombe et al. 2015). Although our results showed that there was no effect of sprint training or diet restriction on offspring incubation time when compared to their respective control situations (Figure 5; Table 5), we did see a difference between the experiments. Year was included as a random effect in our final model so it is possible this difference is due, again, to significantly longer handling time within the sprint training experiment. These effects of the maternal environment on lizard egg phenotypes are seldom explored in reptiles and deserving of more attention.

Maternal effects are key for animals to best prepare their offspring for the environment in which they are being born (Mousseau, 1998; Wolf and Wade, 2009). Our hypothesis that maternal dietary restriction and sprint training would have different consequences for the offspring phenotype in green anoles was supported. Our results show that offspring phenotype changes depending on the energetic environment of the mother, and the manner in which the energetic environment is imposed. These results highlight an important point that ecologically relevant tasks such as locomotion deserve more attention within the context of maternal effects as they clearly impact offspring phenotype. They significantly enhance our understanding of maternal effects within

reptiles and this work is an important piece to understanding maternal effects as a whole.

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Conclusion

My dissertation research tested how the insulin/insulin-like signaling network is affected by varying environments. We did this by measuring hepatic expression of the two primary hormones, insulin-like growth factor one (IGF1) and insulin-like growth factor two (IGF2). We learned that *IGF1* and *IGF2* are both implicated in the response to diet restriction and sprint training, but in different ways. This significantly improved our understanding of the underlying mechanisms facilitating trade-offs involved in response to a low diet or sprint training. We also learned that maternal environment, and the way in which maternal environment is affected, influences offspring phenotype. This experiment led to a greater understanding of maternal effects, especially in response to understudied ecologically relevant tasks such as sprinting. In total, these experiments provide us with a better picture of the underlying mechanisms promoting and facilitating trade-offs.

Appendix

Supplemental Information

Across all animals, regardless of diet, IGF2 was expressed at 100X higher level than IGF1 (Supplemental Figure 3). The High and Low diet treatments created a continuum of energetic states where smaller animals on the High diet were in positive energetic balance and increased body mass whereas bigger animals on the high diet either did not change or slightly lost weight. Smaller animals on the Low diet either maintained their weight or had minimal weight loss, and bigger animals on the Low diet were in negative energy balance and lost weight (Supplemental Figure 5).

Absolute expression statistical analyses

We used the *nlme* package (Pinheiro and Bates 2013) to fit general linear mixed (glm) models, and Box-Cox transformed dependent variables as required to meet model assumptions of normality. In cases where mixed-models still exhibited heteroscedasticity following transformations, we dealt with this by fitting an exponential variance structure (Zuur et al. 2009). We fit glm models to SQ for each gene and set treatment as a fixed factor and individual as a random factor to account for repeated qPCR replicate measures.

To determine the mean difference between genes, within treatments, we ran a linear model to SQ with target (*IGF1* or *IGF2*) as the predictor and treatment was set as a fixed effect. We used package *car* to run a Levine's test on all SQ of genes measured with treatment as a fixed factor.

Absolute expression between IGF1 and IGF2

Body mass and change in body mass were significant factors affecting gene expression in all cases (see fig. 1 and 2 along with table 2 and 3). However, to facilitate comparison with earlier studies, we note here that the absolute relative expression levels of *IGF1* were significantly lower than that of *IGF2* (N=41, $F_{1,220}$=129.7, p<2e-16) (Supplemental fig. 3; table 3). This difference between *IGF1* and *IGF2* is consistent with brown anoles (Cox et al. 2017; Beatty and Schwartz, 2020), and other reptiles based on transcriptomic data (McGaugh et al. 2015). Absolute expression for each gene was not statistically different between treatments (fig. 3 and 4; table 4). The variation between treatments was not significant for *IGF1* (N=41, $F_{1,109}$=2.6322, p> 0.10); *IGF2* (N=41, $F_{1.109}$=1.4577, p>0.22); or *EEF2* (N=41, $F_{1,109}$=2.9522, p>0.088. Variation of *GAPDH* was different between treatments, though (N=41, $F_{1,109}$=12.161, p>0.00071).

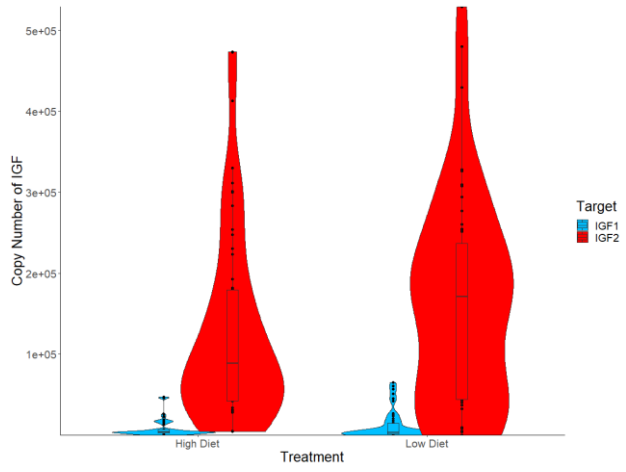

Figure 3. Shown in this figure are violin plots *of IGF1* and *IGF2* expression between HD and LD groups when analyzing traditionally. The visible variation in *IGF2* is due to body size, but for purposes of comparison we measured variation and mean difference between treatments.

(a) (IGF1)	Model term	Coefficient	SE
	Intercept	1.182	0.00697
	Treat (LD)	-0.00666	0.0102
(b) (IGF2)			
	Intercept	1.253	0.00886
	Treat (LD)	-0.00747	0.0130
(c) (GAPDH)			
	Intercept	1.272	0.00830
	Treat (LD)	-0.00934	0.0122
(d) (EEF2)			
	Intercept	1.141	0.00588
	Treat (LD)	0.000476	0.00863

Table 4: Best-fitting models describing the variation in copy number of (a) (insulin-like growth factor 1), (b) (insulin-like growth factor 2), (c) (glyceraldehyde 3-phosphate dehydrogenase), and (d) (eukaryotic elongation factor 2) of absolute expression. Baseline category was HD group.

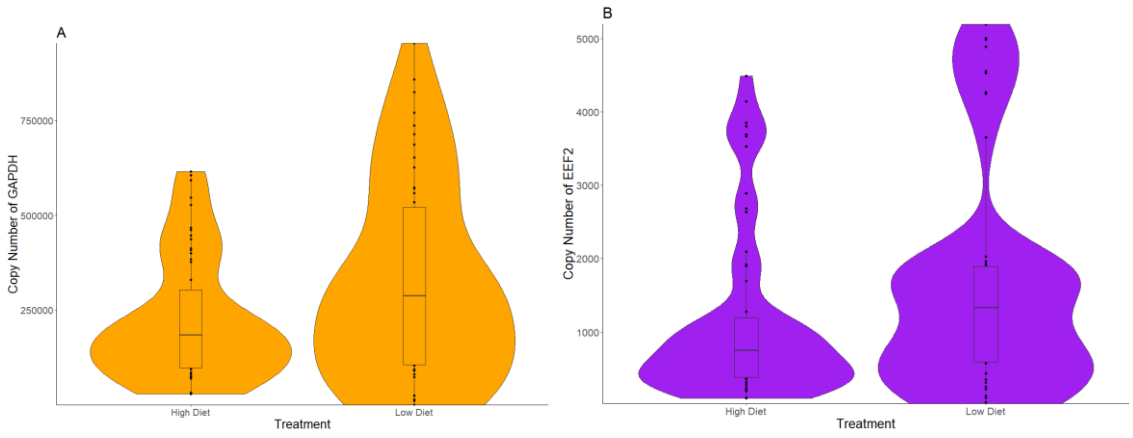

Figure 4. This figure shows a traditional analysis of the absolute data. (A) Variability of *GAPDH* expression was significantly ($p<0.05$) different between treatments. (B) *EEF2* was not different between groups but expression levels were to low to use to normalize data. The variation seen in both genes are due to differences in body size of the lizards. The mixed models (in figures 3-4) better explain the variation.

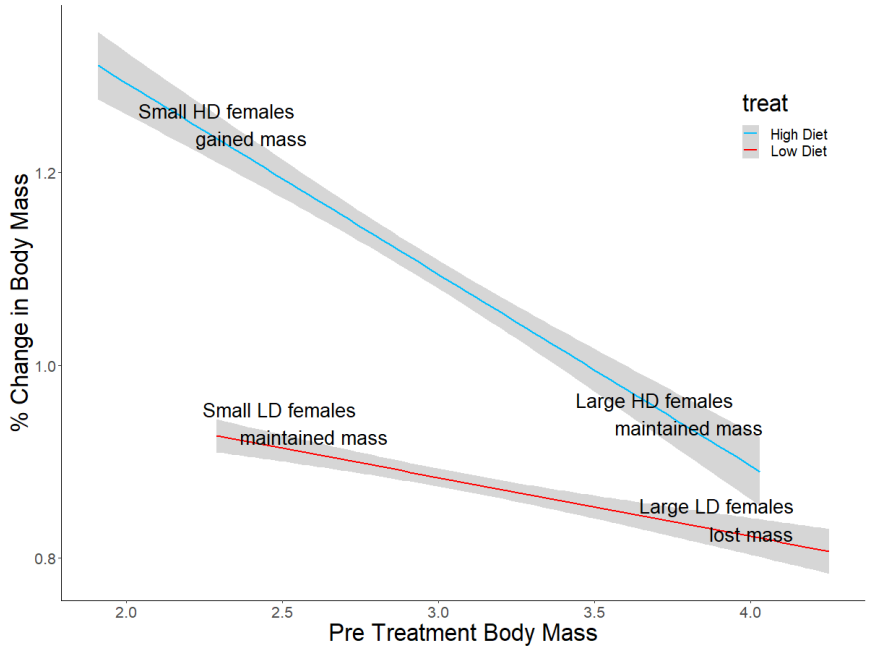

Figure 5. Linear model showing with pre-treatment body mass on x-axis and percent change in mass on y-axis. The blue line denotes low diet lizards and the red line shows high diet lizards.

(a) (Pre-Treatment Mass)	Model term	t value	p - value
	Intercept	92.923	<2e-16
	Treat (LD)	2.701	0.00719
(b) (Post-Treatment Mass)			
	Intercept	122.75	<2e-16
	Treat (LD)	-13.58	<2e-16

Table 5. Linear model describing the difference in average mass of the two experimental groups (a) before diet restriction was implemented and (b) after 8 weeks of diet restriction. The groups did not differ before diet restriction was implemented. At the end of the 8-week experiment, the average mass of the diet restricted group was significantly less than that of the HD group.

(a) (IGF1)	Model term	Coefficient	SE
	Intercept	0.92	0.02
	Treat (LD)	-0.04	0.03
	Final Body Mass	-0.001	0.007
	Treat (LD):Final Body Mass	0.02	0.01
(b) (IGF2)			
	Intercept	-3.76	123.33
	Treat (LD)	250.69	165.12
	Final Body Mass	46.60	37.80
	Treat (LD):Final Body Mass	-84.56	55.16
(c) (GAPDH)			
	Intercept	38.69	191.77
	Treat (LD)	563.08	256.81
	Final Body Mass	64.15	58.77
	Treat (LD):Final Body Mass	-194.74	85.78
(d) (EEF2)			
	Intercept	1.91	2.16
	Treat (LD)	6.37	2.89
	Final Body Mass	0.91	0.66
	Treat (LD):Final Body Mass	-2.19	0.97

Table 6 Best-fitting models describing the variation in copy number of (a) (*IGF1*), (b) (*IGF2*), (c) (*GAPDH*), and (d) (*EEF2*) with change in body mass as a covariate when the four HD individuals who lost mass are removed from the dataset. The reported coefficients give estimated change in the dependent variable between the baseline category and the category named in the table. Baseline category was HD group.

Vita

The author was born and raised in Cleveland, Ohio. She completed her undergraduate studies at Indiana University in 2016. In the Fall of 2018, she began her doctorate at the University of New Orleans. She completes her PhD career under the mentorship of Dr. Simon Lailvaux.