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

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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 quantity of food or high quantity of food) 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 is 100X higher than IGF1 regardless of diet treatment, and *IGF1* and *IGF2* expression interact with post-treatment body mass and treatment, as do 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 to individuals in the High Diet group of similar Δ mass. These results illustrate that expression of *IGF1* and *IGF2,* and housekeeping genes are 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 Swansen, 2012; Smykal and Raikhal, 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., 2019). 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 due to 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). On the other hand, 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) > 40mm) *A. carolinensis* females (N=100) from urban populations in Orleans parish in Louisiana in June of 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 day of capture. The mass range of the lizards was 1.91g to 4.25g and their SVL range was 44.68mm to 56.03mm. 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 36.6cm x 21.6cm x 24.9cm plastic terrariums with a half inch layer of mulch as substrate along with a wooden rod to perch, and kept on a light:dark cycle of 13:11 hours. 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 one week to acclimate before beginning treatment.

Diet Treatments

To alter the energetic environments, the experimental animals were randomly assigned to Low Diet (LD) and High Diet (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 (Supplemental Table 5). All lizards were given ½" crickets (*Acheta domesticus*). The LD group was fed one cricket coated in ZooMed ReptiCalcium powder, three 3 times weekly and the HD group females were fed an *ad libitum* diet of three crickets, three 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 continuum of about size 2g – 4g on set High and Low Diets is expected to create a

continuum of animals in energetic state where small animals on the HD would increase in mass (positive energy balance) whereas bigger animals on the HD either did not change or slightly lost weight. Small animals on the LD would either maintain their weight or have minimal weight loss, and bigger animals on the LD would be in negative energy balance and lose weight (Supplemental Figure 5). In this way, we can test for the effect of the categorical energetic environment (treatment groups), 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 eight weeks and females that lost >33% from initial body weight 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 analyses. 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 eight 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.

Insulin-Like Growth Factor 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 the Illustra RNAspin Mini kit according to manufacturer protocol (GE, Cat. No: 25-0500-70). Briefly, samples were lysed in RNAspin Lysis Buffer (GE, Cat. No. 25-0500-70) with 5mm stainless steel beads (Qiagen Cat. No. 69989) using the Tissuelyser II (Qiagen) at 30Hz for a period of 3 minutes. Proteinase K (Qiagen, Cat. No. 19131) was added post-homogenization to degrade proteins during cell lysis. During RNA isolation, a DNAse digestion was included according to manufacturer protocol. Total RNA was quantified on an Agilent 2200 TapeStation. All samples were standardized by making a 100 ng/µL dilution. Following manufacturer protocols, total RNA (100 ng) was used in cDNA synthesis reactions using qScript XLT cDNA SuperMix (QuantaBio, Cat. No. 95161-500). cDNA for all samples were made on the same 96-well-plate.

Primers were designed for four target genes: *IGF1*, *IGF2*, *GAPDH*, and *EEF2* (Table 1). *IGF1*, *IGF2*, *GAPDH*, and *EEF2* primer and probe pairs 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 Supp. Info). Amplicon regions of the four targets gene regions with a 10bp 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 40ng/µL and 1µg of plasmid was digested using BgIII (NEB, R0144) to a final concentration of 20ng/µL. Total copy number was calculated from concentration and plasmid length

(Staroscik, 2004). The plasmid was diluted to a concentration of 1×10^8 copies per µL and used to produce a serial dilution ranging from 1×10^7 to 1×10^2 copies per µL. In order to standardize the total amount of nucleic acid in each standard, Lambda DNA (NEB, Cat. No. N3011S) was prepared at a concentration of 310ng/µL and used to balance each standard solution.

Real time qPCR was conducted as described in Beatty and Schwartz (2020) to quantify *IGF1*, *IGF2*, *GAPDH* and *EEF2* in a multiplex qPCR reaction containing 1X PrimeTime Gene Expression Mastermix (Integrated DNA Technologies DNA, Cat. No. 1055772), 0.3µM of each primer, 0.2µM of each probe, 3µl of cDNA at a 1:100 dilution in a final volume of 20µl volume. Samples were randomized to 2 plates and ~~run~~ ran 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

CFX Maestro Software (BioRad) was used to calculate PCR efficiency, CQ (quantitation cycle) values, standard deviations, and absolute copy number of each gene. PCR efficiency for *IGF1* was 101.8% (r^2= 0.992); *IGF2* was 106.4% (r^2= 0.987); *GAPDH* was 102.8% (r^2=0.988); and *EEF2* was 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 normalizing samples before cDNA synthesis.

All analyses were run 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. 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

The energetic environments, as defined by High and Low diet treatments, are 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 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 analysed 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 (*Δ* mass, 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 euthanasia 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 (Pinheiro and Bates, 2013) 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 do 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 refit 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 supplemental information; however, in the discussed results we analyse mass-dependent relationships throughout.

1) Final mass analysis

We fit 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 fit exponential variance structure to models for *IGF1*, *IGF2*, and *GAPDH* to deal with heteroscedasticity.

2) Change in mass analysis

To understand how energetic state affects IGF1 and IGF2 gene expression, we fit general linear models to absolute copy number for each gene measured with treatment, Δ mass, 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

1) 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 2A), 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 2B) 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 is also retained in the models for housekeeping genes (Fig. 1C and 1D; Table 2C and 2D) (*GAPDH* and *EEF2*). Within the housekeeping genes, the LD group had a negative correlation with expression levels and final body mass while the HD group had positive direct correlations between expression levels and fnal body mass.

2) Change in mass analysis

The change in mass is indicative of the energetic state of the animals at the time of gene expression. The final model for *IGF1* retained only an effect of *Δ* mass, indicating no treatment effects on gene expression (Table 3A). We included an interaction between treatment and *Δ* mass 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 Δ mass, indicating that LD lizards expressed *IGF2*, *GAPDH* and *EEF2* at higher levels compared to those individuals in the HD groups of similar Δ mass (Table 3). Furthermore, the positive relationship between Δ mass and gene expression observed for *IGF1* was also seen in *IGF2*, *GAPDH*, and *EEF2*.

Although HD lizards were fed in such a way as to increase energetic environment, four individuals lost weight over the course of the experiment. Because we do not know why those lizards lost mass, we included them in our main analyses here and interpret the results of modelling all of the data, but we also analysed the data with those four lizards removed (see supplemental Table 6). When those lizards

are removed, change in mass is no longer retained as a significant factor in the final minimum adequate models for *IGF2*, *GAPDH* and *EEF2*.

Discussion

The responsiveness of IGF1 to 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 the 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 2A). Larger animals in resource-limiting environments (Low Diet Treatment) may express *IGF1* to a lesser extent than smaller animals, in that same environment, due to 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 who are maintaining mass, when resources are scarce. Our data also show that lizards who gained mass over the course of the experiment, regardless of treatment, had higher expression of *IGF1* relative to those animals that maintained or lost weight (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 2B). Further, when change in body mass over the experiment was accounted for, we found that animals in a low energetic environment (Low Diet) exhibited higher expression of *IGF2* relative to ~~HD~~ animals in a high energetic environment (High Diet) (Fig. 2B). Our data therefore implicate *IGF2* being 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 to 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 and 1D; Table 2C and 2D) and when accounting for change in body mass (Fig. 2C and 2D; Table 3C and 3D). 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's possible that the females receiving less 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 both within 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 et al., 2002; Kaul et al., 2011). The increase in *EEF2* expression seen in the LD animals (Fig. 1D; Table 2D and Fig. 2D; Table 3D) 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 change in the 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 and species outside of a biomedical context in these organisms, and thus for reptiles specifically.

A final constraint to using oviparous organisms, such as green anoles, as a model organism is that green anoles lay eggs every 7 to 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 data set, change in mass was no longer included as a significant factor (see supplemental table 6). Although our results reveal 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, 2016); 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 ~~growth,~~ nutrient signalling of the energetic environment to the regulate, cell proliferation and differentiation in a nearly all animal species. In this paper, we demonstrated that both *IGF1* and *IGF2* gene expression are 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 of 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.

Acknowledgements

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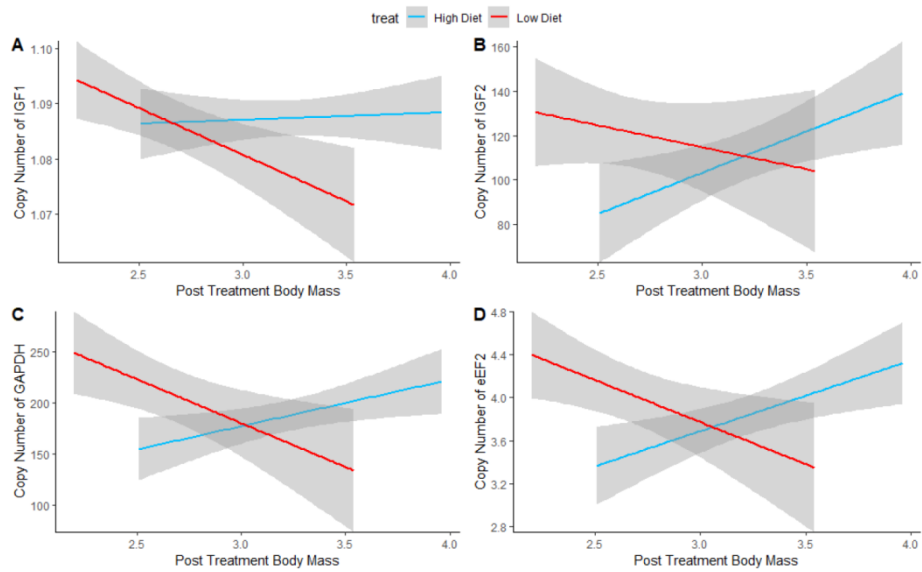
Figures

Figure 1. Partial residuals illustrating expression of (A) *IGF1*; (B) *IGF2*; (C) *GAPDH*; and (D) *EEF2* in HD (n=22) and LD (n=19) individuals after accounting for effects of 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 and it still fit well with our models.
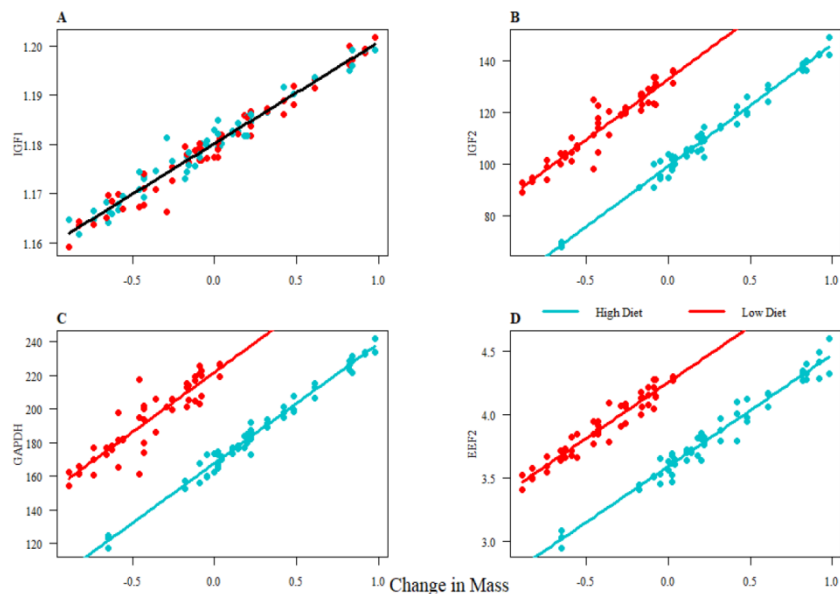

Figure 2. Estimated marginal means for gene expression when accounting for change in body mass. Treatment was included in the models for (B) *IGF2*; (C) *GAPDH*; and (D) *EEF2* when conditioned with change in body mass. Treatment was not included in the final model for (A) *IGF1*. (For High Diet, n=22 and for Low Diet, n=19.)

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

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 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.

(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 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.

(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

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).
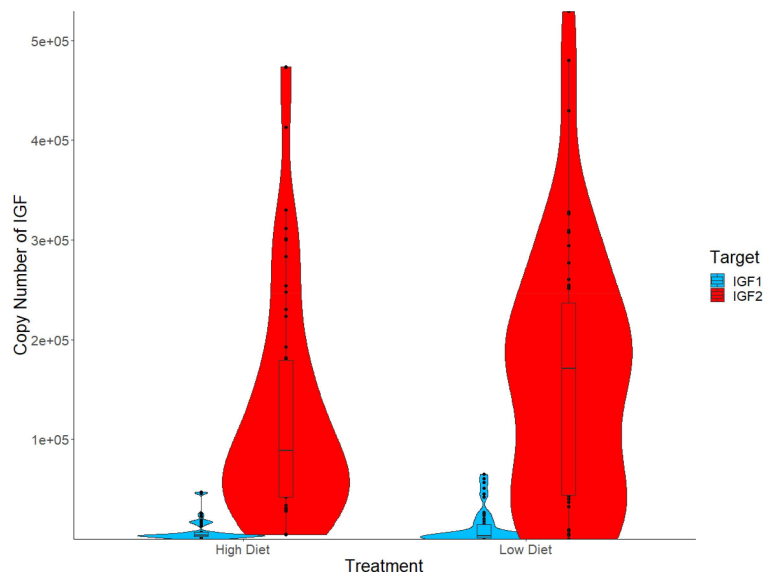
Fig. S1. Shown in this figure are violin plots of IGF1 and IGF2 expression between HD and LD groups when analysing traditionally. The visible variation in IGF2 is due to final body size, but for purposes of comparison we plot the raw data to show the variation and mean difference between the genes and treatments
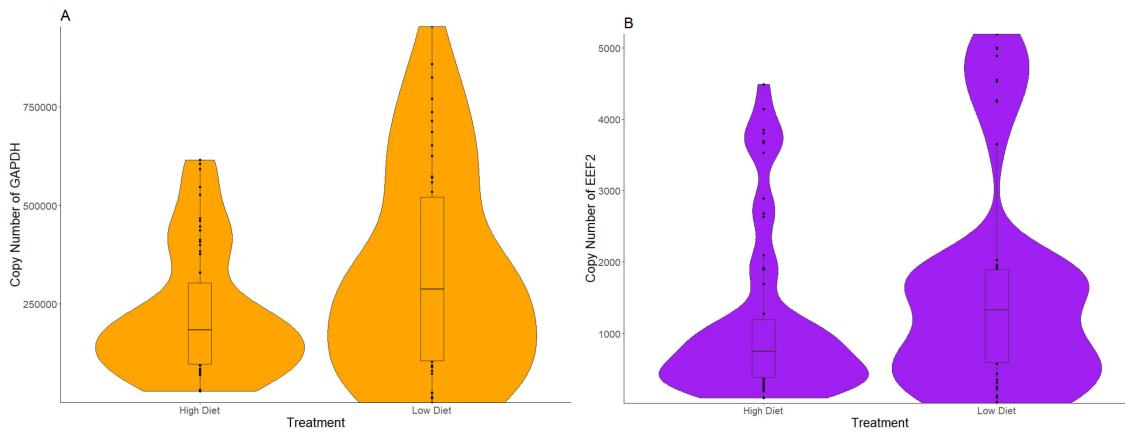
Fig. S2. This figure shows a traditional analysis of the absolute data. (A) Variability of GAPDH expression was significantly (p<0.05) different between treatments. (B) EEF2 was not different between groups but expression levels were to low to use to normalize data. The variation seen in both genes are due to differences in body size of the lizards. The mixed models (in figures 3-4) better explain the variation.
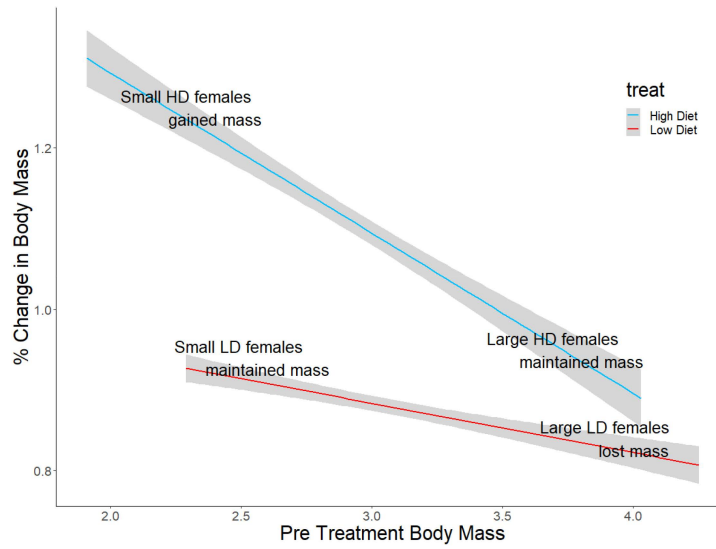
Fig. S3. Linear model showing with pre-treatment body mass on x-axis and percent change in mass on y-axis. The blue line denotes low diet lizards and the red line shows high diet lizards.

Table S1. 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.

(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 S2. 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 differed before diet treatment was implemented with the LD group being greater than HD group. At the end of the 8-week experiment, the average mass of the LD group was significantly less than that of the HD group

	Model term	t value	
		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

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Table S3: 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 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.

(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