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Research paper

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

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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.

1. Introduction

Each day, animals are required to conduct a variety of dynamic, ecologically relevant tasks that can directly affect survival and reproductive success (Bennett and Huey, 1990; Irschick and 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 and 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 and Carrier, 2003; Lailvaux and Husak, 2014; Husak and 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 and Van Noordwijk, 1992; Roff and Fairbairn, 2007). 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, Lailvaux et al., 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, for instance via exercise training (Husak et al., 2015, 2016; Careau and 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

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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 and Lailvaux, 2017) and likely also elicit activity in disparate metabolic and biochemical pathways (Chung et al., 2021; Husak and Lailvaux, 2022). Despite the attention paid to the physiological and genetic factors underlying locomotor performance (Sorci et al., 1995; Bouchard, 2012; Sharman and 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 and Husak, 2014, Husak and 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 often expressed at a higher level than *IGF1* (Beatty et al. 2022). This and other studies have shown that reptiles express both *IGF1* and *IGF2* post-natally (McGaugh et al., 2015; Reding et al., 2016; Schwartz and Bronikowski, 2016; Beatty and Schwartz, 2020). Furthermore, 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, though frequently used as housekeeping genes, are also affected by resource limitation (Marks et al., 2021), and 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 (Sarbassov 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 and 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.

2. Materials and methods

2.1. 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) $>$ 40 mm) *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 h. Lizards were individually held in 36.6 cm × 21.6 cm × 24.9 cm 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 ~1.25 cm 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., 2015). 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.

2.2. 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 and Lailvaux, 2019; Wang and 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 25 cm. 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 and Lailvaux, 2019; Wang and 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 and Pickering, 2008) and similar training experiments (Husak et al., 2015; Lailvaux et al., 2020; Wang and 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).

2.3. Post-treatment

The green anoles were rapidly euthanized via decapitation 24 h 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 2.0 mL screw top microcentrifuge tubes that contained ~ 250 μl of RNAlater. These were then stored at 4 °C for 4 weeks prior to gene expression analysis.

2.4. 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 5 mm stainless steel beads (Qiagen Cat. No. 69989) using the Tissuelyser II (Qiagen) at 30 Hz for a period of 3 min. 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), 0.3 μM of each primer, 0.2 μM of each probe, 3 μl of 1:100 dilution of cDNA (or standard) in a final reaction volume of 20 μl 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 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.

2.5. 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 (7th) 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 <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.

2.6. 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 and 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 et al., 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, 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).

3. Results

3.1. Final body mass analysis

The final model for *IGF1* (Fig. 1A and B; Table 1A) and *IGF2* (Fig. 2A and B; 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 and Schwartz, 2020; Marks et al., 2021).

3.2. 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 B; 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).

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.

(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

4. 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 B; Table 1A), albeit not in the

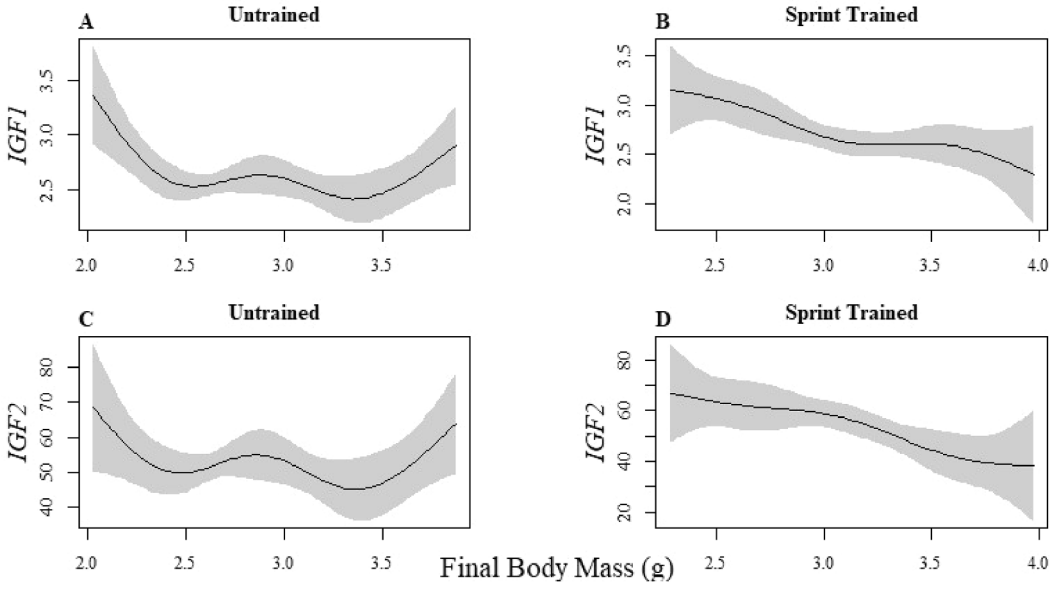

Fig. 1. Absolute values of gene expression conditioned with final body mass (in grams) showing expression of (A) *IGF1* in the untrained lizards; (B) *IGF1* in the sprint-trained lizards; (C) *IGF2* in the untrained lizards; (D) *IGF2* in the sprint-trained lizards. Nonlinear interactions between treatment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

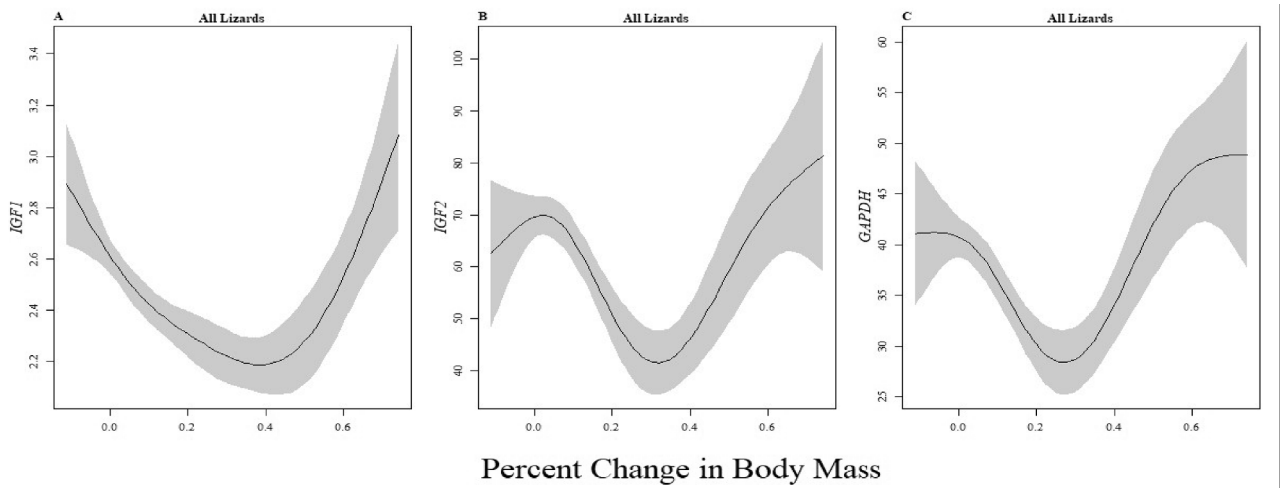

Fig. 2. Absolute values of gene expression conditioned with % change in body mass of (A) *IGF1* in the untrained and sprint-trained lizards; (B) *IGF2* in the untrained and sprint-trained lizards; (C) *GAPDH* in the untrained and sprint-trained lizards. There was no effect of treatment on *IGF1*, *IGF2*, 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.

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.

(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

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 compared to 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 expression 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 as these lizards were wild caught 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 D; 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

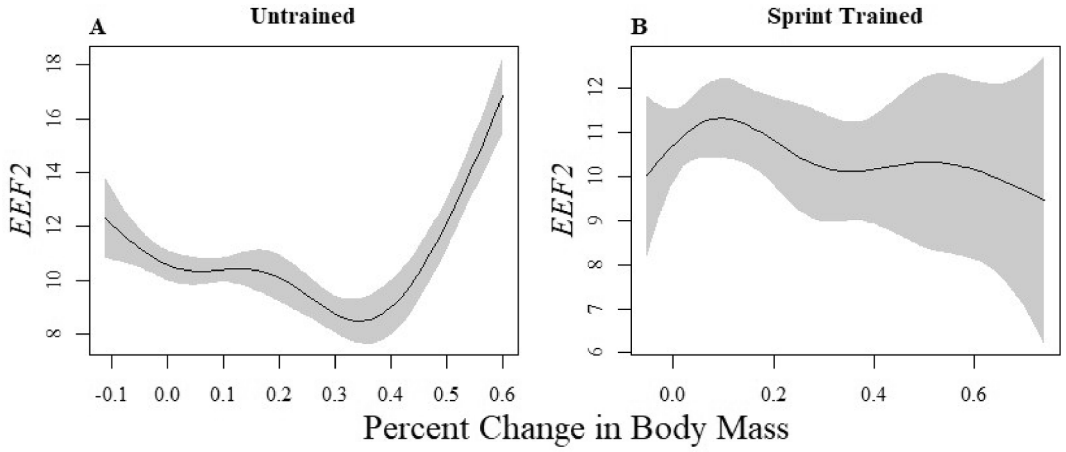

Fig. 3. Absolute values of *EEF2* gene expression conditioned with % change in body mass for (A) untrained lizards and (B) sprint-trained lizards. There is a nonlinear interaction between treatment and percent change in body mass for *EEF2*.

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 see Duncan et al. 2015 for such an assay in Sceloporus lizards), but validating the relationship between gene expression and circulating hormone levels at the whole-organism level in these animals 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 B; 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 and 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.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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