Nutritional Modulation of IGF-1 in Relation to Growth and Body Condition in *Sceloporus*
Lizards

Christine A. Duncan ^a

Amanda E. Jetzt ^b

Wendie S. Cohick ^{a,b}

Henry B. John-Alder ^{a,c,*}

^a Graduate Program in Endocrinology and Animal Biosciences, Rutgers University, 84 Lipman Drive, New Brunswick, NJ 08901, USA, Christine.a.duncan@gmail.com

^b Department of Animal Sciences, Rutgers University, 84 Lipman Drive, New Brunswick, NJ, 08901, USA, cohick@aesop.rutgers.edu; jetzt@aesop.rutgers.edu

^c Department of Ecology, Evolution, and Natural Resources, Rutgers University, 14 College Farm Road, New Brunswick, NJ 08901, USA

* Corresponding author: H. John-Alder

E-mail: henry@aesop.rutgers.edu

Fax: 732-932-8746

Phone: 848-932-1064

Abstract

Nutrition and energy balance are important regulators of growth and the growth hormone/insulin-like growth factor (GH/IGF) axis. However, our understanding of these functions does not extend uniformly to all classes of vertebrates and is mainly limited to controlled laboratory conditions. Lizards can be useful models to improve our understanding of the nutritional regulation of the GH/IGF-1 axis because many species are relatively easy to observe and manipulate both in the laboratory and in the field. In the present study, the effects of variation in food intake on growth, body condition, and hepatic IGF-1 mRNA levels were measured in (1) juveniles of *S. jarrovi* maintained on a full or 1/3 ration and (2) hatchlings of *S. undulatus* subjected to full or zero ration with or without re-feeding. These parameters plus plasma IGF-1 were measured in a third experiment using adults of *S. undulatus* subjected to full or zero ration with or without re-feeding. In all experiments, plasma corticosterone was measured as an anticipated indicator of nutritional stress. In *S. jarrovi*, growth and body condition were reduced but lizards remained in positive energy balance on 1/3 ration, and hepatic IGF-1 mRNA and plasma corticosterone were not affected in comparison to full ration. In *S. undulatus*, growth, body condition, hepatic IGF-1 mRNA, and plasma IGF-1 were all reduced by zero ration and restored by refeeding. Plasma corticosterone was increased in response to zero ration and restored by full ration in hatchlings but not adults of *S. undulatus*. These data indicate that lizards conform to the broader vertebrate model in which severe food deprivation and negative energy balance is required to attenuate systemic IGF-1 expression. However, when animals remain in positive energy balance, reduced food intake does not appear to affect systemic IGF-1. Consistent with other studies on lizards, the corticosterone response to reduced food intake is an unreliable indicator of nutritional stress. Further studies on ecologically

relevant variation in food intake are required to establish the importance of nutrition as an environmental regulator of the GH/IGF axis. Within the range of positive energy balance, the potential involvement of molecular signals in growth regulation requires further investigation.

Keywords: Insulin-like growth factor-1, cDNA cloning, Nutrition, Food intake, Growth, Lizard

1. Introduction

Growth rate is expected to be under strong directional selection because large body size is adaptive in most organisms (Blanckenhorn, 2000; Kingsolver and Pfennig, 2004). For a variety of reasons, however, animals do not always grow at their maximal rate. In many species, for example, selection on body size is asymmetric between males and females (Bonnet et al., 2000), causing adults of one sex to grow faster than the other (Fairbairn et al., 2007). Furthermore, throughout development, especially in the wild, animals may encounter periods of insufficient food to support positive growth, in which case growth is traded-off against functions more critical for survival (Le Galliard et al., 2005; Mugabo et al., 2010). In these conditions, food availability is a prime environmental regulator of growth, but whether the underlying mechanism is simply a limitation of nutrients and energy or a more complicated homeorhetic process (Bauman et al., 1982) is not usually known. The lizard genus *Sceloporus* provides a well-suited system for comparative studies on growth regulation because many species are both amenable to laboratory experimentation and accessible in the wild, and the phylogeny has been characterized

using morphological and molecular data (Leache, 2010; Wiens et al., 2010). The present study reports properties of the basic growth regulatory system in lizards and thus represents a starting point for further studies of growth regulation in this group.

The central growth-regulatory system in vertebrates is the growth hormone/insulin-like growth factor-1 (GH/IGF-1) axis, which integrates much of the genetic, hormonal, and nutritional regulation of growth (Fox et al., 2006). Among species that have been studied, the structure and function of this endocrine growth axis have been well-conserved (Duan, 1997). Briefly, under anabolic conditions, plasma GH stimulates transcription of IGF-1 in the liver, leading to an increase in plasma IGF-1, which acts at target tissues to mediate many of the growth-promoting effects of GH. In contrast, complete food deprivation induces a dramatic decline in circulating IGF-1 levels in all species studied to date. This results from a decrease in hepatic GH receptors (mammals: (Thissen et al., 1994) birds: (Scanlon, 1997) fishes: (Duan, 1998), leading to an uncoupling of the GH/IGF-1 axis (Breier, 1999; Pierce et al., 2001; Rhoads et al., 2007). This allows GH to exert direct effects on lipid mobilization in support of survival while preventing IGF-1 from diverting nutrients to non-essential processes such as growth.

Nutrition has been described as having a “profound effect on the GH/IGF axis” (Duan, 1998; Moriyama et al., 2000), leading some investigators to conclude that “the principal environmental regulator of the GH/IGF axis is nutritional status” (Pedroso et al., 2006; Pierce et al., 2005). However, the degree of nutritional deprivation that is required to alter IGF-1 levels and whether there is a direct correlation between IGF-1 levels and growth rate varies between studies (Beckman, 2011). Thus, extrapolation of this model of growth regulation to undescribed species requires caution, especially in field active animals (e.g., (Sparkman et al., 2009), and

characterization of growth regulation is a necessary first step in studies in groups of animal that have not previously been studied.

The IGF system and its regulation have not been well-characterized in lizards and other reptiles to date (Crain et al., 1995; Sparkman et al., 2010; Sparkman et al., 2012; Sparkman et al., 2009). Therefore, the present study was designed as an initial step to develop and validate methodologies to assess hepatic IGF-1 expression and plasma IGF-1 in lizard species. The objective of the current study was to characterize effects of food intake on IGF-1 expression and circulating hormone. We present the results of three experiments performed in the laboratory involving (1) yearling *S. jarrovi* (Yarrow's Spiny Lizard) on full or 1/3 ration; (2) hatchling *S. undulatus* (Eastern Fence Lizard) subjected to full or zero ration with and without re-feeding; and (3) adult *S. undulatus* subjected to the same conditions as the hatchlings.

2. Methods

2.1 Animal collection and care

Hatchling male and female *S. undulatus* were collected in late summer or early fall at 1 to 2 months of age in the New Jersey Pinelands in the vicinity of Rutgers University Pinelands Research Station in New Lisbon, Burlington County, NJ (41°N, 74°35'W) under permit from the NJ Department of Environmental Protection, Division of Fish and Wildlife (SC28068).

Reproductively mature *S. undulatus* males (at least two years old) were collected in the spring from the same field site as the hatchlings (SC 2909C). Following collection, all lizards were transported to Rutgers University and housed individually (permits SH 25086, SH 28094, SH 29097) in plastic cages (59.1 x 43.2 x 45.7 cm) that contained sand or Marcal® KaoBed™ granular bedding and two bricks, which were stacked to create shelter and a basking site. Cages

were separated by opaque dividers to prevent social interactions. Each cage was illuminated by an incandescent spotlight (Philips 65 W BR-40SP, Royal Philips Electronics, Netherlands) suspended above the basking site to provide a 10 h basking period. Cages were arranged under fluorescent bulbs (General Electric Chroma 50, General Electric Company, Fairfield, CT) for full-spectrum radiation on a 12:12 h light:dark photoperiod. Water was provided *ad libitum* in a shallow dish lined with aquarium gravel. All experiments were approved by the Rutgers University Animal Care and Facilities Committee (protocol no. 01-019).

Livers and blood from *S. jarrovi* collected in a previously published study (Cox et al., 2008) were also analyzed for IGF-1 expression and corticosterone levels, respectively, in the present work. Briefly, male and female yearling lizards (2 to 3 months of age) had been captured in the fall from the Chiricahua Mountains, Coronado National Forest, AZ (31°54'–31°55'N, 109°16'W) under permit from the Arizona Game and Fish Department (SP 553889).

2.2 Experimental design

Prior to dietary manipulation of *S. undulatus*, snout-vent length (SVL) was measured to the nearest 1 mm and body mass was measured to the nearest 0.02 g. These data were used to assign lizards to size-matched treatment groups (Table 1). During experiments, SVL and body mass were measured to monitor growth rate, which was calculated by dividing an individual's change in SVL by the number of days of experimental treatment. Treatment was not expected to affect growth rates of adult *S. undulatus* because, unlike rapidly growing hatchling *S. undulatus*, adults were on the asymptotic part of the predicted growth curve for this species (Haenel and John-Alder, 2002; John-Alder et al., 2007). For *S. undulatus*, the number of uneaten crickets was counted at the conclusion of the experimental periods; in the previous study conducted with *S.*

jarrovii, the number of uneaten crickets was measured weekly (Cox et al., 2008). Feeding rate for each experiment was reported as crickets/day. It should be noted that *S. jarrovii* and adult *S. undulatus* were fed larger crickets than hatchling *S. undulatus*. Therefore, feeding rate is a unit of measure that can only be used to make comparisons within an experiment.

To monitor energy balance, the relationship between body mass and SVL (i.e., body condition) was measured at both the beginning and the end of each experiment (Cox and John-Alder, 2007). $\log_{10}$ body mass was regressed against $\log_{10}$ SVL with separate regressions performed for each experiment. A single regression line was estimated from a dataset that included two sets of size measurements for each individual: one measurement from the beginning of the experiment and one from the end of the experiment. Residuals were obtained from this pooled regression line, and a single value was calculated for each animal by subtracting the residual of its initial measurement from the residual of its own final measurement. The resultant values facilitate a direct statistical test for change in the relationship between mass and SVL. If mass and length change isometrically (i.e., parallel to the regression line), these values should average zero. If change in mass is disproportionate to change in length, these values should be positive (body condition improves) or negative (body condition declines). Therefore, these values were examined using a two-tailed t-test with a null hypothesis that change in body condition over the course of each experiment would equal zero.

2.2.1 Experiment 1: *S. jarrovii*

Details of this experiment have been previously reported (Cox et al., 2008). In brief, *S. jarrovii* yearlings were divided into two dietary treatment groups that were balanced for body size and sex: full ration (3 crickets/day for 10 wk; males: $n = 11$; females: $n = 8$) and 1/3 ration (1

cricket/day for 10 wk; males: n = 10; females: n = 12). Individuals in the full ration group were essentially fed to satiety. Data for *S. jarrovi* were reproduced with permission from Cox et al. 2008, © 2008 Canadian Science Publishing or its licensors.

2.2.2 Experiment 2: *S. undulatus* hatchlings

Hatchling *S. undulatus* were assigned to one of three experimental dietary treatment groups that were balanced for body size and sex: full ration (6 crickets/day for 10 days; males: n = 4; females: n = 7), zero ration (0 crickets/day for 10 days; males: n = 3; females: n = 8), and zero ration with re-feeding (zero ration for 7 days followed by full ration for 3 days; males: n = 5; females: n = 6). An additional group of field fresh hatchlings (males: n = 3; females: n = 8) was included to determine IGF-1 expression in free-living lizards. Livers were collected from field fresh lizards immediately upon arrival in the laboratory. In the three experimental groups, livers were collected at the conclusion of dietary manipulation for analysis of IGF-1 expression and plasma was collected for corticosterone assays. Hatchlings were too small to provide sufficient plasma for IGF-1 assays. Males and females were combined for statistical analyses.

2.2.3 Experiment 3: *S. undulatus* adults

To enable measurement of both plasma IGF-1 and hepatic IGF-1 expression, adult *S. undulatus* were used in the third experiment. Adult male *S. undulatus* were assigned to one of three dietary treatment groups that were balanced for body size: full ration (6 crickets/day for 15 days; n = 9), zero ration (0 crickets/day for 11 days; n = 10), and zero ration with re-feeding (zero ration for 11 days followed by re-feeding for 4 days; n = 10).

2.3 Cloning of IGF-1 from *S. undulatus* and *S. jarrovi*

At the time this work was initiated, no lizard IGF-1 cDNA sequences had been published. Therefore, it was necessary to first clone *Sceloporus* IGF-1 in order to design primers for RT-qPCR. Liver tissue from *S. undulatus* was homogenized in TRIzol® (Life Technologies, Grand Island, NY) and isolated according to manufacturer's recommendations. RNA integrity was verified by electrophoresis on a 1% formaldehyde denaturing gel as previously described (Cohick et al., 2000). RNA (2 µg) was treated with DNase I (DNA-free™; Life Technologies) and reverse transcribed using SuperScript™ II Reverse Transcriptase (Life Technologies). Primers (forward: 5'-GCGGGGCTGAGTTGGTGGAT-3'; reverse: 5'-CGCGCAGTACATCTCCAGCC-3') were designed based on the nucleotide sequence for bovine IGF-1 to generate an amplicon of 170 bp in length. The amplicon spans exons 3 and 4, which code for the mature IGF-1 peptide, thus allowing for the quantification of all IGF-1 mRNA transcripts. cDNA was amplified using touchdown PCR conditions that included a decrement in annealing temperature of 0.5°C/cycle for the first 30 cycles and a constant annealing temperature for the last 15 cycles. The cycling conditions were as follows: 10 min at 94°C; cycles 1–30: 1 min at 94°C, 1 min at 50–35°C, 1 min at 72°C; cycles 31–45: 1 min at 94°C, 1 min at 35°C, 1 min at 72°C; 10 min at 72°C. The PCR products were visualized on a 2% agarose gel and a band of the expected length was subsequently purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia CA). The purified PCR product was cloned into pCR™4-TOPO® TA vector (Life Technologies). Plasmid DNA from one clone was purified using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced in both directions. This sequence was used to design primers using Primer Express® software (Life Technologies) to generate an amplicon of 135 bp in length. Primers were forward: 5'-TTGGTGGATGCTCTTCAGTTTG-3' and reverse:

5'-CAGGTCACAGCTTTGGAAACAA-3'. The cloning was repeated using these primers and four additional clones were sequenced in the forward and reverse directions. These primers were also used to clone a 135 bp fragment of IGF-1 from *S. jarrovi* with the following modifications: RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed using the TaqMan® Reverse Transcription Reagents Kit (Life Technologies). Plasmid DNA from two clones was sequenced in both forward and reverse directions. The IGF-1 sequences obtained for *S. undulatus* and *S. jarrovi* were compared to the GenBank database using BLAST™ programs (National Library of Medicine).

2.4 Quantitative RT-PCR (RT-qPCR)

To quantify changes in IGF-1 mRNA, an RT-qPCR assay was validated using the IGF-1 primers described above. Forward (5'-GAAGAGGAAGCAGCTGTGGC-3') and reverse (5'-GCTATGTTGCCTTGGACTTCG-3') primers were also designed to amplify a 52 bp region of the β -actin gene. The cycling conditions were 10 min at 95°C followed by 40 cycles with 15 sec at 95°C and 1 min 60°C. Samples were diluted 1:4, and 5 μ l was amplified in a 20 μ l reaction mix containing 10 μ l Power SYBR Green PCR Master Mix (Life Technologies), 4 μ l ultrapure H₂O, and 0.5 μ l (2 μ M stock) of each forward and reverse gene-specific primer. The threshold cycle (C_T) of each sample was recorded on an ABI 7900HT. IGF-1 and β -actin cDNA values were calculated using a standard curve consisting of serial dilutions of pooled cDNA ranging from 0.025 to 250 ng. Amplification efficiency of standard curves ranged from 88 to 96%. IGF-1 was normalized to β -actin (unaffected by dietary intake, data not shown) and expressed relative to a calibrator (pooled RNA from experimental animals). Negative controls (no reverse transcriptase and no template) were run to ensure that specific amplification occurred.

Dissociation curves were run for each sample to ensure that a single specific product was obtained.

2.5 Radioimmunoassays (RIA)

At the conclusion of the experimental periods, blood was collected from the post-orbital sinus as well as the neck and trunk wounds using heparinized microcapillary tubes (Thermo Fisher Scientific, Waltham MA). Plasma was separated via centrifugation and stored at -20° or -80°C until further analysis.

For RIA of corticosterone, 30 µL of plasma were extracted twice in diethyl ether, dried under a stream of ultra-filtered air, and reconstituted in phosphate buffered saline with gelatin (Smith and John-Alder, 1999). Samples were assayed using a 1:50 dilution of corticosterone antiserum (Esoterix Laboratory Services, Calabasas Hill, CA). Inter-assay variation was 7.5% for *S. jarrovi*. Intra-assay variation was 8% for *S. jarrovi*, 19% for hatchling *S. undulatus*, and 16% for adult *S. undulatus*. Limited volumes of plasma samples (here and below for IGF-1), precluded the possibility of replicating the RIAs.

IGF-1 RIAs were conducted in Dr. Gordon Grau's laboratory at the Hawaii Institute of Marine Biology, University of Hawaii using previously published methods (Shimizu et al., 1999) with the following modifications: to separate IGF-1 from IGF binding proteins, 100 µL plasma were precipitated with 400 µL acid-ethanol (12.5% 2 M HCl:87.5% ethanol) for 30 min at room temperature, neutralized with 200 µL 0.855 M Tris base and centrifuged at 3,000 rpm for 30 min at 4°C. For the RIA, recombinant salmon/trout (*Oncorhynchus* sp.) IGF-1 (Novozymes Biopharma Adelaide, SA, AUS) was used as standard and ¹²⁵I-radiolabeled tracer and rabbit anti-barramundi (*Lates calcarifer*) IGF-1 was used as the primary antiserum (Novozymes

Biopharma). The bound antibody-hormone complexes were precipitated from free radiolabel by the addition of goat anti-rabbit secondary antibody (Sigma-Aldrich, St. Louis, MO). Parallelism was demonstrated between the standard curve and an *S. undulatus* plasma pool (a gift from Dr. Dan Warner, University of Alabama at Birmingham) and Mozambique tilapia (*Oreochromis mossambicus*) plasma. Extracted plasma samples (80 μ L) were assayed in duplicate with all samples analyzed in one assay. The intra-assay coefficient of variation was 16%.

It is worth noting that intra-assay variation in the range of 16% to 19% is considerably higher than we, and most other investigators, usually find acceptable. In the present case, we were unable to repeat assays due to limitations in plasma volume. A potential problem of high intra-assay variation is not that it introduces systematic bias but that it increases the likelihood of a type II error (false negative) in statistical hypothesis testing. Fortunately, however, visual suggestions of statistically significant differences in figures 4B and 5B are corroborated by the statistical analyses.

2.6 Statistical analyses

All statistical analyses were performed using SAS 9.2 (version 9.2, SAS Institute Inc., Cary, NC). Comparisons were made between control and treated groups within experiments. Growth rate and feeding rate were analyzed using one-way ANOVA with initial SVL as a covariate when necessary followed by an LS means test. Change in body condition residuals, hepatic IGF-1 mRNA, and plasma corticosterone were analyzed using one-way ANOVA. Values for hepatic IGF-1 mRNA were log₁₀-transformed to normalize their distributions prior to analysis via ANOVA. When significant treatment effects were observed ($P < 0.05$), post hoc separation of groups was determined using the Ryan-Einot-Gabriel-Welsch test, a powerful step-down

multiple-stage test available as a SAS option. Plasma IGF-1 was analyzed by non-parametric Kruskal-Wallis ANOVA.

3. Results

3.1 Partial cDNA sequence of lizard IGF-1

As predicted, the length of the amplicon generated for *S. undulatus* was 170 bp. This partial sequence aligned with 78% homology to bovine IGF-1 (GenBank accession no. NM_001077828). During the course of this work, *S. undulatus* IGF-1 was cloned and sequenced (Sparkman et al., 2012). A comparison of our sequence with theirs showed 100% agreement (nucleotides 1-135 of accession no. JN315420). We also report for the first time the sequence of *S. jarrovi* IGF-1, which was 100% homologous with *S. undulatus*.

3.2 Feeding rate, body condition, and growth rate

As previously reported (Cox et al., 2008), *S. jarrovi* on full ration consumed an average of 2.62 crickets/day versus 0.98 crickets/day on 1/3 ration (Table 1). In the *S. undulatus* hatchling experiment, animals on full ration consumed an average of 4.48 crickets/day throughout the experimental period (days 1–10). Hatchling *S. undulatus* on zero ration for 7 days followed by full ration for 3 days consumed 3.09 crickets/day during the re-feeding period on days 8–10 ($F_{2,29} = 182.44$, $P < 0.0001$; Table 1). Adult *S. undulatus* on full ration consumed an average of 1.83 crickets/day (days 1–15) versus 3.21 crickets/day during the re-feeding period on days 12–15 ($F_{2,26} = 41.45$, $P < 0.0001$; Table 1).

In all three experiments, body condition was lower in lizards on restricted rations compared with body condition of the corresponding full ration groups (*S. jarrovi*: $F_{1,39} = 8.69$, P

= 0.0054, *S. undulatus* hatchlings: $F_{2,30} = 29.38$, $P < 0.0001$, *S. undulatus* adults: $F_{2,26} = 29.91$, $P < 0.0001$; Fig. 1). In both hatchling and adult *S. undulatus*, re-feeding for a brief period of time was sufficient to restore body condition to that of the group on full ration. In *S. jarrovii*, lizards on 1/3 ration continued to grow, as measured by increases in SVL, but their growth rate was approximately 50% lower than that observed in animals fed the full ration (Fig. 2A). In hatchling *S. undulatus*, zero ration dramatically reduced growth rate by about 80% ($F_{2,30} = 10.85$, $P = 0.0003$; Fig. 2B) and three days of re-feeding failed to restore growth to its pre-treatment level. In adults of *S. undulatus*, manipulations of food ration did not affect growth rate ($F_{2,26} = 2.26$, $P = 0.1244$; Fig. 2C).

3.3 Hepatic IGF-1 mRNA and plasma IGF-1

Long-term food restriction (1/3 ration) for 10 weeks did not affect expression of hepatic IGF-1 mRNA in *S. jarrovii* ($F_{1,35} = 0.34$, $P = 0.5616$; Fig. 3A). In hatchling *S. undulatus*, hepatic IGF-1 mRNA was not affected by 10 days of captivity on full ration (Fig. 3B). Hepatic IGF-1 mRNA was decreased 5-fold by zero ration and was restored by re-feeding to levels observed in field fresh animals and those fed full ration ($F_{3,39} = 14.36$, $P < 0.0001$; Fig. 3B). Similarly, in adults of *S. undulatus*, zero ration decreased hepatic IGF-1 mRNA by 3-fold, while re-feeding restored IGF-1 mRNA to levels observed in animals fed full ration ($F_{2,26} = 22.19$, $P < 0.0001$; Fig. 3C).

Using a heterologous system of commercial reagents available for teleost fish described above, we validated an IGF-1 RIA for use in *Sceloporus* lizards. The serial dilutions of *S. undulatus* plasma yielded a curve parallel to the salmon/trout standard curve as well as serial dilutions of plasma from *Oreochromis mossambicus* (Fig. 4A). Parallelism was tested explicitly

by regressing %B/B₀ against IGF-1 dose for extracted lizard plasma and salmon standards in a multiple regression model, with full interaction between species and dose to allow for non-parallel curve fits. This model rejected significance of the interaction term ($p = 0.4524$), indicating parallelism between curves fit to extracted lizard plasma and salmon standards. Using this RIA, we found that zero ration significantly decreased plasma IGF-1 levels of adult *S. undulatus*. Plasma IGF-1 was fully restored by re-feeding ($P = 0.0093$; Fig. 4B).

3.4 Plasma corticosterone

At the conclusion of the experimental periods, plasma corticosterone was about an order of magnitude higher in hatchling *S. undulatus* than in adults of this species and juvenile *S. jarrovi*. Plasma corticosterone was not affected by dietary treatment in juvenile *S. jarrovi* ($F_{1,37} = 1.10$, $P = 0.3002$; Fig. 5A) and adult *S. undulatus* ($F_{2,22} = 0.55$, $P = 0.5830$; Fig. 5C). In contrast, in *S. undulatus* hatchlings, plasma corticosterone was elevated substantially by zero ration and restored by re-feeding ($F_{2,29} = 4.08$, $P = 0.0274$; Fig. 5B). Corticosterone was not correlated with plasma IGF-1 in adults of *S. undulatus* ($R^2 = 0.0059$, $p = 0.8059$), the only group for which both hormones were measured.

4. Discussion

The GH/IGF-1 axis is the central growth-regulatory endocrine system in vertebrates. In the present study, we exploited the expected effects of variation in food intake to develop methodologies and characterize the IGF-1 system in lizards, and to compare these effects to those reported in other species. To the extent that we can generalize from the parameters we measured, we found that nutritional regulation of GH/IGF-1 in lizards conforms to the broader

vertebrate model. In yearling (i.e., pre-reproductive) males and females of *S. jarrovi*, ten weeks on 1/3 ration did not affect hepatic IGF-1 mRNA levels but decreased growth rate compared to lizards on full ration. This level of food restriction caused a decrease in body condition (i.e., lizards on 1/3 ration had less body mass for any given body length), but the continuation of growth indicates that lizards remained in positive energy balance even on the reduced ration. In fact, a comparison of growth rate in the present experiment to that of free-living *S. jarrovi* of similar size (Cox and John-Alder, 2007) suggests that the full ration administered here likely exceeds typical food intake in the wild, and that 1/3 ration may in fact be closer to a natural rate of food intake (Cox et al., 2008; Smith et al., 1994). Similarly, long-term variation in food intake had no consistent effect on plasma IGF-1 in the brown house snake (*Lamprophis fuliginosus*), another squamate reptile (Sparkman et al., 2010), and experimental studies on birds and fishes have found no consistent effect of moderate food restriction on IGF-1 (e.g., Pérez-Sánchez et al., 1995; Pierce et al., 2001; Beckman et al., 2004; Rønning et al., 2009). Overall, these studies indicate that when animals remain in positive energy balance, variation in food intake is not a predictable environmental regulator of IGF-1.

Several experiments have reported evidence of transient reductions in IGF-1 expression and plasma IGF-1 in response to moderate food restriction. In Japanese quail (*Coturnix japonica*), a restricted, weight-maintenance diet initially lowered hepatic IGF-1 mRNA compared to an *ad libitum* diet after 2 days, but this effect of food restriction disappeared after 5 days due to a decline in the *ad libitum*-fed group (Rønning et al., 2009). Furthermore, in Coho salmon (*Oncorhynchus kisutch*), a time course experiment measured the effects of three different ration sizes on plasma IGF-1 throughout 8 weeks of treatment (Pierce et al., 2001). After 4 weeks, animals consuming the high ration had the highest levels of plasma IGF-1. However,

animals fed high and medium rations had equally high levels of plasma IGF-1 after 8 weeks compared to animals fed the low ration. Therefore, we cannot rule out the possibility of an early, transient response in hepatic IGF-1 mRNA in *S. jarrovi*, which would not have been evident at the end of our 10-week experiment. In any case, when food intake remains at or above the weight-maintenance level, IGF-1 shows no lasting response.

Since 1/3 ration did not reduce the expression of hepatic IGF-1 mRNA, we conducted two experiments involving severe food restriction (i.e., zero ration) with or without re-feeding. In both hatchling and adult *S. undulatus*, zero ration caused hepatic IGF-1 mRNA to decrease, and this was accompanied by a decrease in plasma IGF-1 in adults. Both of these parameters were restored by refeeding. In addition, zero ration caused growth to cease (mean growth rate: 0.05 mm/day; lower and upper 95% confidence limits: -0.07 to 0.17 mm/day) in hatchlings and caused body condition to decrease in both hatchlings and adults, indicating that food-deprived lizards were in negative energy balance in both of these experiments. We did not expect zero ration to reduce growth in adults because all lizards were growing very slowly on the asymptotic part of their growth trajectories (Haenel and John-Alder, 2002; John-Alder et al., 2007).

Our rationale for measuring plasma corticosterone was that functional parameters of the hypothalamo-pituitary-adrenal (HPA) axis can be strongly influenced by variation in food intake and nutrition (Dallman et al., 1999). Therefore, we anticipated that corticosterone would be a reliable hormonal index of energy balance. Our results call attention to the fact that these relationships are not always straightforward. We observed the expected increase in corticosterone in response to food restriction only in hatchling *S. undulatus*. Corticosterone was unaffected by both severe and moderate food restriction in adults of *S. undulatus* and in yearling *S. jarrovi*, respectively. Studies on mammals, fishes, and birds have generally shown that

diminished food intake is associated with elevated levels of plasma glucocorticoid (cortisol or corticosterone; (Dallman et al., 1999; Kelley et al., 2001; Lynn et al., 2003; MacKenzie et al., 1998; Romero and Remage-Healey, 2000). In contrast, plasma corticosterone was reported to be unaffected by 20 days of food restriction in tree lizards (*Urosaurus ornatus*) (French et al., 2007a). In addition, seven days of food restriction actually caused a reported decrease in plasma corticosterone in the same species (French et al., 2007b). The failure of zero ration to cause an increase in plasma corticosterone in adult *S. undulatus* might be attributable to the time of year when this experiment was conducted, which was at the end of the breeding season when adult *S. undulatus* have relatively low levels of plasma corticosterone (John-Alder et al., 2009), low adrenocortical cellular sensitivity to adrenocorticotropin (ACTH) (Carsia and John-Alder, 2003), and reduced stress responsiveness (Smith and John-Alder, 1999). In any case, severe food restriction can cause an increase in corticosterone in lizards, as occurred in our hatchling *S. undulatus*, but this response is apparently highly condition-dependent and clearly requires further characterization. Furthermore, the difference in corticosterone response between juveniles and adults of *S. undulatus* indicates that effects of zero ration on systemic IGF-1 do not require intervening mediation of corticosterone.

In summary, the responses we observed with severe food restriction (i.e., zero ration) and re-feeding are very similar to reports on other species (Anthony et al., 1999; Beccavin et al., 2001; Fox et al., 2010; Gabillard et al., 2006; Schew et al., 1996), which indicate that food restriction severe enough to result in a negative energy catabolic state is required to reduce the expression of hepatic IGF-1 mRNA and plasma IGF-1. Furthermore, the results of our experiment with 1/3 ration are similar to the body of evidence in other species, which indicates that within the range of positive energy balance, a substantial reduction in food intake can

decrease growth without affecting IGF-1. This series of experiments suggests that normal variation in food consumption in free-living animals may not be a key environmental regulator of systemic IGF-1. Thus, further studies on ecologically relevant variation in food intake are required to establish the importance of nutrition as an environmental regulator of the GH/IGF axis. Furthermore, within the range of positive energy balance, the involvement of molecular homeorhetic signals (e.g., IGF-1; (Bauman et al., 1982; Dantzer and Swanson, 2012; Leroi, 2001; Sparkman et al., 2009) in growth regulation requires further investigation.

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Figure legends

Figure 1. Effect of ration on change in body condition residuals in A) *S. jarrovi*, B) *S. undulatus* hatchlings, and C) *S. undulatus* adults. Values represent mean (± 1 SE) changes in body condition residuals. Lowercase letters denote statistical significance between treatment groups. Log₁₀ body mass was regressed against log₁₀ SVL with each lizard's initial and final measurements included in one model. A single pooled regression line was fit to these data, then the initial residual was subtracted from the final residual for each individual. Data for *S. jarrovi* are reproduced with permission from Cox et al. 2008, © 2008 Canadian Science Publishing or its licensors.

Figure 2. Effect of ration on normalized growth rate in A) *S. jarrovi*, B) *S. undulatus* hatchlings, and C) *S. undulatus* adults. Normalized growth rate was calculated by dividing the growth rate (change in SVL over time) of each individual by the highest growth rate within each experiment. Mean (± 1 SE) normalized growth rate. Lowercase letters denote statistical significance between treatment groups. Data for *S. jarrovi* are reproduced with permission from Cox et al. 2008, © 2008 Canadian Science Publishing or its licensors.

Figure 3. Effect of ration on hepatic IGF-1 mRNA in A) *S. jarrovi*, B) *S. undulatus* hatchlings, and C) *S. undulatus* adults. Lines represent median values within treatment group. Lowercase letters denote statistical significance between treatment groups.

Figure 4. Validation of a heterologous radioimmunoassay for use in *Sceloporus* lizards. A) Percent ¹²⁵I-IGF-1 binding (%B/B₀) is plotted against recombinant *Oncorhynchus* sp. IGF-1 and

against volumes of extracted plasma for *S. undulatus* and *O. mossambicus*. B) Effect of ration on plasma IGF-1 in *S. undulatus* adults. Mean $\pm$ 1 SE plasma IGF-1. Kruskal-Wallis ANOVA determined that ration affected plasma IGF-1 ($P = 0.0093$).

Figure 5. Effect of ration on plasma corticosterone in A) *S. jarrovi*, B) *S. undulatus* hatchlings, and C) *S. undulatus* adults. Lines represent median values within treatment group. Values are plotted on a $\log_{10}$ scale to facilitate the graphical presentation, but statistical analyses used untransformed values (see text for details). Lowercase letters denote statistical significance between treatment groups. Note the differences in scaling of the y-axes. Data for *S. jarrovi* were reprinted from Carsia et al., 2012 with permission from Elsevier.

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Table 1. Age class, initial body size, experimental food ration, and feeding rate in *S. jarrovii*, *S. undulatus* hatchlings, and *S. undulatus* adults. Values are means $\pm$ 1 SE; na = not applicable. In *S. undulatus*, feeding rate differed significantly among the three hatchling treatment groups ($F_{2,29} = 182.44$, $P < 0.0001$) and the three adult treatment groups ($F_{2,26} = 41.45$, $P < 0.0001$). Superscripted lower-case letters denote statistical groupings for comparisons of feeding rates among treatment groups; see text for additional details. Data for *S. jarrovii* are reproduced with permission from Cox et al. 2008, © 2008 Canadian Science Publishing or its licensors.

Species	Age Class	N	Initial SVL (mm)	Initial Body Mass (g)	Ration	Time Fed/Re-fed	Feeding Rate (crickets/d)
<i>S. jarrovii</i>	Yearlings	19	49.2 $\pm$ 0.4	2.92 $\pm$ 0.09	Full	10 weeks	2.62 $\pm$ 0.07 ^a
		22	49.2 $\pm$ 0.5	2.93 $\pm$ 0.10	1/3	10 weeks	0.98 $\pm$ 0.01 ^b
<i>S. undulatus</i>	Hatchlings	11	32.9 $\pm$ 1.1	1.17 $\pm$ 0.15	Field Fresh	na	na
		11	32.5 $\pm$ 0.9	1.06 $\pm$ 0.11	Full	10 days	4.48 $\pm$ 0.14 ^a
		11	32.5 $\pm$ 1.0	1.18 $\pm$ 0.10	Zero	10 days	0.00 $\pm$ 0.00 ^b
		11	32.4 $\pm$ 1.0	1.00 $\pm$ 0.09	Zero/Re-fed	7 days/3 days	3.09 $\pm$ 0.30 ^c
<i>S. undulatus</i>	Adults	9	62.9 $\pm$ 1.0	7.84 $\pm$ 0.34	Full	15 days	1.83 $\pm$ 0.13 ^a
		10	62.7 $\pm$ 1.6	7.53 $\pm$ 0.53	Zero	11 days	0.00 $\pm$ 0.00 ^b
		10	63.3 $\pm$ 1.2	7.86 $\pm$ 0.43	Zero/Re-fed	11 days/4 days	3.21 $\pm$ 0.41 ^c

Figure2

Figure 1

B. *S. undulatus* adults