NUTRITIONAL AND HORMONAL MODULATION OF INSULIN-LIKE GROWTH FACTOR-1 WITH RESPECT TO GROWTH IN SEXUALLY DIMORPHIC LIZARDS

by

CHRISTINE A. DUNCAN

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And approved by

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ABSTRACT OF THE DISSERTATION

Nutritional and Hormonal Modulation of Insulin-Like Growth Factor-1

with Respect to Growth in Sexually Dimorphic Lizards

By CHRISTINE A. DUNCAN

Dissertation Director:

Dr. Henry B. John-Alder

In many species of animals, adults of one sex grow faster or for a longer period of time to become larger than the other. Sex differences in growth rate can often be attributed to differences in androgenic versus estrogenic hormonal effects on the growth hormone/insulin-like growth factor-1 (GH/IGF-1) axis. In this paradigm, testosterone (T) stimulates hepatic IGF-1 message, resulting in an increase in plasma IGF-1, while estrogenic hormones have the opposite effect. However, this generality is inherently biased because studies have focused almost entirely on male-larger species. Previous work in lizards has demonstrated that T inhibits growth in female-larger species, while stimulating growth in male-larger species. Thus, the effect of T on IGF-1 may not be universal but may depend on a species' pattern of sexual size dimorphism. Since IGF-1 had not previously been characterized in lizards, my research required the development of novel assay techniques. To this end, a partial sequence of IGF-1 was cloned. Comparison of the deduced amino acid sequences of IGF-1 confirmed high sequence

identity (72 – 80%) between lizards and the corresponding region in human. These sequences supported the development of assays to characterize the response of IGF-1 to variation in food intake. In *S. undulatus*, zero ration decreased hepatic IGF-1 message and plasma IGF-1, while re-feeding restored levels to that of full ration. In yearling *S. jarrovii*, 1/3 ration had no effect on hepatic IGF-1 message compared to full ration. Altogether, results from nutritional manipulation in *Sceloporus* lizards are consistent with previous work but suggest that food restriction short of starvation may have little effect on IGF-1. Following the validation of assays, we investigated the effects of T on IGF-1 in a female-larger species, *S. undulatus*. Contrary to published studies on male-larger species, T decreased hepatic IGF-1 message in adult males and juvenile males and females. However, T did not affect plasma IGF-1. Our results challenge the widespread belief that males grow faster than females by increasing their production of IGF-1.

DEDICATION

To my dear friend, Megan Dietz.

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LIST OF ABBREVIATIONS

11-KT	11-Ketotestosterone
1/3R	1/3 Ration Treatment Group
ANOVA	Analysis of Variance
ANCOVA	Analysis of Covariance
bp	Base Pair
CAST	Castrated Treatment Group
cDNA	Complementary Deoxyribonucleic Acid
CON	Control Treatment Group
DHT	5α-Dihydrotestosterone
DMSO	Dimethyl Sulfoxide
E_2	17β-Estradiol
FR	Full Ration Treatment Group
GH	Growth Hormone
GHR	Growth Hormone Receptor
GHRH	Growth Hormone Releasing Hormone
kDa	Kilodalton
IGF-1	Insulin-like Growth Factor-1
IGFBP	Insulin-like Growth Factor Binding Protein
mRNA	Messenger Ribonucleic Acid
PBSG	Phosphate-buffered Saline with Gelatin
RIA	Radioimmunoassay
RT-PCR	Reverse Transcription Polymerase Chain Reaction

SE	Standard Error
SSD	Sexual Size Dimorphism
SST	Somatostatin
SVL	Snout-Vent Length
T	Testosterone
TEST	Testosterone Treatment Group
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
ZR	Zero Ration Treatment Group

CHAPTER I.

INTRODUCTION

Sex-Specific Regulation of Growth

Body size is one of the most important traits of an organism because of its pervasive influence on behavior, physiology, and ecology. Relative body size affects access to mates, fecundity, predator avoidance, and survival (Shine, 1994; Haenel et al., 2003; Cox et al., 2003, 2007; Arendt, 1997). While much attention has focused on correlations between large body size and mating (e.g., male-male competition, female choice) and reproductive success (e.g., number and quality of offspring), disadvantages do exist. These disadvantages include viability costs (e.g., more visible to predators, require more resources) as well as sexual selection against large body size (e.g., less agile with respect to acquiring mates and defending territories) (Blanckenhorn, 2000). Being large can be advantageous, however variation in body size occurs between species (interspecific variation), within species (intraspecific variation), and even between sexes of the same species (sexual size dimorphism, SSD). The widespread phenomenon of SSD often arises when sex differences in age-specific growth patterns cause members of one sex to become larger than members of the opposite sex in a given species.

Many authors have focused on adaptive hypotheses to explain SSD. The most prevalent hypotheses are sexual selection on male body size and natural selection on female body size (Darwin, 1871; Andersson, 1994). Sexual selection favors large male body size when males compete for access to mates (intrasexual selection hypothesis) or when there is female choice (intersexual selection hypothesis). Under this selection, large body size should confer an advantage when large males out-compete smaller males in

agonistic encounters or when female choose larger mates. Natural selection favors large female body size when the number of offspring increases with maternal size (fecundity advantage hypothesis). Clutch size and quality of offspring are known to positively correlate with female body size (Andersson 1994). Current findings in lizards suggest that selection can explain only a small fraction of the interspecific variation in SSD (Cox et al., 2003). Therefore, other mechanisms must be involved.

Selection favors differences in morphological appearance between males and females, however, both sexes presumably share the same fundamental genetic architecture that regulates somatic growth. During ontogeny, sex-biased expression of genes that control growth leads to sex differences in growth rate (Fairbairn, 1997; Badyaev, 2002).

Allocation Trade-Offs

Data from the literature suggest that maximal growth can be traded in favor of competing life history traits such as age at first reproduction and fecundity (Arendt, 1997). Not only does growth affect these traits, but these traits also affect growth because they demand energy from the same limited resource. In order to understand the evolution of different growth rates, the proximate mechanisms that regulate trade-offs must be elucidated. Understanding the correlations between growth and its regulatory mechanisms will reveal how life history strategies evolve.

The term homeorhesis was first introduced by Kennedy (1967) to differentiate between the regulation of long-term growth and the homeostatic control of body temperature. Homeorhesis was later adopted by Bauman et al. (1980) and defined as the

orchestrated changes for the priorities of a physiological state. Furthermore, homeorhetic mechanisms function on a chronic basis to coordinate physiological processes and tissue metabolism, whereas homeostatic mechanisms provide short-term regulation to maintain physiological equilibrium. One of the most well-characterized homeorhetic controls is growth hormone (GH). Growth hormone plays a significant role in partitioning energy to tissues to support development throughout the growth process. Besides its role in growth, GH is known to have important functions during pregnancy and lactation. Exogenous GH has been shown to improve the ability of the mammary gland to synthesize milk, mobilize fatty acids from adipose tissue, and accumulate muscle protein (Bauman et al., 1982). Overall, complex interactions during growth are regulated by homeorhetic mechanisms that facilitate nutrient partitioning to tissues according to priority.

Studies conducted on a wide range of taxonomic groups provide evidence for a trade-off between resistance to fluctuating patterns of food availability and the capacity for rapid growth. In order to survive harsh conditions such as low food availability or crowding, *Caenorhabditis elegans* larvae can enter into a stress-resistant stage known as dauer (Cassada and Russell, 1975). While in this stage, larvae will not eat or grow, instead metabolites are accumulated in the gut and stored for later use when conditions are more favorable (Hu, 2007). Additionally, adult insects can enter a similar stage known as diapause (Tatar et al., 2003). Formation of this stage enables insects to avoid adverse conditions. Episodic feeders such as *Python molurus* (Burmese python) have adapted to infrequent feeding by decreasing their energy expenditure between meals (Secor and Diamond, 1998). Subsequently, in response to food consumption, the gut undergoes a remarkable bout of tissue growth and remodeling.

Maximization of Growth

Following periods of decreased food availability and slow growth, animals can undergo a period of "catch up" growth, where their growth rates exceed that of individuals of the same species that continuously feed. This biological phenomenon, known as compensatory growth, has been observed in invertebrates and vertebrates. Studies have revealed that this period of accelerated growth enables these animals to grow to the same size as conspecifics that experienced more favorable conditions (Wilson and Osbourn, 1960; Ali et al., 2003).

Studies that draw comparisons between free-living and captive populations also provide additional support for the idea that individuals do not always maximize their growth. For example, in laboratory environments, individuals can grow at rates faster than those measured under natural field conditions (Woolbright, 1989; Ferguson and Talent, 1993; Taylor and DeNardo, 2005). Further evidence is documented in groups of free-living *Sceloporus undulatus* (eastern fence lizard) and *Uta stansburiana* (side-blotched lizard) that received supplemental feeding and exhibited higher growth rates compared to control groups (Ferguson et al., 1982; Ferguson and Fox, 1984). Differences in local food availability and temperature have been associated with variable growth rates such that populations with access to less food and shorter daily activity periods grow more slowly than populations with access to more food and longer daily activity periods (Niewiarowski and Roosenberg, 1993; Sinervo and Adolph, 1994).

Growth Hormone/Insulin-Like Growth Factor-1 Axis

The growth hormone/insulin-like growth factor-1 (GH/IGF-1) axis is the central growth-regulatory endocrine system in vertebrates. Among vertebrate species that have been studied, the components and regulation of the GH/IGF-1 axis are well-conserved in structure and function. The amino acid sequence of IGF-1 is highly conserved among vertebrate species. The range of similarity for IGF-1 between human and other vertebrates is 71% (shark, *Squalus acanthias*) to 100% (bovine, *Bos sp.*; dog, *Canis sp.*; horse, *Equus sp.*; guinea pig, *Caveus porcellus*; and pig, *Sus scrofa*).

Under the control of hypothalamic hormones, growth hormone releasing hormone (GHRH) and somatostatin (SST), GH is released from the anterior pituitary in a sex- and species-specific manner (Shapiro et al., 1995). For example, in male rats, GH is released in a pulsatile manner approximately every 3.5 – 4 hours. In females, GH is released more often but has an irregular pattern that produces peaks of lower magnitude than males. Growth hormone can exert direct effects on tissues by acting through its receptor (GH receptor, GHR) to regulate growth and metabolism. In addition, GH can indirectly stimulate growth by increasing transcription of IGF-1 in the liver, and this GH-induced increase in IGF-1 message is associated with an increase in circulating IGF-1 (Fig. 1). In circulation, IGF-1 binds to high-affinity IGF binding proteins (IGFBPs). Once at the target tissues, the IGF-1 ligand stimulates growth and mediates much of the growth-promoting effects of GH. Furthermore, IGF-1 inhibits the secretion of GH by through negative feedback mechanisms.

The IGFBP family extends the half-life, transports, and modulates biological responses of IGF-1. Six homologous IGFBPs have been identified and characterized in mammals, and more recently, IGFBPs have been identified and characterized in non-

mammalian vertebrates (Kelley et al., 2002). These IGFBPs are mainly produced in the liver and are subsequently secreted into circulation. In circulation, IGF-1 forms complexes with IGFBPs at a greater affinity than that of the type 1 IGF receptor (Rechler and Clemmons, 1998). The most abundant IGFBP in circulation is IGFBP-3 at approximately100 nM, while the remaining IGFBPs are found at 2 – 15 nM. Both IGFBP-3 and -5 can bind to an acid labile subunit in addition to IGF-1 to form a ternary structure that further extends the half-life of IGF-1. Free IGF-1 has a half-life of approximately eight minutes, which can be increased to approximately 30 minutes when bound to IGFBP-3 and to approximately 15 hours when in a ternary complex with IGFBP-3 and ALS (Rechler and Clemmons, 1998). The concentrations of IGFBPs are regulated by hormones (GH, IGFs, insulin, and sex steroid hormones) and nutritional status, which will be discussed in the following sections.

Nutritional Modulation of the GH/IGF-1 Axis

Nutrition is a major regulator of the GH/IGF-1 axis. The conclusion that nutritional status is a principal environmental regulator of the GH/IGF-1 axis is based on numerous studies involving starvation (Duan, 1998; Moriyama et al., 2000; Pierce et al., 2005; Pedroso et al., 2006). Under anabolic conditions, GH increases IGF-1 production, leading to cell proliferation, differentiation, and somatic growth. Under catabolic conditions such as starvation, the axis is uncoupled, meaning that GH no longer stimulates hepatic IGF-1 message. Due to the development of GH resistance, likely caused by a decline in GH binding sites in the liver, GH increases in the face of constant or decreasing IGF-1 and exerts direct effects on lipid mobilization for energy (mammals:

Thissen et al., 1994; birds: Scanes, 1997; fishes: Duan, 1998). Additionally, catabolic conditions increase levels of low molecular weight binding proteins in circulation (mammalian IGFBP-1; piscine ≤ 31 kDa IGFBPs) that sequester IGF-1 and inhibit energy-expensive growth until conditions are more favorable (e.g., with resumed feeding; Lee et al., 1997; Kelley et al., 2001).

Hormonal Modulation of the GH/IGF-1 Axis

Sex steroid hormones have a major impact on the IGF-1 system, either indirectly by affecting the GH/IGF-1 axis, or directly by affecting IGF-1 production at the tissue level. Studies have shown that androgens promote male growth in male-larger species and estrogens inhibit male growth in male-larger species, in part through effects on the GH/IGF-1 axis. However, these data are inherently biased because studies focus almost entirely on male-larger species (mammals: Borski et al., 1996; Gatford et al., 1998; Ford and Klindt, 1989; Wehrenberg and Giustina, 1992; birds: Fennel and Scanes, 1992; fishes: Holloway and Leatherland, 1998; Huggard et al., 1996; Kuwaye et al., 1993). Several studies have analyzed the effects of exogenous sex steroid hormones on the GH/IGF-1 axis by quantifying the molecular and cellular responses (Gatford et al., 1998). In male-larger species, androgens indirectly increase hepatic IGF-1 message and levels of plasma IGF-1 by increasing the magnitude of GH released into the circulation. However, the effects of estrogens are variable among species and throughout different stages of life.

Results from male-larger species are also evident in the piscine literature. In male Atlantic salmon, exogenous estradiol (E_2) inhibited growth rate and plasma IGF-1 (Arsenault et al., 2004). In tilapia, cultured hepatocytes treated with E_2 decreased plasma

25 kDa IGFBP and increased plasma 30 kDa IGFBP in males, while E_2 increased plasma 25 and 30 kDa IGFBP in females (Riley et al., 2004). In the same study, cultured hepatocytes from males treated with 5α-dihydrotestosterone (DHT) had higher expression of IGF-1 message, lower plasma 25 kDa IGFBP and the same level of plasma 30 kDa IGFBP, whereas hepatocytes from females treated with 5α-DHT had lower expression of IGF-1 message and increased 25 and 30 kDa IGFBPs compared to controls. In male coho salmon, injections of T and 11-ketotestosterone stimulated plasma IGF-1 and 41-kDa IGFBP (putative IGFBP-3) levels, while plasma GH levels were not altered in either treatment (Larsen et al., 2004). Overall, in male-larger species, the administration of exogenous estrogen inhibits male growth rate and decreases circulating IGF-1, and exogenous androgens stimulate male growth rate and circulating IGF-1.

Few studies have characterized the effects of androgens on growth in female-larger species. In a study with female-larger golden hamsters, the removal of the primary source of androgen production (castration) led to increased body mass relative to intact male hamsters (Swanson, 1967). Similarly, in female-larger American kestrels, injections of exogenous yolk-androgens (T, androstenedione) decreased growth in males but not females relative to controls (Sockman and Schwabl, 2000; Sockman et al., 2005). Additionally, in female-larger red-spotted garter snakes, exogenous T and an anti-estrogen, tamoxifen, decreased female growth rates to rates comparable to intact males (Lerner and Mason, 2001). In the same study, E*2* decreased male growth to rates even lower than that of intact males. However, an exogenous anti-androgen, cyproterone acetate, had no effect on female or male growth with respect to intact females and males, and exogenous anti-estrogen had no effect on male growth compared to intact males. To

summarize, in female-larger species, both exogenous androgens and E_2 inhibit male growth rate.

Sceloporus Lizards: A Model System to Study Growth Regulation

The lizard genus *Sceloporus* provides a well-suited system to conduct comparative studies on growth regulation. In this genus, closely related species exhibit opposite patterns of SSD (male-larger species vs. female-larger species). The direction of SSD is documented, and the phylogeny of *Sceloporus* lizards has been well-characterized using morphological and molecular data (Wiens et al., 2010). Male-larger SSD is the ancestral condition, however, female-larger species have independently evolved at least three times. *Sceloporus* lizards exhibit SSD that varies from males with 25% longer snout-vent length (SVL) than female conspecifics to females with 15% longer SVL than male conspecifics (Fitch, 1978; Cox et al., 2003).

In our laboratory, we have studied species with approximately 10% difference in SVL between sexes (Haenel and John-Alder, 2002; Cox et al., 2005a; Cox and John-Alder, 2005). This difference in body size between sexes allows for consistent contrasts between sexes. In addition to having opposite patterns of SSD, *Sceloporus* lizards are well-suited to conduct comparative studies because they are accessible and tractable for experimental work both in the field and in the laboratory. Importantly, the life histories of these species are well-established so that experimental studies can be conducted during periods of maximal difference in growth rates between males and females. Previous work has determined the characteristics of growth and survivorship, magnitude of SSD, and

profiles of plasma T in three species of *Sceloporus* lizards: *S. undulatus*, *S. virgatus*, and *S. jarrovii* (Haenel and John-Alder, 2002; Cox et al., 2005a; Cox and John-Alder, 2005).

In New Jersey, male and female *S. undulatus* hatch in early August at similar SVL. This population is typically active from April until October and hibernates for the remainder of the year. Sexual size dimorphism develops in *S. undulatus* during the first full season of activity and is fully expressed by one year of age because females grow faster than males (Haenel and John-Alder, 2002). At approximately 11 months of age, plasma T peaks in males and is higher than levels measured in females (Cox et al., 2005a). This sexual divergence in plasma T coincides with increases in male aggression, movement, and ventral coloration (Skelly and John-Alder, 2002; Cox et al., 2005a). Males and females reach reproductive maturity at 20 months, mate after emerging from hibernation, and lay eggs in late May through early June.

Two consecutive experiments conducted in a field enclosure in the New Jersey Pinelands were set up to investigate the role of T on organismal growth in male *S. undulatus* (Cox et al., 2005a). In field-active *S. undulatus*, exogenous T inhibits male growth while increasing daily activity period, daily movement, home range area, and ectoparasite load. If these factors influence growth regulation, then they must affect the total amount of energy acquired and/or the amount of energy allocated to growth. In other words, energy available for growth may be traded off in favor of competing functions such as maintenance and reproduction. In support of this hypothesis, calculations of hypothetical energy costs show that the energetic cost of increased daily activity period accounts for approximately 79% of the estimated difference in energy allocated to growth between control and T-replaced males. If energetic cost of parasitism is factored in to this

estimated difference, then this estimate is nearly sufficient to explain the inhibition of growth observed in T-replaced males.

Additional studies used two sympatric species, *S. virgatus* and *S. jarrovii*, indigenous to the southwestern part of the United States. In Arizona, male and female *S. virgatus* hatch in September at similar SVL. This species is typically active from April until October and hibernate for the remainder of the year. Sexual size dimorphism develops in *S. virgatus* during the first year of life before the age of first reproduction (7 mo.) because females grow faster than males (Cox and John-Alder, 2005). Male and female *S. jarrovii* are born in June and mate in October through November after the seasonal monsoon rains. Males grow at faster rates than females regardless of seasonal changes in plasma T, which leads to the development of male-larger SSD before the age of first reproduction (4 mo.) in low-altitude populations (Cox and John-Alder, 2005).

In free-living *S. virgatus*, a direct comparison of castrated and T-replaced males showed that T inhibited growth in castrated males. Additionally, castration had no effect on growth rate compared to control males, which is likely due to the short treatment period (mean 42 days). In free-living *S. jarrovii*, castration decreased growth rates, while castration accompanied by T replacement restored growth rates compared to control males. Overall, these data reveal the potential for T to act as a bi-potential regulator of organismal growth.

Together, field and laboratory experiments demonstrate that the regulation of sex differences in growth depend on environmental factors. When male and female *S. undulatus* were raised in a controlled environment, the development of SSD was delayed because captive males grew faster than free-living males and at comparable rates to

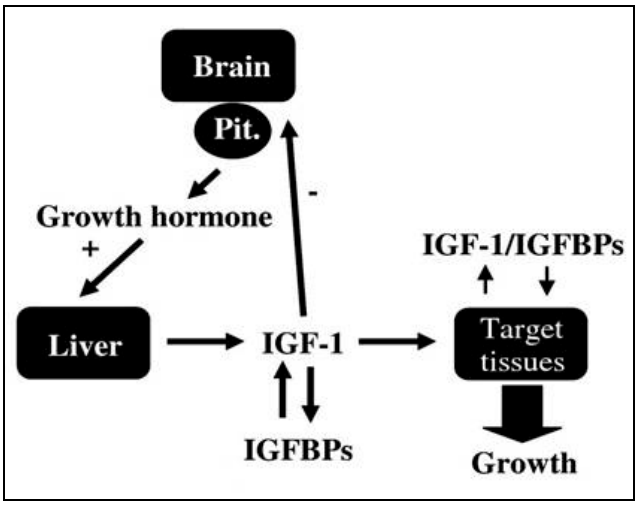
females (John-Alder et al., 2007). Following castration and T replacement in captive male *S. jarrovii*, castrated males grew at similar rates to control and T-replaced males (Cox et al., 2006). This result is in stark contrast to the inhibitory effect of castration measured in free-living *S. jarrovii* (Cox and John-Alder, 2005). In a captive environment, the development of SSD was suppressed because males grew more slowly than free-living males of similar size, while females grew at comparable rates compared to free-living females of similar size. Subsequent to this study, Cox et al. (2008) hypothesized that male-larger SSD develops in *S. jarrovii* due to a sexual difference in energy allocation to growth versus competing functions, but that an ad libitum diet provides a surplus of energy which overwhelms this energetic trade-off. To test this hypothesis, male and female *S. jarrovii* were offered a full ration (3 crickets/d) or a 1/3 ration (1 cricket/d) for a 10-week treatment period (Cox et al., 2008). Food restriction inhibited growth rate by about 50% in snout-vent length (SVL) and decreased mass of fat bodies relative to high food availability, however, sex differences in growth rate were absent. At the conclusion of the treatment period, levels of plasma corticosterone did not differ between treatment groups, but 1/3 ration increased the production of basal corticosterone in adrenocortical cells relative to full ration (Carsia, McIlroy, Cox, Barrett, and John-Alder, unpublished). These data demonstrate that 1/3 ration did not lead to chronic stress. Altogether, these data collected from both field and laboratory experiments indicate that sex differences in growth rate and the development of SSD rely on cues from the environment.

Taken together, results from previous research that has investigated the effects of sex steroid hormones on growth and the GH/IGF-1 axis suggest that T may differentially

regulate growth of male-larger versus female-larger species via its ability to modulate the IGF-1 system. To understand why one sex becomes larger than the opposite sex, we need to first understand how SSD develops. Therefore, studies in our laboratory focus on physiological mechanisms that regulate growth and the development of SSD. In *Sceloporus* lizards, surgical castration accompanied by T replacement restores male growth in a male-larger species, while castration alone inhibits male growth (Cox and John-Alder, 2005). However, in female-larger species the opposite is found – i.e., surgical castration accompanied by T replacement inhibits male growth and castration alone promotes male growth (Cox et al., 2005a; Cox and John-Alder, 2005). Therefore, based on the limited number of species studied in our laboratory, hormonal regulation of growth may differ between male-larger and female-larger species. Furthermore, the mechanism by which T affects the GH/IGF-1 axis in male-larger versus female-larger species is unknown. The long-term goal of this research is to understand proximate mechanisms that cause growth differences that lead to SSD. The specific goal of this dissertation is to determine whether T regulates growth in a female-larger species of *Sceloporus* lizard via its ability to modulate the IGF-1 system.

Figure

Figure 1.1. The growth hormone/insulin-like growth factor-1 axis. Adapted from Duan, 1997.



CHAPTER II.

MOLECULAR CLONING OF HEPATIC IGF-1 cDNA AND SEQUENCE ANALYSIS IN LIZARDS

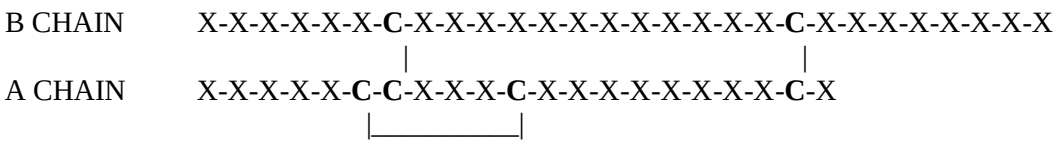
Abstract

Insulin-like growth factor-1 (IGF-1) is a critically important hormone involved in growth regulatory mechanisms. Previous work in lizards has demonstrated that testosterone inhibits growth in female-larger species, while stimulating growth in male-larger species. In mammals and fishes, androgenic steroids stimulate organismal growth and IGF-1, while estrogenic compounds have the opposite response. The opposing effects of androgens and estrogens documented in male-larger species suggest that the opposite effects of testosterone in lizards may reflect species differences in how testosterone affects IGF-1. Since the IGF-1 system has not been characterized in lizards to date, we needed to first develop and validate novel assay techniques in order to measure the response of IGF-1 to experimental treatments. To this end, we designed primers using conserved regions of IGF-1 from species with known sequences. Through the use of molecular cloning, we cloned a partial nucleotide sequence of IGF-1 from the liver of five species of lizard (*Sceloporus undulatus, Sceloporus jarrovii, Anolis sagrei, Coleonyx elegans,* and *Goniurosaurus lichtenfelderi*). We obtained deduced amino acid sequences that contain 55 amino acids for *S. undulatus* and 43 amino acids for *S. jarrovii*, *A. sagrei*, *C. elegans*, and *G. lichtenfelderi*. These species share a high percent of sequence similarity that ranges from 86 – 100%. Furthermore, compared to human IGF-1, the positions of nucleotides in lizard sequences vary 72 – 77%. Based on the identification of

IGF-1 sequences in these species, we can develop quantitative real-time PCR assays that are species-specific.

Introduction

Despite its functional diversity, the insulin/IGF superfamily is an ancient family that shares a conserved peptide structure. Thus far, functions of insulin or insulin-like peptides have been described in a wide range of taxonomic groups. These peptides are involved in growth, development, and metabolism. Furthermore, they can act through endocrine, paracrine, and autocrine mechanisms. The primary peptide sequence of each member of the family is characterized by three domains comprised of an amino terminal B chain joined to a carboxyl A chain by an intervening C chain (Blundell and Humbel, 1980). Highly conserved cysteine residues link the A and B domains by two interdomain sulfide bonds as well as form an intradomain disulfide bond within the A chain. The B and A domains are relatively invariant and exhibit a pattern of distinct and highly conserved cysteine motifs.



In humans, the IGF-1 gene consists of six exons. Transcription of this gene is initiated by exons 1 and 2 and produce IGF-1 mRNA that includes either exon 1 (class 1 transcript) or exon 2 (class 2 transcript) as the leader exon (Lund, 2011). Both classes of transcript include exons 3 and 4, which code for the mature IGF-1 peptide (Rotwein, 1991). The mature peptide contains domains B, C, A, and D and is approximately 70

amino acids, depending on the species. Among vertebrate species that have been studied, the components and regulation of the GH/IGF-1 axis are well-conserved in structure and function. Under the control of hypothalamic hormones, growth hormone releasing hormone (GHRH) and somatostatin (SST), GH is released from the anterior pituitary in a sex- and species-specific manner (Shapiro et al., 1995). Circulating GH stimulates transcription of IGF-1 in the liver, and this GH-induced increase in IGF-1 message is associated with an increase in circulating IGF-1. In circulation, IGF-1 binds to high-affinity IGF binding proteins (IGFBPs). These binding proteins extend the half-life, transport, and modulate biological responses of IGF-1. Once at the target tissues, IGF-1 stimulates growth and mediates much of the growth-promoting effects of GH.

Since the IGF-1 system has not been characterized in lizards to date, we needed to first develop and validate novel techniques to assay IGF-1. Because our species of interest are not well-studied with respect to molecular biology, we relied on regions with high sequence similarity among species to clone IGF-1. To this end, a partial sequence of IGF-1 was generated through the use of molecular cloning. Based on this fragment, primers for species-specific quantitative real-time PCR (qRT-PCR) were designed and used to measure the relative expression of the IGF-1 gene. To our knowledge, we are the first to clone IGF-1 from any species of reptile. Additionally, to measure plasma levels of IGF-1, we took advantage of reagents and assay systems that have been developed for aquaculture species to complete our analyses.

Methods

Using RNA isolated from livers, cDNA was generated and then amplified with primers that were designed against a conserved region of IGF-1. Subsequently, this amplicon was cloned into the pCR™4-TOPO® TA vector and sequenced.

All experimental procedures were approved by the Rutgers University Animal Care and Facilities Committee (protocol number 01-019) and the Czech Central Commission for Animal Protection (protocol number 18847/2003-1020).

RNA Isolation

Following rapid decapitation, fresh liver tissue was harvested and homogenized in TRIzol® (Invitrogen Corp., Carlsbad, CA, USA) using an RNase-free pestle and passed through an 18-gauge needle to shear cellular components with high molecular weight (*Sceloporus undulatus*). Alternatively, frozen liver tissue was homogenized with TRIzol® using a Teflon pestle and a glass homogenizing tube (*Sceloporus jarrovii*, *Anolis sagrei*, *Coleonyx elegans*, and *Goniurosaurus lichtenfelderi*). Total RNA was isolated using chloroform extraction and isopropanol precipitation, followed by DNase I treatment with DNA-free™ (*S. undulatus*; Ambion Inc., Austin, TX, USA) or using the Qiagen RNeasy Mini Kit according to the manufacturer's protocol (*S. jarrovii*, *A. sagrei*, *C. elegans*, and *G. lichtenfelderi*; Qiagen Inc., Valencia, CA, USA). RNA yield was quantified by using the ratio of absorbance at 260 and 280 nm on the GeneQuant II™ spectrophotometer (*S. undulatus*; Pharmacia Biotech Inc., Piscataway, NJ, USA) or on the NanoDrop® ND-1000 spectrophotometer (*S. jarrovii*, *A. sagrei*, *C. elegans*, and *G. lichtenfelderi*; Thermo Fisher Scientific Inc., Waltham, MA, USA). RNA integrity was

verified on a 1% formaldehyde denaturing gel stained with ethidium bromide as previously described (Cohick et al., 2000).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA (2 μg) was reverse transcribed according to the manufacturer's protocol using SuperScript™ II Reverse Transcriptase (*S. undulatus*; Invitrogen Corp.), the TaqMan® Reverse Transcription Reagents Kit (*S. jarrovii*; Applied Biosystems Inc., Carlsbad, CA, USA), or the High Capacity cDNA Reverse Transcription Kit (*A. sagrei*, *C. elegans*, and *G. lichtenfelderi*; Applied Biosystems Inc.).

Polymerase Chain Reaction (PCR) and Complementary DNA (cDNA) Cloning

Complementary DNA was amplified under low stringency 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: initial denaturation at 94°C for 10 minutes; cycles 1 – 30: 1 minute denaturation at 94°C, 1 minute annealing at 50 – 35°C, and 1 minute extension at 72°C; cycles 31 – 45: 1 minute denaturation at 94°C, 1 minute annealing at 35°C, and 1 minute extension at 72°C; final extension at 72°C for 10 minutes. For *S. undulatus*, primers (forward: 5'-GCGGGGCTGAGTTGGTGGAT-3'/reverse: 5'-CGCGCAGTACATCTCCAGCC-3') were designed based on the nucleotide sequence for bovine IGF-1 (*Bos sp.*; accession number X15726) to generate an amplicon of 170 bp in length. For *S. jarrovii*, *A. sagrei*, *C. elegans*, and *G. lichtenfelderi*, primers (forward: 5'-TTGGTGGATGCTCTTCAGTTTG-3'/reverse: 5'-

CAGGTCACAGCTTTGGAAACAA-3') were designed based on the sequence for *S. undulatus* IGF-1 to generate an amplicon of 135 bp in length. These primers amplify a region of IGF-1 that spans exons 3 and 4, which code for the mature IGF-1 peptide, and is present in all four transcripts of IGF-1, thus allowing the quantification of IGF-1 message in all transcripts. Following PCR, samples were visualized on a 2% agarose gel that was stained with ethidium bromide. Samples that expressed a band of the expected product size were subsequently purified using the QIAquick Gel Extraction Kit or the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, USA).

PCR products were cloned into the pCR™4-TOPO® TA vector using Subcloning Efficiency™ DH5α™ Competent *Escherichia coli* cells (*S. undulatus*; Invitrogen Corp.) or One Shot® TOP10 Chemically Competent *E. coli* cells (*S. jarrovii*, *A. sagrei*, *C. elegans*, and *G. lichtenfelderi*; Invitrogen Corp.). Bacterial cultures were grown overnight. Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen Inc.) and sequenced with T3 and T7 universal primers. Sequencing was done in Dr. Zylstra's laboratory at Rutgers University (*S. undulatus*) or using the ABI 3730*xl* DNA Analyzer (Applied Biosystems Inc.) for capillary electrophoresis and fluorescent dye terminator detection by GeneWiz DNA sequencing services (GeneWiz Inc., South Plainfield, NJ, USA) (*S. jarrovii*, *A. sagrei*, *C. elegans*, and *G. lichtenfelderi*). GeneWiz produced DNA trace chromatograms for the forward and reverse nucleotide sequences, which were analyzed and then aligned using ApE (A Plasmid Editor; Davis, 2009). If peaks on the chromatograms were difficult to read, especially at the beginning of the sequencing reaction, then these nucleotides were not included in subsequent analyses. The resulting sequences were compared to the GenBank database using the BLASTn

program. To obtain the amino acid sequences, sequential sets of three DNA bases were read as codons. The resultant amino acid sequences were aligned with IGF-1 sequences from other species of vertebrates.

Results

The length of the amplicon generated for *S. undulatus* was 167 nucleotides (additional nucleotides were removed from the beginning of the sequence, see *PCR and cDNA Cloning*). This partial nucleotide sequence aligned with 77% identity to bovine IGF-1 (Fig. 1) and contained the C domain as well as portions of the B and A domains of the full IGF-1 gene. The BLASTn search results confirmed that the amplicon had high sequence identity to IGF-1 from other species of vertebrates. Comparison of the deduced 55 amino acid sequence of IGF-1 demonstrated that the identity between *S. undulatus* IGF-1 and the analogous region in human, bovine, dog (*Canis sp.*), horse (*Equus sp.*), guinea pig (*Caveus porcellus*), and pig (*Sus scrofa*) is 80% (Fig. 3). Furthermore, amino acid alignments show that the C domain of the *S. undulatus* IGF-1 sequence contains the most variation when compared to human, with only 42% sequence identity (Table 1).

In addition to *S. undulatus*, partial IGF-1 was cloned from *S. jarrovii*, *A. sagrei*, *C. elegans*, and *G. lichtenfelderi*. The length of these amplicons was approximately 135 nucleotides. These sequences also contained the C domain as well as portions of the B and A domains. Comparison of the deduced 43 amino acid sequence of IGF-1 from these species confirmed high sequence identity between *S. jarrovii* (74%), *A. sagrei* (72%), *C. elegans* (74%), and *G. lichtenfelderi* (77%) and the corresponding region in human

(Table 1, Fig. 3). Sequence identity between species of lizards ranged from 86 – 100% (Table 2, Fig. 4).

Discussion

The insulin/IGF family of hormones is an ancient family that is conserved in structure and function. This high degree of conservation is likely due to the essential roles that these hormones play in development, growth, and metabolism. Pro-insulin, IGF-1, and IGF-2 share similarities in three domains that consist of an amino terminal B domain coupled to a carboxyl A domain by an intervening C domain (Blundell and Humbel, 1980). The sequence of amino acids in the B and A domains is well-conserved and contains cysteine residues that maintain tertiary structure as well as facilitate the binding of IGF-1 with its receptor (Cascieri et al., 1988; Bayne et al., 1990) and IGFBPs (Clemmons et al., 1992; Magee et al., 1999). The sequence of amino acids in the C domain is relatively variable between hormones and even between species for the same hormone. The C domain of human IGF-1 aids in receptor binding affinity by modulating the interaction of the B and A domains with its receptor (Bayne et al., 1987).

Not surprisingly, the sequences of IGF-1 that were cloned from *S. undulatus, S. jarrovii, A. sagrei, C. elegans,* and *G. lichtenfelderi* share the greatest identity with one another compared to published sequences from non-reptilian species (Fig. 3). Additionally, the highest percent of sequence identity is found between species of the same suborder of Squamata (Iguania: *S. undulatus*, *S. jarrovii*, and *A. sagrei*; Gekkota: *C. elegans* and *G. lichtenfelderi*; Table 2, Fig. 4). Furthermore, the two species of *Sceloporus* lizards (*S. undulatus* and *S. jarrovii*) share 100% sequence similarity. The

percent of sequence similarity is associated with the degree of relatedness between species.

Interestingly, the B and A domains of these lizard species are identical, which is likely attributed to their essential functions in maintaining tertiary structure and binding to the type 1 IGF receptor and IGFBPs. However, a high percent of variation in the amino acid sequence is found in the C domain (Table 1, Fig. 3). Our data corroborate the findings from previous reports that the most variation in the IGF-1 sequence is located in the C domain. In general, the variability in the C domain is likely due to the secondary role that it plays in receptor binding affinity.

To our knowledge, we are the first to clone IGF-1 cDNA from any species of reptile. The partial IGF-1 cDNA sequences that we have cloned from five species of lizards contribute to the growing body of literature that indicates that the mature IGF-1 peptide sequence is highly conserved across taxonomic groups. In addition, the generation of these IGF-1 cDNA sequences will aid in the development of species-specific primers for quantitative real-time PCR assays to measure the expression of IGF-1 message. Since there are no commercial reagents available for lizards, researchers can use these sequences to identify reagents that were developed for other species and share the highest percent of sequence similarity with their species of lizard.

Tables

Table 2.1. Similarity of IGF-1 amino acid sequence for each domain between human and species of lizards. The number in the numerator indicates the number of amino acids that were similar between human and lizard IGF-1 within a domain. The number in the denominator indicates the total number of amino acids that were cloned for lizard IGF-1 within a domain. Note that the C domain in *A. sagrei* contains 11 amino acids compared to 12 amino acids in human IGF-1. Percentage (%) of sequence similarity is provided in parentheses.

	B domain	C domain	A domain
S. undulatus	16/19 (84)	5/12 (42)	11/12 (92)
S. jarrovii	16/19 (84)	5/12 (42)	11/12 (92)
A. sagrei	16/19 (84)	4/12 (33)	11/12 (92)
C. elegans	16/19 (84)	5/12 (42)	11/12 (92)
G. lichtenfelderi	16/19 (84)	6/12 (50)	11/12 (92)

Table 2.2. Similarity of the IGF-1 amino acid sequence between species of lizards. Values in table represent the percent (%) of sequence similarity for IGF-1 between two species.

	S. undulatus	*S. jarrovii*	*A. sagrei*	*C. elegans*	*G. lichtenfelderi*
S. undulatus		100	91	86	88
S. jarrovii	100		91	86	88
A. sagrei	91	91		88	86
C. elegans	86	86	88		95
G. lichtenfelderi	88	88	86	95	

Figures

Figure 2.1. Alignment of the partial nucleotide sequence for IGF-1 cDNA from *Bos sp.* (accession number X15726) and *Sceloporus undulatus*. The cloned fragment of *S. undulatus* cDNA includes 167 nucleotides.

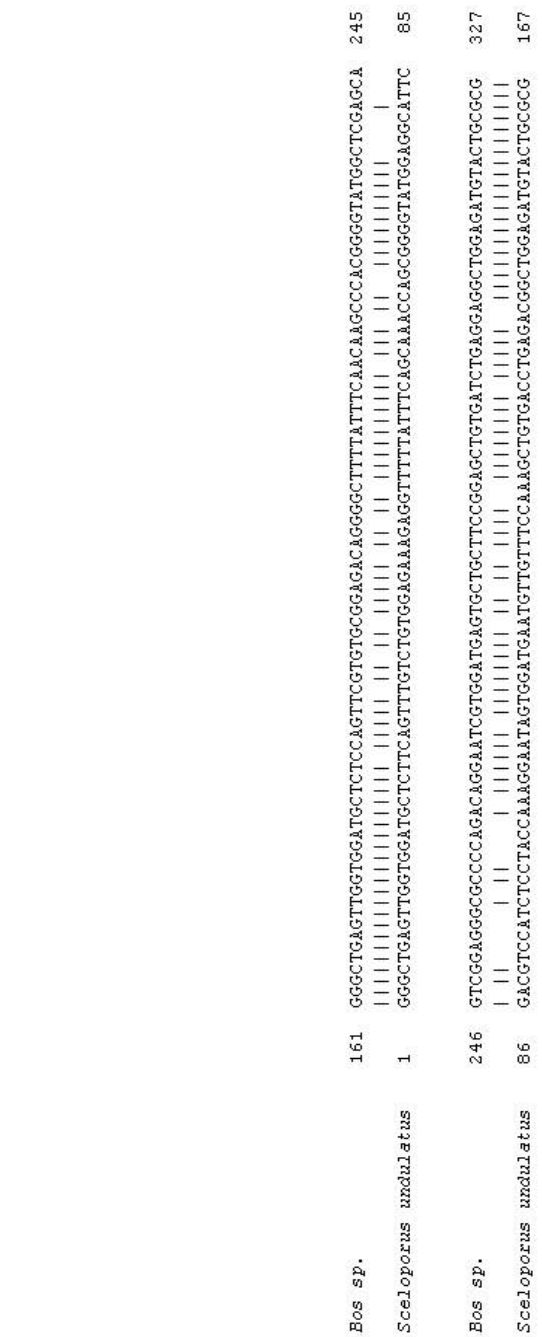


Figure 2.2. Alignment of the partial nucleotide sequence for IGF-1 cDNA from five species of lizard. Gray letters in the *Sceloporus undulatus* sequence indicate that at least one species differs from the others at this site. In all other sequences, dashed lines represent similarities and letters indicate differences in amino acid sequence compared to *Sceloporus undulatus* IGF-1. Periods were used to maximize alignment between species.

IGF-1 Nucleotide Sequence Chart

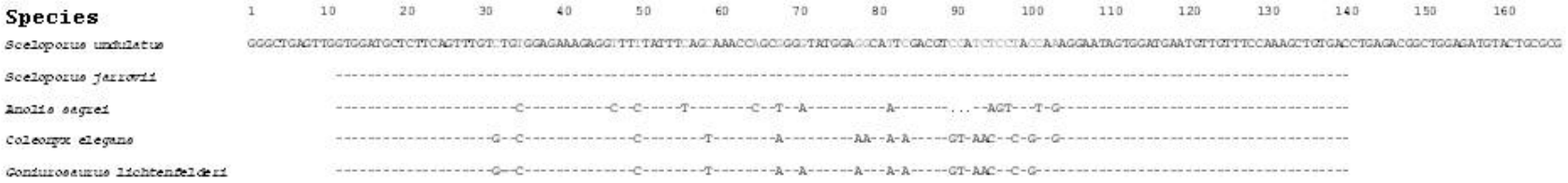


Figure 2.3. Alignment of the IGF-1 amino acid sequence for several vertebrate species. Gray letters in the human sequence indicate that at least one species differs from the others at this site. In all other sequences, dashed lines represent similarities and letters indicate differences in amino acid sequence compared to human IGF-1. Periods were used to maximize alignment between species. Box highlights the IGF-1 sequence from species of lizards. References: [1] Rinderknecht and Humbel, 1978; [2] Francis et al., 1988; [3] Shimatsu and Rotwein, 1987; [4] Bell et al., 1986; [5] Ballard et al., 1990; [6] Kajimoto and Rotwein, 1990; [7] Duguay et al., 1996; [8] Cao et al., 1989; [9] Hashimoto et al., 1997; [10] Degger et al., 2000; [11] Reinecke et al., 1997; [12] McRory and Sherwood, 1994; [13] Duguay et al., 1995.

IGF-1 Amino Acid Sequence Chart

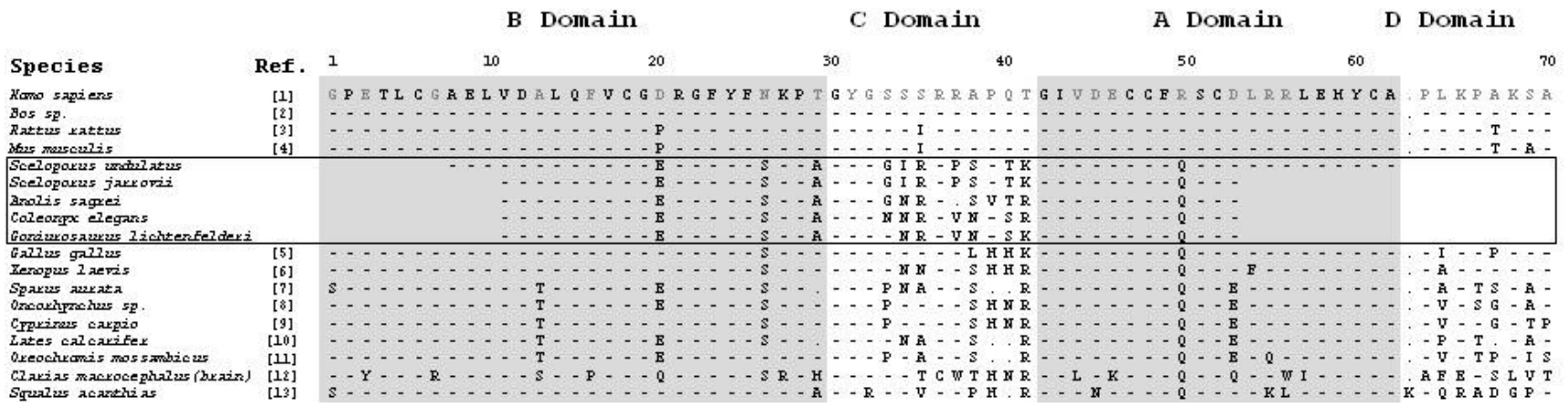
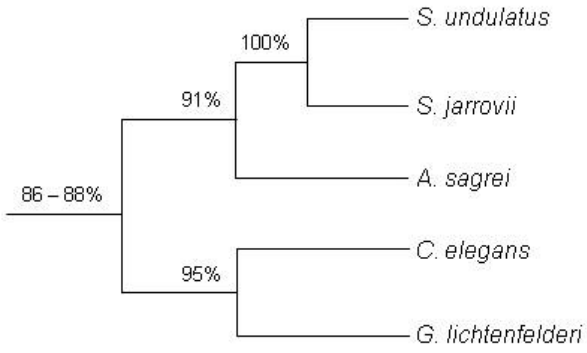


Figure 2.4. Percent of sequence similarity plotted on an abbreviated phylogeny of lizard species. The percentages (%) on the phylogeny indicate the sequence similarity to *S. undulatus*. The branch lengths are arbitrary.



CHAPTER III.

NUTRITIONAL MODULATION OF HEPATIC IGF-1 MESSAGE AND PLASMA IGF-1 IN RELATION TO GROWTH AND BODY CONDITION IN *SCELOPORUS* LIZARDS

Abstract

Previous investigators have concluded "the principal environmental regulator of the growth hormone/insulin-like growth factor (GH/IGF) axis is nutritional status" (Pierce et al., 2005; Pedroso et al., 2006). However, reliable data on food intake and the nutritional status of free-living animals – especially those upon which the conclusion is based – are scarce and notoriously difficult to obtain. Furthermore, discrepancies in the literature (e.g., lack of an effect of ration size on hepatic IGF-1 message; discordant regulation of hepatic IGF-1 message and plasma IGF-1) indicate that correlations between nutritional status, hepatic IGF-1 message, and plasma IGF-1 are more complex than generally acknowledged. Studies on lizards can help to fill these gaps in our understanding of 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, we report results of three experiments on two species of *Sceloporus* lizards, which were designed to determine if nutritional status regulates the GH/IGF-1 axis in lizards. Hepatic IGF-1 message levels were determined in (1) juveniles of *S. jarrovii* maintained on a full or 1/3 ration (FR vs. 1/3R) for 10 weeks, (2) hatchlings of *S. undulatus* subjected to total food deprivation (zero ration, ZR) with or without re-feeding (RF) for a period of 10 days and (3) adults of *S. undulatus* subjected to total food

deprivation with or without re-feeding for a period of 15 days. Plasma IGF-1 levels were also measured in the later study. In *S. jarrovii,* hepatic IGF-1 message was unchanged by 1/3R, which is probably representative of food intake in the wild. In *S. undulatus*, however, ZR caused a reduction in both hepatic IGF-1 message and plasma IGF-1, and RF restored these parameters to levels of FR. Our results demonstrate that complete food deprivation, not simply a reduction in food intake, is required to depress hepatic transcription and production of IGF-1, a finding consistent with previous work in other classes of vertebrates. In free-living animals, the extreme nutritional deficit of prolonged periods of fasting without food is likely to decrease the activity of the GH/IGF-1 axis. However, even under nutritionally suboptimal conditions, as long as animals can maintain positive energy balance, variation in their food intake probably has little effect on the hepatic production of IGF-1. In these circumstances, IGF-1 has an important permissive role, but variation in food intake itself is the primary determinant of growth rate.

Introduction

Throughout development, animals encounter conditions (e.g., food abundance and scarcity) that may or may not support positive growth rates. In turn, growth rate, as it is manifested in body size, may influence the ability of individuals to compete, reproduce, and escape predation (Arendt, 1997; Blanckenhorn, 2000). Variable growth rate in free-living animals is a classic example of phenotypic modulation, where a phenotypic trait (growth rate) is modulated by environmental factors. In this case, the availability of food is one of the prime environmental regulatory factors. Previous investigators have

concluded that “the principal environmental regulator of the GH/IGF axis is nutritional status” (Pierce et al., 2005; Pedroso et al., 2006). However, the applicability of this conclusion to free-living animals in the wild has not been examined. Studies on lizards can help to fill this gap in knowledge because many species are relatively easy to observe and manipulate both in the laboratory and in the field.

Growth is a long-term process that requires an orchestration of highly complex interactions. During this process, homeorhetic control partitions nutrients to maintain a higher order of endocrine regulation that supersedes homeostasis (Bauman et al., 1982). Furthermore, homeorhetic factors “operate on a ‘chronic’ basis and involve a ‘coordination’ of physiological processes and tissue metabolism” (Bauman, 1999). Perhaps most notably, Bauman et al. (1982) described growth hormone (GH) as a homeorhetic factor due to its involvement in processes including growth and milk production as well as its effects on the metabolism of nutrients.

The GH/insulin-like growth factor-1 (IGF-1) axis is the central growth-regulatory endocrine system in vertebrates. Among species that have been studied, the components and regulation of the GH/IGF-1 axis are well conserved in structure and function (Duan, 1998). Briefly, under anabolic conditions, plasma GH stimulates transcription of IGF-1 in the liver, and this GH-induced increase in IGF-1 message leads to an increase in plasma IGF-1. In circulation, more than 99% of IGF-1 binds to high-affinity IGF binding proteins (IGFBPs) which have multiple functions (Frystyk et al., 1994). These binding proteins serve as transporters for IGF-1 and modulate its half-life and biological effects. At target tissues, IGF-1 mediates much of the growth-promoting effect of GH. Under catabolic conditions (e.g., food deprivation), the axis is uncoupled, meaning that GH no

longer stimulates hepatic IGF-1 message. Instead, GH increases (except in rats; Thissen et al., 1994) and exerts direct effects on lipid mobilization for energy, while IGF-1 remains constant or even decreases due to the development of GH resistance at the level of the liver (mammals: Thissen et al., 1994; birds: Scanes, 1997; fishes: Duan, 1998). Furthermore, the presence of low molecular weight IGFBPs is stimulated during catabolism.

Nutrition has been described as having a "profound effect on the GH/IGF axis" (Duan, 1998; Moriyama et al., 2000). That conclusion is based on studies in which complete food deprivation led to increases in plasma GH, while having the opposite effect on hepatic IGF-1 message and plasma IGF-1. Complete food deprivation also elevates levels of corticosteroids, which are known to have an inhibitory effect on growth. This negative effect on growth may be modulated, at least in part, by the GH/IGF-1 axis (Beckman, 2011).

Few studies have reported the effects of more modest constraints on food intake, as might commonly be observed under natural conditions. In immunocastrated boars, a 50% decrease in ration size led to significantly lower levels of plasma IGF-1 (Bauer et al., 2009). Furthermore, in young Japanese quails (*Coturnix japonica*), a weight-maintenance diet decreased hepatic IGF-1 message after 2 days but had similar effects as ad libitum food intake after 5 days of food restriction (Rønning et al., 2009). When various ration sizes (ranging from zero ration to ad libitum) were offered to young *Sparus aurata* (gilthead bream), plasma IGF-1 increased as ration size increased, with the exception that fingerlings on 50% ration had the highest level (Pérez-Sánchez et al., 1995). When *Oncorhyncus kisutch* (coho salmon) were offered a high, medium, or low

ration (1.6, 1.1, and 0.6% body weight/day, respectively), the low ration significantly decreased plasma IGF-1 with respect to the comparable levels measured in medium and high ration at the end of the 8-week treatment period (Pierce et al., 2001). Another study with *O. kisutch* investigated the link between constant and varying levels of food availability on plasma IGF-1 and found that plasma IGF-1 increased with increasing food intake (Beckman et al., 2004). Additional studies have found that a reduction in dietary protein causes a decrease in plasma IGF-1 (Pérez-Sánchez et al., 1995; Crain et al., 1995).

Nevertheless, discrepancies among studies indicate that relationships involving nutrition, growth, and IGF-1 are complex. For instance, in *S. aurata*, the size and composition of the food ration affected growth rate but did not affect hepatic IGF-1 message (Metón et al., 2000). Furthermore, in *Morone chrysops* x *Morone saxatilis* (hybrid striped bass), responses of hepatic IGF-1 message in response to a reduction in diet and re-feeding were inversely related to changes in plasma IGF-1. Limited food availability led to an increase in hepatic IGF-1 message and a decrease in plasma IGF-1, whereas hepatic IGF-1 message decreased and plasma IGF-1 increased during the compensatory growth response relative to the ad libitum fed controls (Picha et al., 2006). Although numerous experiments have investigated the effects of complete food deprivation on the IGF-1 system, little information is known regarding the effects of ecologically relevant levels of food ration on IGF-1. Therefore, we designed a series of experiments to provide additional insight into relationships between nutritional status, growth rate, hepatic IGF-1 message, and plasma IGF-1. The primary objective of our study was to characterize the effects of ration size – moderate and complete food

deprivation – on growth and the IGF-1 system in *Sceloporus* lizards. We present the results of three experiments performed in the laboratory involving 1) yearling *Sceloporus jarrovii* (Yarrow's spiny lizard) on full or 1/3 ration (FR versus 1/3R), 2) hatchling *S. undulatus* (eastern fence lizard) subjected to total food deprivation (zero ration, ZR) with and without re-feeding (RF), and 3) adult *S. undulatus* subjected to the same conditions as the hatchlings of this species.

Methods

Animal Collection and Care

The collection and care of *S. jarrovii* have previously been described by Cox et al. (2008). Briefly, in September 2004, male and female yearling lizards (2 – 3 mo. of age) were captured in the Chiricahua Mountains, Coronado National Forest, AZ, USA (31°54'–31°55'N, 109°16'W) under permit from the Arizona Game and Fish Department (SP 553889). In September 2008, hatchling male and female *S. undulatus* (1 – 2 mo. of age) were collected from the New Jersey Pinelands, New Jersey, USA under permit from the New Jersey Department of Environmental Protection, Division of Fish and Wildlife (SC28068). Hatchling lizards were captured by hand in the vicinity of Rutgers University Pinelands Research Station in New Lisbon, Burlington County, NJ (41°N, 74°35'W). In May 2009, reproductively mature *S. undulatus* males (at least two years old) were collected by hand and hand-held noose from the same field site as the hatchlings (SC 2909C). 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 10h basking period. Cages were arranged under fluorescent bulbs (General Electric Chroma 50, General Electric Company, Fairfield, CT, USA) for ultraviolet radiation on a 12:12 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).

Experimental Design

Prior to dietary manipulation, 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. During experiments, body condition and growth rate were monitored via measurements of SVL and body mass. Change in body condition residuals were calculated by regressing $\log_{10}$ body mass against log SVL_{10} using data collected prior to the beginning of the experiment (initial) and at the conclusion of the experiment (final) (Cox and John-Alder, 2007). A single pooled regression line was estimated from all experimental lizards at both time points to calculate initial and final residuals for each individual. Based on this regression, a directional change for each individual was determined by subtracting the initial residual from the final residual. Growth rate was calculated by dividing an individual's change in SVL by the number of days on the experimental treatment. To provide a more direct comparison between experiments, we also calculated normalized growth rate for each

lizard by dividing the growth rate of each individual by the highest growth rate within the experiment. Unlike rapidly growing hatchling *S. undulatus* (initial SVL = 27 – 39 mm), we did not expect treatment to affect growth rates of adult *S. undulatus* because these lizards were on the asymptotic part of the predicted growth curve for this species (initial SVL = 54 – 70 mm; Haenel and John-Alder, 2002; John-Alder et al., 2007). Feeding rate (reported as crickets/d) was measured by counting uneaten crickets weekly (*S. jarrovii*) or after the conclusion of the experimental periods (*S. undulatus*). 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 measurement that can only be used to make comparisons within an experiment.

Experiment 1: *S. jarrovii*

S. jarrovii yearlings were divided into one of two size-matched dietary treatment groups that were also balanced for sex: full ration (FR; 3 crickets/d x 10wks; males: n = 11; females: n = 8) and 1/3 ration (1/3R; 1 cricket/d x 10wks; males: n = 10; females: n =12). Individuals in the full ration group were essentially fed an ad libitum diet (see Fig. 2A in Cox et al., 2008).

Experiment 2: *S. undulatus* hatchlings

Hatchling *S. undulatus* were assigned to one of four size-matched dietary treatment groups that were also balanced for sex: field fresh (FF; males: n = 3; females: n = 8), full ration (FR; 6 crickets/d x 10d; males: n = 4; females: n = 7), zero ration (ZR; 0 crickets/d x 10d; males: n = 3; females: n = 8), and re-fed (RF; zero ration x 7d and full

ration x 3d; males: n = 5; females: n = 6). At the time of sacrifice, hatchlings were too small to provide sufficient blood plasma for assay of plasma IGF-1 and were used only for measurements of hepatic IGF-1 message. Males and females were combined for statistical analyses.

Experiment 3: *S. undulatus* adults

Adult *S. undulatus* were used in the third experiment so that we would be able to collect blood plasma for measurements of plasma IGF-1, even though we did not anticipate a measureable response in growth over the brief experimental period. Adult male *S. undulatus* were assigned to one of three size-matched dietary treatment groups: full ration (FR; 6 crickets/d x 15d; n = 9), zero ration (ZR; 0 crickets/d x 11d; n = 10), and re-fed (RF; ZR x 11d and FR x 4d; n = 10).

RNA Isolation, Reverse Transcription Polymerase Chain Reaction (RT-PCR), and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Following rapid decapitation, liver tissues were collected, flash frozen on dry ice, and stored at -80°C until analysis. Liver tissue was homogenized with TRIzol® using a Teflon pestle and a glass homogenizing tube. Total RNA was isolated from experimental samples according to the manufacturer's protocol using Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Carlsbad, CA, USA). RNA yield was quantified at an absorbance of 260/280 nm using the NanoDrop® ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA), and integrity was verified by electrophoresis

on a 1% formaldehyde denaturing gel as previously described (Cohick et al., 2000). Gene-specific primers (IGF-1: forward: 5'-TTGGTGGATGCTCTTCAGTTTG-3'/reverse 5'-CAGGTCACAGCTTTGGAAACAA-3'; β-actin: forward: 5'-GAAGAGGAAGCAGCTGTGGC-3'/reverse: 5'-GCTATGTTGCCTTGGACTTCG-3') were designed based on the nucleotide sequence of clones using Primer Express® software (Applied Biosystems Inc.) to amplify 135 bp and 52 bp, respectively. Complementary DNA (cDNA) was fluoresced with SYBR® Green (Applied Biosystems Inc.), and the threshold cycle (C_T) of each sample was recorded on an ABI 7900HT. Standard curves of pooled cDNA were serially diluted so that the curve ranged from 250 ng to 25 pg. Amplification efficiency of standard curves ranged from 88-96%. Relative quantification of IGF-1 was normalized to β-actin (for *S. undulatus* adults, normalized IGF-1 was calculated by dividing the C_T for IGF-1 by the average C_T for β-actin), which was unaffected by dietary intake, and then compared to a calibrator (i.e., pooled RNA from experimental animals). The data were $\log_{10}$ transformed and were expressed as a fold-change of expression levels. The appropriate negative controls were run (i.e., No-RT control and master mix of reagents without template) to ensure the absence of DNA and non-specific amplification. The presence of a specific product was verified by visually inspecting the dissociation curves for each sample.

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

(Fisher Scientific, Pittsburgh, PA, USA). Plasma was separated via centrifugation and stored at -20° or -80°C until further analysis.

For RIA of corticosterone, plasma samples 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, Inc., Calabasas Hill, CA, USA). Inter-assay variation was 7.5% for *S. jarrovii*. Intra-assay variation was 8% for *S. jarrovii*, 19% for hatchling *S. undulatus*, and 25% for adult *S. undulatus*.

Assays for IGF-1 were conducted in G. Grau's laboratory at the Hawaii Institute of Marine Biology, University of Hawaii. For RIA of IGF-1, proteins were extracted from plasma with acid-ethanol followed by cryoprecipitation to separate IGF-1 peptide from the IGFBPs (Shimizu et al., 1999). Reconstituted samples were assayed in duplicate with ^{125}I-recombinant salmon/trout (*Oncorhynchus sp.*) IGF-1 and rabbit anti-recombinant barramundi (*Lates calcarifer*) IGF-1 as the antiserum (Novozymes Biopharma AU Ltd., Adelaide, SA, AUS). The hormone complexes bound to antibody were precipitated from free radiolabel by the addition of goat anti-rabbit (Sigma-Aldrich, St. Louis, MO, USA). This assay was validated by demonstrating parallel binding to anti-recombinant barramundi IGF-1 in *S. undulatus* plasma (pool from intact *S. undulatus*; gift from Dan Warner) compared to a salmon/trout standard (Novozymes Biopharma AU Ltd.) and *Oreochromis mossambicus* plasma. The intra-assay variation, based on four aliquots of *S. undulatus* pooled plasma included in the assay, was 16%.

Statistical Analyses

All statistical analyses were performed using SAS 9.2 (version 9.2, SAS Institute Inc., Cary, NC, USA), and comparisons were made between control and treated groups within an experiment. 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, log-transformed values for hepatic IGF-1 message, and plasma corticosterone were analyzed using one-way ANOVA. When significant treatment effects were observed ($P < 0.05$), post hoc separation of groups were determined using the Ryan-Einot-Gabriel-Welsch test (REGWQ; SAS Institute 2002). Plasma IGF-1 was analyzed by non-parametric Kruskal-Wallis ANOVA.

Results

Feeding Rate, Body Condition, and Growth Rate

In the *S. jarrovii* experiment, animals on the FR consumed an average of 2.62 crickets/day versus 0.98 crickets/day on 1/3R (Table 1; see Fig. 1 in Cox et al., 2008). In the *S. undulatus* hatchling experiment, animals on the FR consumed an average of 4.48 crickets/day throughout the experimental period (days 1 – 10) versus 3.09 crickets/day in RF during the re-feeding period on days 8 – 10 ($F_{3,29} = 182.44$, $P < 0.0001$; Table 1). Adult *S. undulatus* offered FR consumed an average of 1.83 crickets/day (days 1 – 15) versus 3.21 crickets/day in RF on days 12 – 15 ($F_{2,26} = 41.45$, $P < 0.0001$; Table 1). Additionally, ZR consumed 0 crickets/day.

In all three experiments, body condition was lower in lizards on restricted rations than in the corresponding FR groups (*S. jarrovii*: $F_{1,39} = 8.69$, $P = 0.0054$, *S. undulatus* hatchlings: $F_{2,30} = 29.38$, $P < 0.0001$, *S. undulatus* adults: $F_{2,26} = 29.91$, $P < 0.0001$; Fig.

1). Additionally, in both hatchling and adult *S. undulatus*, re-feeding for a brief period of time was sufficient to restore body condition relative to the full ration group.

In *S. jarrovii*, lizards in the 1/3R group continued to grow, as measured by increases in SVL, but their growth rate was approximately 50% lower than FR (Table 1, Fig. 2A; see Fig. 2A in Cox et al., 2008). In hatchling *S. undulatus*, ZR dramatically reduced growth rate by about 80% ($F_{2,30} = 10.85$, $P = 0.0003$; Table 1, Fig. 2B) and was without effect on *S. undulatus* adults ($F_{2,26} = 2.26$, $P = 0.1244$; Table 1, Fig. 2C).

Hepatic IGF-1 Message and Plasma IGF-1

Long-term food restriction (1/3R) did not affect expression of hepatic IGF-1 message in *S. jarrovii* ($F_{1,35} = 0.34$, $P = 0.5616$; Fig. 3A). However, a shorter experimental period with a more extreme dietary manipulation (ZR) led to a 5-fold decrease in hepatic IGF-1 message in *S. undulatus* hatchling, while RF was comparable to FF and FR ($F_{3,39} = 14.36$, $P < 0.0001$; Fig. 3B). Similarly, in adults of *S. undulatus*, ZR decreased hepatic IGF-1 message by 3-fold, which RF restored to the same level as FR ($F_{2,26} = 22.19$, $P < 0.0001$; 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). Using this RIA, we found that ZR decreased plasma IGF-1 in adult *S. undulatus*. After re-feeding, plasma IGF-1 in RF was similar to FR ($P = 0.0093$; Fig. 4B).

Plasma Corticosterone

At the conclusion of the treatment period, levels of plasma corticosterone were similar among treatment groups in *S. jarrovii* ($F_{1,37} = 1.10$, $P = 0.3002$; Fig. 5A; Carsia, McIlroy, Cox, Barrett, and John-Alder, unpublished) and *S. undulatus* adults ($F_{2,22} = 0.55$, $P = 0.5830$; Fig. 5C). In contrast, ZR elevated plasma corticosterone in *S. undulatus* hatchlings and RF returned corticosterone to the same level as FR ($F_{2,29} = 4.08$, $P = 0.0274$; Fig. 5B).

Discussion

Contrary to prediction, in *S. jarrovii*, 1/3R did not decrease expression of hepatic IGF-1 message (Fig. 3A). Although 1/3R reduced body condition and decreased growth rate compared to FR, this dietary treatment still allowed for positive growth and did not affect levels of plasma corticosterone measured at the end of the experiment (Fig. 1A, 2A 5A; Carsia, McIlroy, Cox, Barrett, and John-Alder, unpublished). Compared to free-living *S. jarrovii* of similar SVL, 1/3R grew more slowly and FR grew more quickly during the treatment period (Cox and John-Alder, 2007). Therefore, lizards on FR were consuming a nutritional surplus, which indicates that this dietary manipulation involved a ration greater than what might be available in a natural environment. Altogether, these results support the conclusion that food restriction in *S. jarrovii* probably did not impose the intended nutritional stress and should not have been expected to affect hepatic IGF-1 message because the literature draws conclusions from extremes of food intake.

The conclusion that nutritional status is a principal environmental regulator of the GH/IGF-1 axis is based on numerous studies involving dietary manipulations under

controlled laboratory conditions (Duan, 1998; Pierce et al., 2005; Pedroso et al., 2006). However, after a more careful analysis of the experiments, it becomes apparent that complete food deprivation, not a modest constraint, may be required to affect hepatic IGF-1 message. Additional studies have found evidence to suggest that expression of hepatic IGF-1 message is stable within a range of food intake (Pérez-Sánchez et al., 1995; Pierce et al., 2001; Beckman et al., 2004; Bauer et al., 2009; Rønning et al., 2009). In other words, above a nutritional threshold, hepatic IGF-1 message is transcribed and further increases in food intake have little if any effect. Alternatively, in food-restricted (1/3R) *S. jarrovii*, an early and transient response in hepatic IGF-1 message may have occurred during this long-term experiment but went unsubstantiated since hepatic IGF-1 message was only measured once. In *Coturnix japonica*, the expression of hepatic IGF-1 message in response to food restriction was measured over the course of 5 days. In that experiment, the group on a weight-maintenance diet expressed less hepatic IGF-1 message compared to the ad libitum group following 2 days of food restriction, however these groups had similar levels of expression after 5 days of food restriction due to a decline in the ad libitum-fed group (Rønning et al., 2009). Furthermore, in *Oncorhyncus kisutch*, a time course experiment measured the effects of three different ration sizes on plasma IGF-1 throughout 8 weeks of treatment. After 4 weeks, high ration had the highest levels of plasma IGF-1, however high and medium rations had equally high levels of plasma IGF-1 after 8 weeks compared to the low ration (Pierce et al., 2001). These findings are important in the context of our experiment with *S. jarrovii* since it provides evidence for the transient expression of hepatic IGF-1 message. In this vein, food restriction might have been initiated in a downward step function which took the lizards

several days or weeks to make the necessary metabolic adjustments. During this transition, hepatic IGF-1 message might have decreased and then eventually rebounded.

Building on the results from *S. jarrovii*, we set up two subsequent experiments to administer a more severe dietary restriction over a shorter period, which involved complete food deprivation with or without re-feeding. In hatchling *S. undulatus*, ZR was sufficient to decrease hepatic IGF-1 message, body condition, and growth rate as well as increase plasma corticosterone, while RF restored these parameters to FR (Fig. 3B, 1B, 2B, 5B). Additionally, we manipulated the diets of adult *S. undulatus* in order to collect adequate volumes of blood plasma to measure IGF-1 hormone in circulation, which allowed us to evaluate whether hepatic IGF-1 message is a reliable indicator of plasma IGF-1. At the conclusion of the treatment period, ZR decreased hepatic IGF-1 message, plasma IGF-1, and body condition, which was restored by RF (Fig. 3C, 4B, 1C), however food intake did not affect growth rate or plasma corticosterone (Fig. 2C, 5C). Since we used lizards that were at least two years old for this experiment, we did not expect diet to affect growth rate, especially in such a short period of time, because these lizards were on the asymptotic part of their growth trajectories (Haenel and John-Alder, 2002; John-Alder et al., 2007). Furthermore, this experiment was conducted during the end of the breeding season (May) when these lizards have relatively low levels of plasma corticosterone compared to earlier in the breeding season (April) (John-Alder, et al., 2009). Previous work in *S. undulatus* has also demonstrated that males are less sensitive to stress during the non-breeding season compared to the breeding season as measured by a decline in sensitivity to adrenocorticotropin (ACTH) (Carsia and John-Alder, 2003). Altogether,

these data support the idea that adult males were less sensitive to complete food deprivation due to a decrease in sensitivity to ACTH.

Traditionally, corticosterone is known as a stress hormone that aids in survival by mobilizing energy stores. More recently, studies have emerged that suggest that long-term elevation of corticosterone has a detrimental effect. For example, in Gálapagos marine iguanas (*Amblyrhynchus cristatus*), males that survived an El Niño-induced famine had a greater ability to terminate the response by reducing their levels of plasma corticosterone compared to males with a less robust response (Romero and Wikelski, 2010). In populations of species with limited breeding opportunities, such as garter snakes that inhabit northern ranges (*Thamnophis sirtalis parietalis*), males can modify their stress response based on the time of year (i.e., breeding versus non-breeding seasons) (Moore et al., 2001). During the breeding season, these males do not experience a change in corticosterone in response to capture stress, while males from southern populations with an extended breeding season (*T.s. concinnus*) had increase levels of corticosterone in response to capture stress.

Based on the results from our experiments on *Sceloporus* lizards, we have developed a model to illustrate the relationships between food ration, growth rate, hepatic IGF-1 message, and plasma IGF-1 (Fig. 6). Data from young, rapidly growing lizards (i.e., yearling *S. jarrovii* and hatchling *S. undulatus*) demonstrate that differing levels of food availability led to dramatic differences in growth between treatment groups. Under ZR, lizards cannot support positive growth and had relatively low expression of hepatic IGF-1 message as well as plasma IGF-1. The inflection in growth rate illustrates the point where energy intake becomes positive and sufficient to support growth. When offered

1/3R, lizards maintained positive growth and hepatic IGF-1 message was similar to FR, even though food intake and growth rate were lower. These data demonstrate that hepatic IGF-1 message is permissive across a range of food intake and is not continuously scaled to food intake. Furthermore, our results suggest that a nutritional threshold affects the association of hepatic IGF-1 message and growth regulation, and complete food deprivation is necessary to couple hepatic IGF-1 message and growth.

Throughout development, animals experience periods of rapid growth, which are influenced by external variables. During periods of growth, animals are met with challenges, such as food shortage, that may disrupt their growth patterns. How an animal responds to this fluctuation may affect size-dependent life history traits. In order to combat this disturbance, animals have developed mechanisms to cope with and even overcome unfavorable conditions (e.g., dauer formation, adult diapause, extreme temperatures, food shortage, compensatory growth). Authors have suggested that such life history trade-offs may be regulated by molecular signals (Leroi, 2001). Of particular interest is the potential for IGF-1 to mediate the trade-off between resistance to fluctuating patterns of food availability and the capacity for rapid growth.

Altogether, the results from nutritional manipulation in *Sceloporus* lizards indicate that IGF-1 in lizards responds to food intake in a similar pattern as observed in other vertebrates. These data contribute to an ever-expanding body of literature that illustrates the importance of nutrition as a regulator of IGF-1. However, this series of experiments, which involved varying levels of food intake, suggest that the natural significance of nutritional regulation of IGF-1 is not well understood. Ecologically

realistic levels of nutritional manipulation may not support the conclusion that normal variation in food consumption is a key environmental regulator of IGF-1.

Table

Table 3.1 Effect of food ration on feeding rate and growth rate in *S. jarrovii*, *S. undulatus* hatchlings, and *S. undulatus* adults. Lowercase letters denote statistical significance between treatment groups.

Species	Age Class	Food Ration	Amount of Time Fed	Mean Feeding Rate (crickets/d) ± 1 SE	Mean Growth Rate (mm/d) ± 1 SE
S. jarrovii	Yearlings	Full	10 weeks	2.62 ± 0.07 [a]	0.21 ± 0.01 [a]
		1/3	10 weeks	0.98 ± 0.01 [b]	0.10 ± 0.01 [b]
S. undulatus	Hatchlings	Full	10 days	4.48 ± 0.14 [a]	0.32 ± 0.03 [a]
		Zero	0 days	0.00 ± 0.00 [b]	0.06 ± 0.06 [b]
		Re-fed	3 days	3.09 ± 0.30 [c]	0.08 ± 0.05 [b]
S. undulatus	Adults	Full	15 days	1.83 ± 0.13 [a]	0.13 ± 0.04
		Zero	0 days	0.00 ± 0.00 [b]	0.05 ± 0.03
		Re-fed	4 days	3.21 ± 0.41 [c]	0.10 ± 0.02

Figures

Figure 3.1. Effect of food ration on change in body condition in A) *S. jarrovii*, B) *S. undulatus* hatchlings, and C) *S. undulatus* adults. Mean values ± 1 SE. Lowercase letters denote statistical significance between treatment groups.

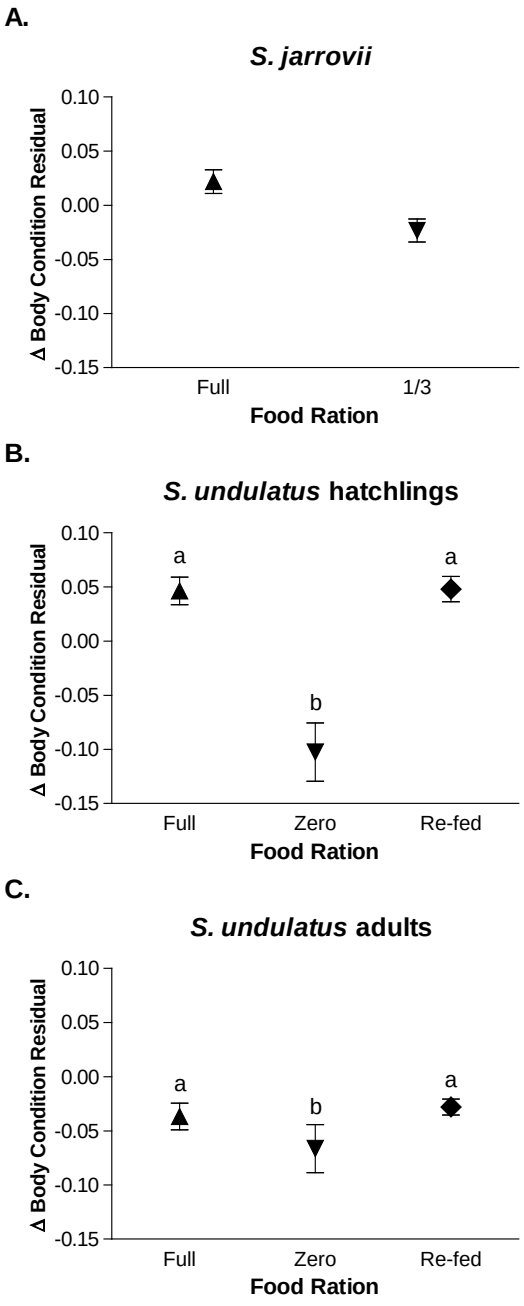


Figure 3.2. Effect of food ration on normalized growth rate in A) *S. jarrovii*, B) *S. undulatus* hatchlings, and C) *S. undulatus* adults. Normalized growth rate for *S. jarrovii* was redrawn from Cox et al., 2008. Mean (+1 SE) normalized growth rate. Lowercase letters denote statistical significance between treatment groups.

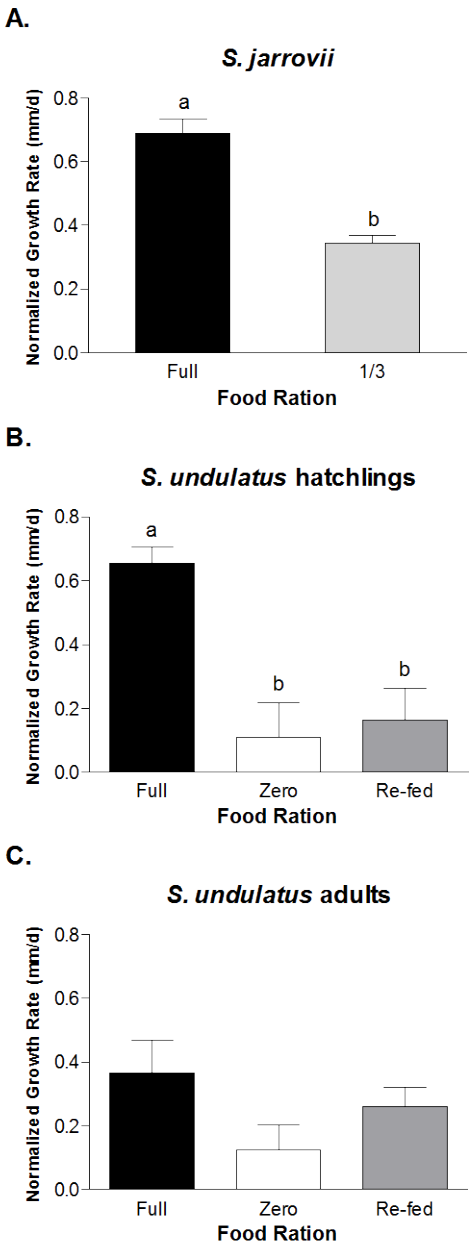


Figure 3.3. Effect of food ration on hepatic IGF-1 message in A) *S. jarrovii*, B) *S. undulatus* hatchlings, and C) *S. undulatus* adults. Lines represent median values within treatment group. Lowercase letters denote statistical significance between treatment groups.

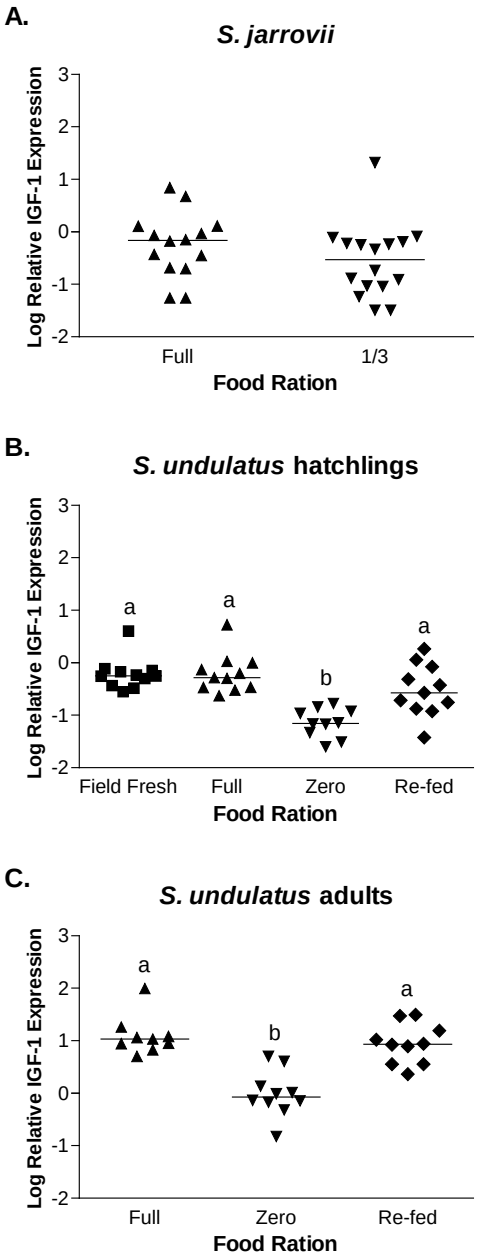
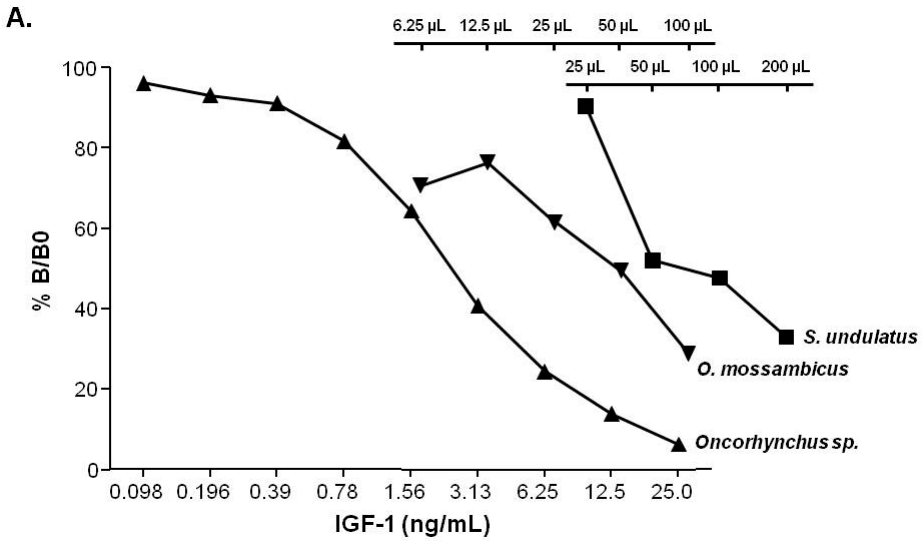


Figure 3.4. Validation of a heterologous radioimmunoassay for use in *Sceloporus* lizards. A) Percent ^{125}I-IGF-1 binding (%B/B0) is plotted against known concentrations of plasma IGF-1 for *Oncorhynchus sp.*and against volumes of extracted plasma for *S. undulatus* and *O. mossambicus*. B) Effect of food ration on plasma IGF-1 in *S. undulatus* adults. Mean (+1 SE) plasma IGF-1.



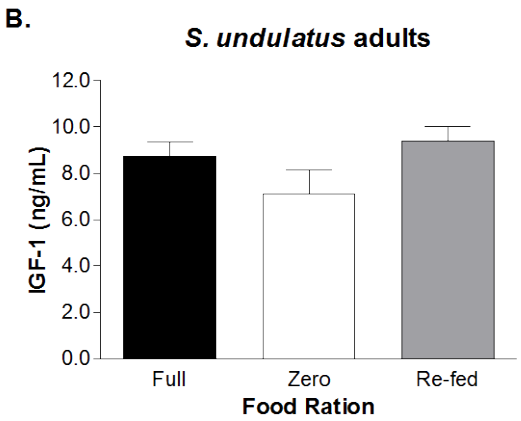


Figure 3.5. Effect of food ration on plasma corticosterone in A) *S. jarrovii*, B) *S. undulatus* hatchlings, and C) *S. undulatus* adults. Lines represent median values within treatment group. Lowercase letters denote statistical significance between treatment groups. Note the differences in scaling of the y-axes and that data are plotted on a $\log_{10}$ scale.

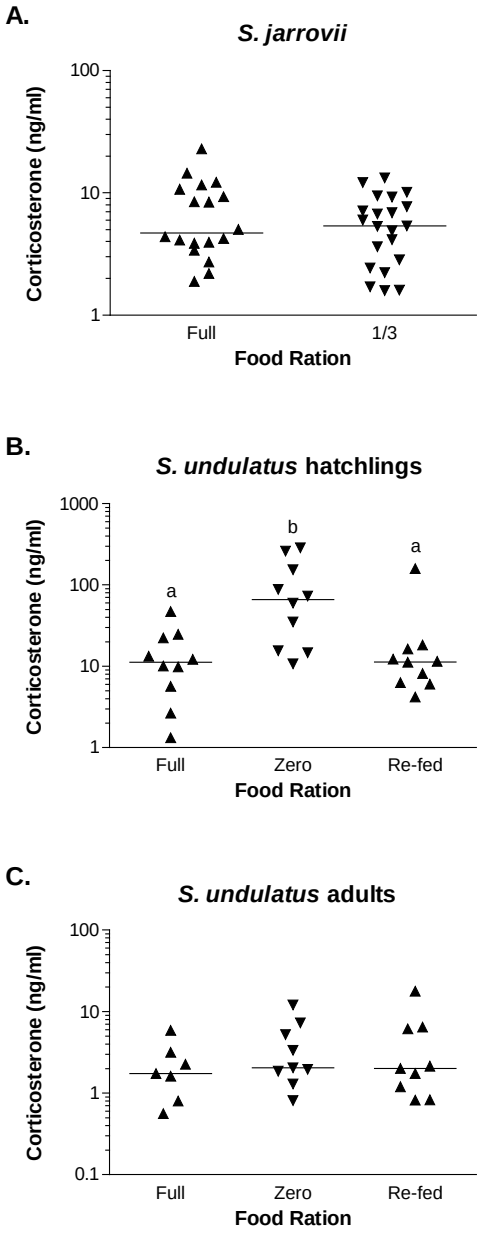
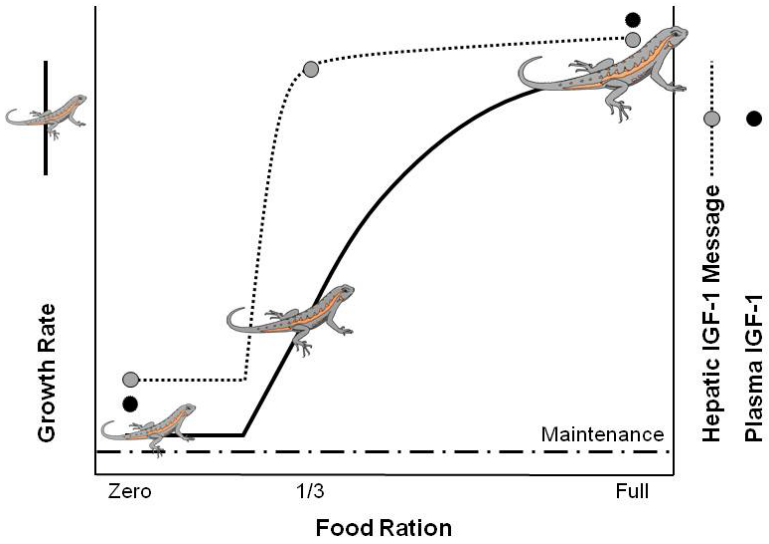


Figure 3.6. Graphical model to illustrate the relationships between food ration, growth rate, hepatic IGF-1 message, and plasma IGF-1.



CHAPTER IV.

TESTOSTERONE INHIBITS HEPATIC IGF-1 MESSAGE BUT NOT PLASMA IGF-1 IN *SCELOPORUS UNDULATUS*, A FEMALE-LARGER SPECIES OF LIZARD

Abstract

In many animal species, adults of one sex are larger than the other. Sexual size dimorphism (SSD) can arise when members of one sex grow faster than the other. Thus, as a starting point to understanding how SSD arises within a species, we first need to understand how sexes differ in growth regulation. Sex differences in growth rate are often attributed to the opposite effects of androgenic versus estrogenic hormones on the growth hormone/insulin-like growth factor-1 (GH/IGF-1) axis. In this paradigm, testosterone (T) stimulates hepatic IGF-1 message, resulting in an increase in plasma IGF-1, while estrogenic hormones have the opposite effect. However, these conclusions are inherently biased because studies focus almost entirely on male-larger species. In lizards, T promotes growth in males of male-larger species and inhibits growth in female-larger species. Thus, the effect of T on IGF-1 may not be universal but may depend on a species' pattern of SSD. In the present study, we investigated the effects of T on the expression and production of IGF-1 in eastern fence lizards (*Sceloporus undulatus*). In this species, females grow faster to become larger than males, and T inhibits growth in both sexes. We manipulated circulating T via surgical castration with and without implantation of Silastic® tubules loaded with T. We found that T decreased hepatic IGF-1 message in adult males as well as juvenile males and females, while plasma IGF-1 was

unaffected. We attribute our novel findings to our use of a female-larger species for this kind of study. Our results challenge the widespread belief that males grow faster than females because T promotes the production of IGF-1.

Introduction

Body size is a fundamental determinant of reproductive performance and survival and is therefore a critically important organismal trait (Shine, 1994; Arendt, 1997; Haenel et al., 2003; Cox et al., 2003, 2007). In many species of animals, adults of one sex are larger than the opposite sex. This biological phenomenon, known as sexual size dimorphism (SSD), can arise from sex-specific differences in growth rate and growth duration. The occurrence of SSD is widespread throughout the animal kingdom, however causes of its development and diversity are not well understood. Because of differences in reproduction, selection has often favored different optimal adult body sizes in males and females within species. While ultimate explanations for dimorphism in adult body size typically invoke adaptive hypotheses involving sexual selection (Darwin, 1871; Andersson, 1994), males and females share the same genetic blueprint for determining size (Fairbairn, 1997; Badyaev, 2002). Thus, sex-limited gene expression, not sex-limited genes, gives rise to SSD. Thus, as a starting point to understand how SSD arises within a species, we first need to understand how sexes differ in growth regulation.

Our experiments build on the finding that T has opposite effects on growth in female-larger versus male-larger species of lizards. Testosterone inhibits growth in female-larger species of lizards (*Sceloporus undulatus*, Cox et al., 2005a; *S. virgatus*, Cox and John-Alder, 2005), while stimulating growth in male-larger species (*S. jarrovii*,

Cox and John-Alder, 2005; *Anolis sagrei*, Cox et al., 2009). These opposing effects of T may reflect species differences in the molecular endocrinology of growth regulation.

One of the primary growth-regulatory systems in vertebrates is the GH/IGF-1 axis. Under the coordinate control of hypothalamic hormones, pituitary GH stimulates the transcription of IGF-1 in the liver. This GH-induced increase in hepatic IGF-1 message is associated with an increase in plasma IGF-1. In circulation, IGF-1 binds to high-affinity IGF binding proteins (IGFBPs). These IGFBPs slow the clearance of IGF-1 and modify effects of IGF-1 on target tissues, where IGF-1 stimulates tissue growth and mediates much of the growth-promoting effects of GH. Components of this axis are sexually dimorphic and are regulated, in part, by sex steroid hormones (Gatford et al., 1998). Both androgens and estrogens stimulate the secretion of GH, however androgens and estrogens produce sexually dimorphic temporal patterns of circulating GH that can lead to sex differences in growth rate (Shapiro et al., 1995; Gatford et al., 1998).

In male-larger species of mammals and fishes, androgenic steroids stimulate organismal growth and increase hepatic IGF-1 message and plasma IGF-1, whereas estrogenic compounds have been shown to elicit the opposite response. These seemingly opposite effects of androgens and estrogens were observed in ovariectomized female Sprague-Dawley rats, where body weight gain, hepatic IGF-1 message, and plasma IGF-1 were promoted by the administration of 5α-dihydrotestosterone (DHT) and suppressed by estradiol (E_2) (Borski et al., 1996). Additionally, in tilapia (*Oreochromis mossambicus*), DHT increased IGF-1 message in cultured hepatocytes collected from males, but the highest concentration (100 μM) decreased IGF-1 message in hepatocytes collected from females relative to untreated controls (Riley et al., 2004). In the same study, treatment

with E_2 lowered expression of IGF-1 message in both males and females. Exogenous administration of testosterone (T) and 11-ketotestosterone (11-KT) stimulated plasma IGF-1 in male coho salmon (*Oncorhynchus kisutch*) when measured one and two weeks post-injection (Larsen et al., 2004). Collectively, studies using Atlantic salmon (*Salmo salar*) have demonstrated that both E_2 and 4-nonylphenol (an endocrine disrupting substance) decrease body weight and plasma IGF-1 when exposed during various stages of life (Arsenault et al., 2004; McCormick et al., 2005; Lerner et al., 2007). Altogether, these studies suggest that T stimulates growth by enhancing the output of the GH/IGF-1 axis. However, this hypothesis has not been tested in female-larger species in which T inhibits male growth.

Few studies have characterized the effects of sex steroid hormones on growth and IGF-1 in female-larger species. In American kestrels (*Falco sparverius*), injections of yolk-androgens (T and androstenedione) into eggs decreased growth in males but not females relative to vehicle-treated controls (Sockman and Schwabl, 2000; Sockman et al., 2005). Additionally, treatment with T or an anti-estrogen (tamoxifen) inhibited growth of female red-spotted garter snakes (*Thamnophis sirtalis concinnus*) compared to male neonates, whereas E_2 suppressed male growth to rates slower than control males (Lerner and Mason, 2001). However, in the same study, an anti-androgen (cyproterone acetate) had no effect on growth in either sex and an anti-estrogen did not affect male growth. It should be noted that there were high rates of attrition in this experiment and thus the results should be interpreted with caution. In yellow perch (*Perca flavescens*), males and females that were offered an E_2-treated diet consumed more food and grew to become heavier and longer than controls (Malison et al., 1985, 1988; Jentoft et al., 2005; Goetz et

al., 2009). Furthermore, dietary E_2 increased hepatic IGF-1 message in yellow perch (Goetz et al., 2009) but did not affect plasma IGF-1 (Jentoft et al., 2005). One indirect link between androgens, growth rate, and IGF-1 in a female-larger species can be drawn from data collected from Syrian hamsters (*Mesocricetus auratus*). Following pre- and post-pubertal gonadectomy, growth rate (measured as changes in body length and body mass over time) in males was enhanced but growth rate in females was not altered (Swanson, 1967). In the same species, surgical castration elevated levels of plasma IGF-1 compared to T-replaced and intact control males (Vaughan et al., 1994). Therefore, it is possible that the mechanism by which T causes growth inhibition in female-larger species is through its ability to decrease the transcription and production of IGF-1.

Another difficult question to answer is whether sex steroids directly affect IGF-1. To address this question, Phillip et al. (1992) treated hypophysectomized, castrated male Sprague-Dawley rats with T, GH, or GH + T and found that T did not affect hepatic IGF-1 message and serum IGF-1, nor did it enhance the stimulatory effect of GH. Additionally, studies using hypophysectomized, ovariectomized female Sprague-Dawley rats revealed that chronic treatment (10 d) with E_2 accompanied by GH inhibited body weight gain, hepatic IGF-1 message, and serum IGF-1 compared to rats treated with GH alone (Murphy and Friesen, 1988). Moreover, administration of GH in combination with T replacement therapy in hypopituitary males did not affect serum levels of IGF-1 (Fisker et al., 2001). Taken together, these studies suggest that the effects of sex steroids on IGF-1 are mediated through increases in GH.

The opposing effects of androgens and estrogens documented in mammals and fishes suggest that the opposite effects of T on growth in lizards may reflect species

differences in how T affects IGF-1. Specifically, the effects of T on the GH/IGF-1 axis might differ even between closely related male-larger and female-larger species.

To investigate how T affects hepatic IGF-1 message and plasma IGF-1 in a female-larger species, we surgically manipulated circulating levels of T in the female-larger eastern fence lizard (*S. undulatus*). We hypothesized that T will decrease the output of the GH/IGF-1 axis in female-larger species in which T inhibits growth. Specifically, in *S. undulatus*, we expected T to decrease hepatic IGF-1 message and plasma IGF-1. Alternatively, the effects of T on the GH/IGF-1 axis may be evolutionarily conserved, regardless of how T affects growth and the development of SSD. Under these conditions, we predict that T will increase hepatic IGF-1 message and plasma IGF-1 in *S. undulatus*. The experiments reported here will help to resolve the question of whether androgenic up-regulation of the GH/IGF-1 axis takes precedence over the organismal growth response, or whether an association between IGF-1 and organismal growth has allowed opposite androgenic responses to evolve.

Methods

Animal Collection and Care

In June 2009, reproductively mature *S. undulatus* males and females (at least 2 years old) were collected from the New Jersey Pinelands, New Jersey, USA under permit from the New Jersey Department of Environmental Protection, Division of Fish and Wildlife (SC 2909C). Adult lizards were captured by hand and hand-held noose in the vicinity of Rutgers University Pinelands Research Station in New Lisbon, Burlington County, NJ (41°N, 74°35’W). In September 2009, hatchling *S. undulatus* males and

females (approximately 1 – 2 months of age) were collected by hand from the same field site as the adults. All lizards were subsequently transported to our laboratory facility at Rutgers University. Adults were maintained in our laboratory facility for 6 – 27 days until we collected a sufficient number of lizards to begin treatment. At the time of capture, individual hatchlings weighed less than 2.0 g. We allowed these lizards (hereafter referred to as 'juveniles') to develop and grow for four months until they were large enough to perform surgical manipulations (approximately 5-6 months of age). All lizards were housed individually (permit SH 29097) in plastic cages (59.1 x 43.2 x 45.7 cm) that contained 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, USA) for ultraviolet radiation on a 12:12 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 number 01-019).

Experimental Design

Immediately prior to surgical manipulation, snout-vent length (SVL) was measured to the nearest 0.5 mm and body mass was measured to the nearest 0.02 g. These data were used to assign lizards to size-matched treatment groups. During experiments, changes in body size were monitored via weekly (juveniles) or bi-weekly

(adults) measurements of SVL and body mass. Growth rate was calculated by dividing an individual's change in SVL by the number of days of treatment. Unlike juvenile *S. undulatus* (SVL at time of surgery = 53.5 – 64.0 mm), we did not expect our treatments to affect the growth rates of adults because these lizards were on the asymptotic part of the predicted growth curve for this species (SVL at time of surgery = 56.0 – 75.0 mm; Haenel and John-Alder, 2002; John-Alder et al., 2007). Feeding rate (reported as crickets/d) was measured by counting uneaten crickets every 3 days (adults) or every 7 days (juveniles). It should be noted that adults were fed larger crickets than juveniles; therefore, feeding rate is a unit of measurement that can only be used to make comparisons within an experiment.

Experiment 1: *S. undulatus* adults

Adults were divided into five size-matched treatment groups: intact control males (M-CON; n = 9), surgically castrated males (M-CAST; n = 9), surgically castrated + T replaced males (M-TEST; n = 9), intact control females (F-CON; n = 9), and intact control + T supplemented females (F-TEST; n = 9). The 14-day treatment period began immediately following surgery. During this time, individuals were offered 3 crickets/day.

Experiment 2: *S. undulatus* juveniles

Juveniles were divided into four size-matched treatment groups: surgically castrated males (M-CAST; n = 9), surgically castrated + T replaced males (M-TEST; n = 9), intact control females (F-CON; n = 10), and intact control + T supplemented females (F-TEST; n = 10). A group of intact control males were not included in this experiment

because we were interested in testing the effects of T on IGF-1. Furthermore, previous studies have demonstrated that our method of castration and T replacement achieves a level of plasma T that is within the physiological range for this species (Cox et al., 2005a,b). Throughout the treatment period, individuals were offered 3 crickets/day. This experiment was terminated after 36 days of treatment, which was sufficient time to measure significant differences in feeding rate and growth rate among treatment groups.

Testosterone Implants

Tonic-release implants were constructed from 5 mm lengths of Silastic® tubing (Dow Corning, Midland, MI, USA) as previously described (Cox et al., 2005a). After sealing one end of each tubule with silicone adhesive gel (Dow Corning), a Hamilton® syringe was used to inject a solution of 300 µg of testosterone dissolved in dimethyl sulfoxide (DMSO) into the open end of each implant. Each tubule was sealed with silicone adhesive and after several days, the DMSO evaporated and diffused through the tubing, leaving crystalline testosterone within the lumen of each implant. Placebo implants were constructed in an identical fashion, but injected with pure DMSO, which left an empty tubule after evaporation and diffusion.

Surgical Treatments

Animals were anaesthetized with an intramuscular injection of ketamine (Vetus Animal Health, MFA Inc., Columbia, MO, USA; 200 mg kg^{-1} body mass). The testes were exposed with a single ventral incision and bilaterally castrated in castrated (M-CAST) and T-replaced males (M-TEST) by ligating each spermatic cord with surgical

silk, ablating each testis, and cauterizing each ligated spermatic cord after removal of the testes. For intact control males (M-CON) and females (F-CON) as well as T-supplemented females (F-TEST), sham surgeries were performed in which identical incisions were made to expose and manipulate the gonads while leaving them intact. A T-filled or a placebo implant was inserted into the coelomic cavity, and the incision was closed with Surgi-Lock 2oc™ instant tissue adhesive (Meridian Animal Health, Omaha, NE, USA).

RNA Isolation, Reverse Transcription Polymerase Chain Reaction (RT-PCR), and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

At the conclusion of the treatment periods, lizards were euthanized by rapid decapitation and liver tissue was harvested, flash frozen on dry ice, and stored at -80°C until analysis. Liver tissue was homogenized with TRIzol® (Invitrogen Corp., Carlsbad, CA, USA) using a Teflon pestle and a glass homogenizing tube, and total RNA was isolated according to the manufacturer's protocol using Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). RNA yield was quantified at an absorbance of 260/280 nm using the NanoDrop® ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA), and integrity was verified by electrophoresis on a 1% formaldehyde denaturing gel as previously described (Cohick et al., 2000).

Total RNA (2 μg) was reversed transcribed with random primers according to the manufacturer's protocol using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Carlsbad, CA, USA). Then, gene-specific primers (IGF-1: 5'-TTGGTGGATGCTCTTCAGTTTG-3'/3'-CAGGTCACAGCTTTGGAAACAA-5'; β-

actin: 5'-GAAGAGGAAGCAGCTGTGGC-3'/3'-GCTATGTTGCCTTGGACTTCG-5'), which were previously validated for *S. undulatus* (Duncan et al., unpublished), were used to amplify 135 bp and 52 bp PCR products, respectively. cDNA was fluoresced with SYBR® Green (Applied Biosystems Inc.), and the threshold cycle (C_T) of each sample was recorded on an ABI 7900HT. Standard curves of pooled cDNA were serially diluted so that the curve ranged from 250 ng to 25 pg. Amplification efficiency of standard curves ranged from 95-100%. Relative quantification of IGF-1 was normalized to β-actin, which was unaffected by treatment, and then compared to a calibrator (i.e., pooled RNA from experimental animals). The data are expressed as a fold change of expression levels. The appropriate negative controls were run (i.e., No-RT control and master mix of reagents without template) to ensure the absence of DNA and non-specific amplification. The presence of a specific product was verified by visually inspecting the dissociation curves for each sample.

IGF-1 Radioimmunoassay (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 (Fisher Scientific, Pittsburgh, PA, USA). Plasma was separated via centrifugation and stored at -80°C until used for assays. Assays were conducted in G. Grau's laboratory at the Hawaii Institute of Marine Biology, University of Hawaii. For RIA of IGF-1, proteins were extracted from plasma with acid-ethanol followed by cryoprecipitation to separate IGF-1 peptide from the IGFBPs (Shimizu et al., 1999). Reconstituted samples were assayed in duplicate with ^{125}I-recombinant salmon/trout (*Oncorhynchus* sp.) IGF-1 and

rabbit anti-recombinant barramundi (*Lates calcarifer*) IGF-1 as the antiserum (Novozymes Biopharma AU Ltd., Adelaide, SA, AUS). The hormone complexes bound to antibody were precipitated from free radiolabel by the addition of goat anti-rabbit (Sigma-Aldrich, St. Louis, MO, USA). This assay was previously validated for *S. undulatus* (Duncan et al., unpublished) by demonstrating parallel binding to anti-recombinant barramundi IGF-1 in *S. undulatus* plasma (pool from intact *S. undulatus*) compared to a salmon/trout standard (Novozymes Biopharma AU Ltd.) and *O. mossambicus* plasma. The intra-assay variation, based on four aliquots of *S. undulatus* pooled plasma included in each assay, was 16% for adults and 4% for juveniles.

Statistical Analyses

All statistical analyses were performed using SAS 9.2 (version 9.2, SAS Institute Inc., Cary, NC, USA). For the experiment with adults, comparisons were made between treatment groups within a given sex using one-way ANOVA. For the experiment with juveniles, our calculations for feeding rate and growth rate reflect a two-week recovery period, which was determined by the amount of time the lizards required for their food consumption to return to pre-surgery rates. Feeding rate, growth rate, relative expression of hepatic IGF-1 message, and plasma IGF-1 were analyzed using two-way ANOVA with treatment (placebo implant, testosterone implant) and sex as main effects and sex x treatment as an interaction term. Initial SVL was not included as a covariate because it did not significantly affect any of the parameters measured. These analyses were followed by an LS means test or the Ryan-Einot-Gabriel-Welsch test (REGWQ; SAS Institute 2002) for post-hoc separation of treatment groups. Alpha was set at 0.05.

Results

Feeding Rate and Growth Rate

In *S. undulatus* adults, the male treatment groups consumed a similar number of crickets/day during the 14-day treatment period ($F_{2,24} = 5.43$, $P = 0.0114$; Fig. 1A). Although the statistical test produced a significant *P*-value, we attribute this result to the lack of variation in feeding rate for M-CON. In the same way, adult females had comparable feeding rates ($F_{1,16} = 0.00$, $P = 1.0000$; Fig. 1A). In hatchlings, TEST ate fewer crickets/day than males and females treated without T ($F_{3,34} = 37.20$, $P < 0.0001$; Fig. 1B). Additionally, sex affected feeding rate such that females consumed a greater number of crickets/day than males ($F_{3,34} = 16.24$, $P = 0.0003$). Furthermore, a significant sex x treatment term indicates that the treatment effect was more pronounced in females compared to males ($F_{3,34} = 9.54$, $P = 0.0040$).

Testosterone did not affect growth rate of adults (males: $F_{2,24} = 0.44$, $P = 0.6466$; females: $F_{1,16} = 0.00$, $P = 1.0000$; data not shown). In juveniles, however, exogenous T was sufficient to suppress growth rate by approximately 3-fold ($F_{3,34} = 43.85$, $P < 0.0001$), but we did not measure an effect of sex ($F_{3,34} = 0.81$, $P = 0.3747$) or sex x treatment ($F_{3,34} = 0.00$, $P = 0.9836$) on growth rate (Fig. 2).

Hepatic IGF-1 Message and Plasma IGF-1

After the 14-day treatment period, castration increased hepatic IGF-1 message by 3-fold, while T replacement in castrated males restored hepatic IGF-1 message to the same level as the intact control males ($F_{2,24} = 5.35$, $P = 0.0120$; Fig. 3A). In adult

females, T supplementation did not affect hepatic IGF-1 message ($F_{1,16} = 0.10$, $P = 0.7556$; Fig. 3A). Similar to adult males, T decreased hepatic IGF-1 message by 6–fold in juveniles ($F_{3,33} = 29.97$, $P < 0.0001$; Fig. 3B). In addition, females expressed 2-fold higher levels of hepatic IGF-1 message compared to males ($F_{3,33} = 10.02$, $P = 0.0033$), and the inhibitory effect of T on hepatic IGF-1 message was greater in females (sex x treatment interaction: $F_{3,33} = 6.42$, $P = 0.0162$).

Although we observed an attenuation of hepatic IGF-1 message in response to T, plasma IGF-1 was unaffected by T in adult males ($F_{2,24} = 1.91$, $P = 0.1698$) and females ($F_{1,16} = 0.92$, $P = 0.3514$; Fig. 4A). In juveniles, we did not measure an effect of T ($F_{3,34} = 3.42$, $P = 0.0732$), sex ($F_{3,34} = 0.13$, $P = 0.7202$), or the sex x treatment interaction ($F_{3,34} = 0.38$, $P = 0.5414$) on plasma IGF-1 (Fig. 4B). Furthermore, levels of plasma IGF-1 in juveniles were approximately four times greater than adults (Fig. 4).

Discussion

The main objectives of this study were to determine the influence of T on IGF-1 message in the liver and IGF-1 protein in the plasma of a female-larger species. Our first experiment involved the surgical manipulation of circulating levels of T in adults. At the conclusion of the treatment period, castrated males had the highest expression of hepatic IGF-1 message but T did not affect hepatic IGF-1 message in females (Fig. 3A). One possible explanation for this lack of effect in females might be due to the relatively short timeframe that lizards were exposed to male-typical levels of plasma T. Additionally, T slightly increased plasma IGF-1 (Fig. 4A) without affecting food consumption (Fig. 1A). Although food intake is a known regulator of IGF-1 (Thissen et al., 1994; Pérez-Sánchez

et al., 1995; Crain et al., 1995; Scanes, 1997; Duan, 1998; Moriyama et al., 2000; Pierce et al., 2001; Beckman et al., 2004; Pierce et al., 2005; Pedroso et al., 2006; Bauer et al., 2009; Rønning et al., 2009; Duncan et al., unpublished), we attribute T as the main effect on IGF-1 rather than a T by feeding rate interaction because T did not affect food consumption. Furthermore, a previous study with juvenile *S. undulatus* males demonstrated that T inhibits growth (Cox et al., 2005a); however, our experiment with adults was not designed to measure growth rate since we used lizards that had already reached asymptotic growth (Haenel and John-Alder, 2002; John-Alder et al., 2007). Regardless, we would not expect measureable changes in growth to occur in adults during a restricted timeframe such as two weeks.

Both hepatic IGF-1 message and plasma IGF-1 are the targets of research to assess potential indicators of growth. However, controversy has surrounded the contributions of tissue IGF-1 versus plasma IGF-1 to postnatal growth rates. In a series of experiments, researchers used combinations of transgenic and knockout mice to separate the endocrine and autocrine/paracrine functions of IGF-1. To confirm whether the liver is the primary contributor to plasma IGF-1, the IGF-1 gene was deleted from the liver of mice (Yakar et al., 1999, 2009). Although this deletion caused a 75% decrease in plasma IGF-1, growth was comparable to their wild-type littermates. This result demonstrates the importance of the autocrine/paracrine role of IGF-1 in the growth process. In another mouse model, IGF-1 is only expressed in the liver (Hepatic IGF-1 transgenic; HIT) of IGF-1 null mice (IGF-1 Knockout; KO), thereby exposing these animals to IGF-1 via an endocrine manner alone (Wu et al., 2009). Despite the absence of tissue IGF-1, adult HIT-KO mice had body weights similar to controls and greater than growth-retarded

IGF-1 KO mice. Therefore, the autocrine/paracrine actions of IGF-1 that determine growth can be compensated by the elevation of endocrine IGF-1.

In order to gain a better understanding of the association between T, growth rate, hepatic IGF-1 message, and plasma IGF-1 in *S. undulatus*, we conducted a follow-up experiment using juveniles that were actively growing. In both males and females, T decreased hepatic IGF-1 message (Fig. 3B), which was accompanied by a decrease in growth rate (Fig. 2) and food intake (Fig. 1B) compared to the control groups. Although we have weak statistical support, T had a consistent but small and generally non-significant effect on plasma IGF-1 in adults and juveniles (Fig. 4), which is in contrast to the inhibitory effect of T on hepatic IGF-1 message. In juvenile females, and to a lesser extend in males, the effect of T on growth may have been mediated by a reduction in food intake. Previous data collected from dietary-manipulation studies involving *Sceloporus* lizards demonstrated that complete, not partial, food restriction was necessary to inhibit transcription of IGF-1 in the liver (Duncan et al., unpublished). Regardless of the mechanism, our data on growth responses, in light of no change in IGF-1, are consistent with the idea that growth can be varied substantially with changes in food intake, T, and possibly other factors even while IGF-1 remains above the threshold level required to support growth (e.g., Beckman et al., 2004).

The effects of T on hepatic IGF-1 message and plasma IGF-1 reported here suggest that hepatic IGF-1 message and plasma IGF-1 do not always correlate. Similarly, following a transfer from seawater to freshwater, tilapia (*O. mossambicus*) experienced reduced growth (change in body weight), which was accompanied by a decrease in hepatic IGF-1 message and an increase in plasma IGF-1 compared to their counterparts

that remained in seawater (Riley et al., 2003). In a study with juvenile hybrid striped bass (*Morone chrysops* x *Morone saxatilis*), partial food restriction increased hepatic IGF-1 message by 82% while plasma IGF-1 and growth were substantially decreased compared to ad libitum-fed controls (Picha et al., 2006). Following a period of re-feeding, hepatic IGF-1 message decreased by 61% but plasma IGF-1 and growth increased. The liver is the major contributor of endocrine IGF-1, however extrahepatic tissues also release IGF-1 into the bloodstream. Therefore, in *S. undulatus*, the lack of an effect on plasma IGF-1 might be due to the release of IGF-1 from extrahepatic tissues. Circumstances where hepatic IGF-1 message and plasma IGF-1 do not correlate might be due to alterations in mRNA stability, clearance rates, degradation pathways, or availability of IGF-1 to its receptor. In circulation, IGF-1 forms complexes with IGFBPs at a greater affinity than that of the type-I IGF receptor (Kelley et al., 2002). Exogenous T might have up-regulated IGFBPs that sequestered IGF-1 protein and made it less readily available to its receptor.

Growth is under the control of several hormones such as T and IGF-1 as well as environmental factors including food availability. One study with immunocastrated boars was designed to test the direct effect of food availability on weight gain and plasma IGF-1. Following the suppression of E_2 and T, boars on the high-food diet (3 kg/d) had higher levels of plasma IGF-1 and grew faster (weight gain/d) than boars on the low-food diet (2 kg/d) (Bauer et al., 2009). When male and female yellow perch (*P. flavescens*) were administered dietary E_2, they had higher feeding rates and were larger than controls (Malison et al., 1985, 1988; Jentoft et al., 2005; Goetz et al., 2009). However, this result is in contrast to the growth inhibitory effect that E_2 had in fish maintained on a restricted

diet (Malison et al., 1988). These complex interactions make it difficult to tease apart the mechanism by which T regulates growth rate.

The reduction in hepatic IGF-1 message by T in a female-larger species is an especially interesting finding because most published work draws the conclusion that T is a stimulant of IGF-1. This result has not previously been reported, and the consistent effect in earlier experiments has been that T caused an increase in expression of hepatic IGF-1 message. We attribute our novel findings to the use of a female-larger species for this study since previous experiments have consistently used male-larger species, regardless of the diversity of species of fishes and mammals that have been investigated. Our data suggest that the notion that males grow faster than females by enhancing the production of IGF-1, which may be true in male-larger species in which T stimulates growth, is overly simplistic. Furthermore, our results indicate that in female-larger species in which T inhibits growth, the effect of T seems to be to disrupt the causal effects of IGF-1 on growth. These findings are complicated and challenge simple interpretation, thus further experimental work is needed to elucidate this mechanism.

Analysis of other components of the IGF-1 system such as the IGF-1 receptor and IGFBPs will be required to provide a clearer picture of how T can have opposite effects on IGF-1 message at the level of the liver in male-larger versus female-larger species. To achieve a more in depth and comprehensive understanding of species differences in growth-regulatory mechanisms, comparative studies using male-larger species lizards are needed.

Figures

Figure 4.1. Effect of testosterone on feeding rate in A) adults and B) juveniles. Mean (+ 1 SE) feeding rate.

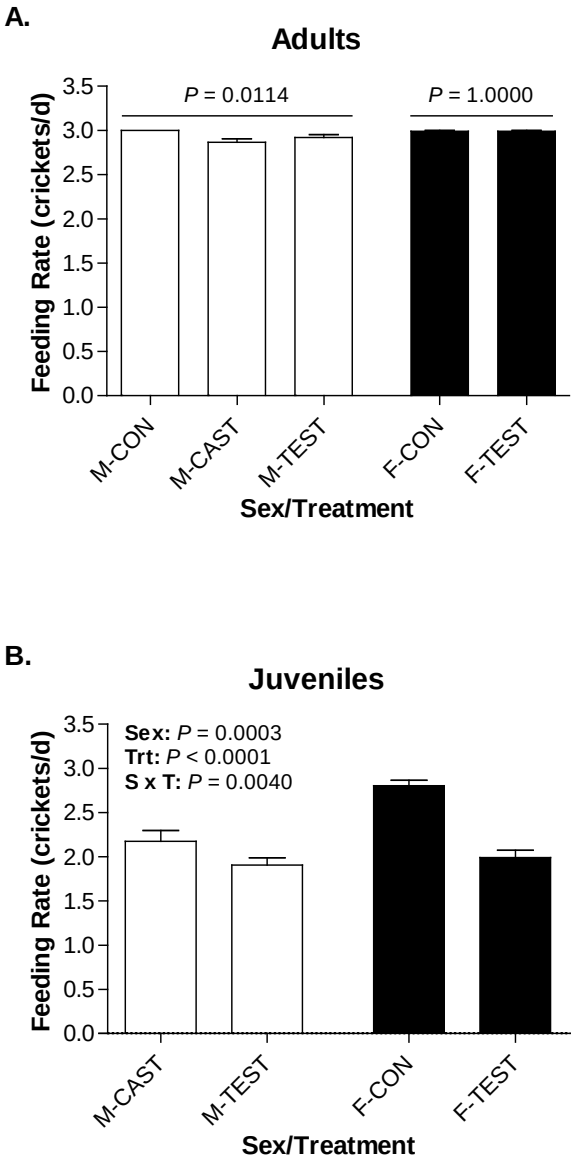


Figure 4.2. Effect of testosterone on growth rate in juvenile males and females. Mean (+ 1 SE) growth rate. Lowercase letters denote statistical significance between treatment groups.

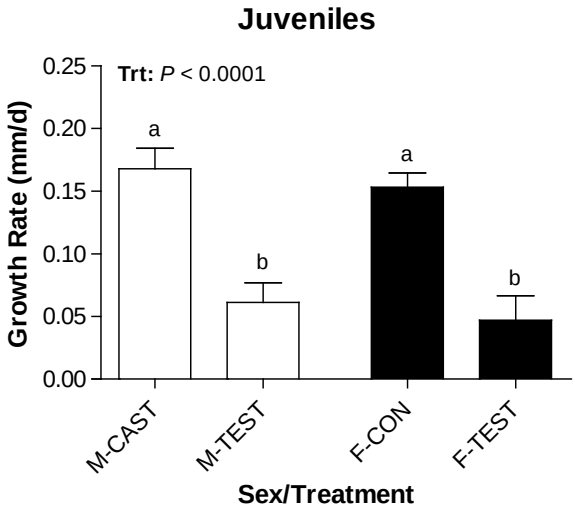


Figure 4.3. Effect of testosterone on the relative expression of hepatic IGF-1 message in A) adults and B) juveniles. Lines represent median values within treatment group. Lowercase letters denote statistical significance between treatment groups. Note that data are plotted on a $\log_{10}$ scale.

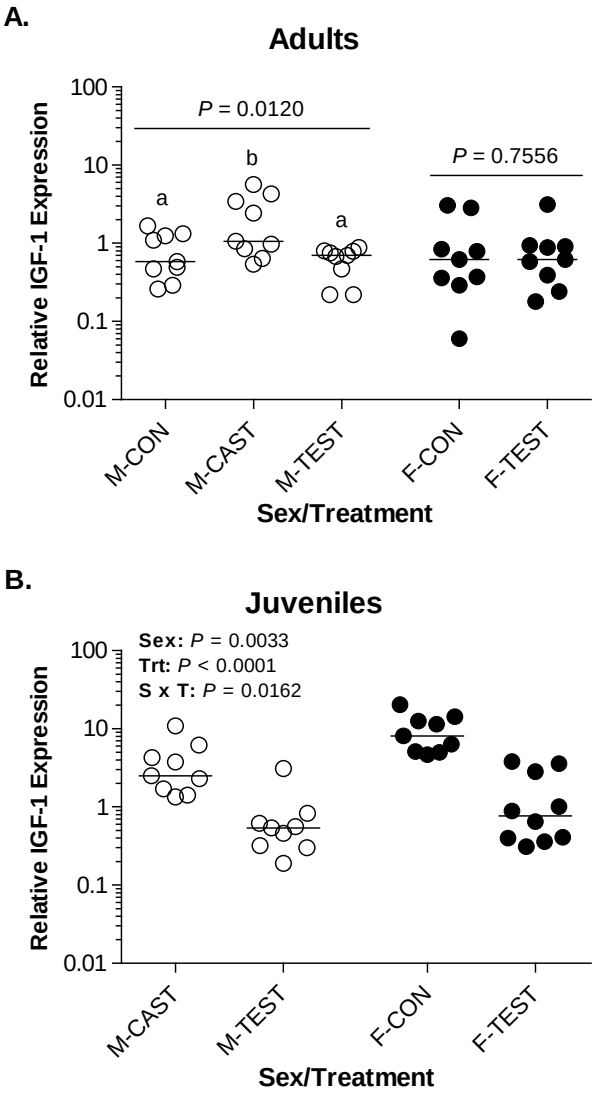
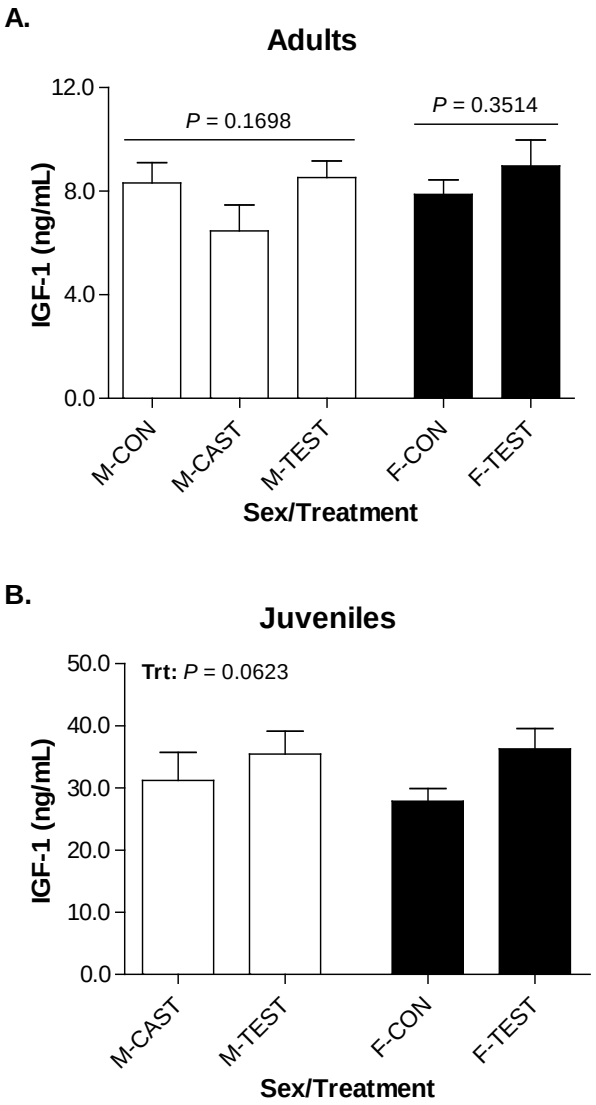


Figure 4.4. Effect of testosterone on plasma IGF-1 in A) adults and B) juveniles. Mean (+ 1 SE) plasma IGF-1. Note the differences in scaling of the y-axes.



CHAPTER V.

CONCLUSIONS

In many species of animals, adults of one sex are larger than the other. This widespread phenomenon of sexual size dimorphism (SSD) often arises when one sex grows faster to become larger than the opposite sex within a given species. To understand how SSD arises within a species, we first need to understand how sex differences in growth regulation lead to male-larger and female-larger species. My dissertation research focused on the growth hormone/insulin-like growth factor-1 (GH/IGF-1) axis, the central growth-regulatory endocrine axis in vertebrates. Of particular interest is the influence of sex steroid hormones on growth in males and females of species with SSD. If sex steroid hormones differentially regulate the production of IGF-1, then growth and the attainment of adult body size would differ between sexes.

Previous work in lizards demonstrated that testosterone (T) inhibits growth in males of female-larger species of lizards, while stimulating growth in males of male-larger species (Cox et al., 2005; Cox and John-Alder, 2005; Cox et al., 2009). In other groups of vertebrates, androgenic steroids stimulate organismal growth, which is accompanied by an increase in hepatic IGF-1 message and plasma IGF-1 (Borski et al., 1996; Riley et al., 2002). In contrast, estrogenic compounds have been shown to inhibit growth and decrease hepatic IGF-1 message and plasma IGF-1 (Borski et al., 1996; Arsenault et al., 2004; McCormick et al., 2005; Lerner et al., 2007). The opposing effects of androgens and estrogens documented in male-larger species suggest that the opposite effects of T in lizards may reflect species differences in how T affects IGF-1. Thus, my overall research goal was to determine how T affects the GH/IGF-1 axis in species of

sexually dimorphic lizards where the growth response is associated with the direction of SSD. The main objectives of the dissertation were to (1) develop methodology to characterize the response of the GH/IGF-1 axis, (2) characterize responses of GH/IGF-1 axis to variation in food intake, and (3) characterize the effect of T on IGF-1 in a female-larger species in which T inhibits growth.

Since the IGF-1 system has not been characterized in lizards to date, the development and validation of novel assay techniques were required in order to measure the response of IGF-1 to experimental treatments. To this end, a partial sequence of IGF-1 was generated from five species of lizard through the use of molecular cloning. To our knowledge, we are the first to clone IGF-1 from any species of reptile. The sequences of IGF-1 that were cloned from *Sceloporus undulatus*, *S. jarrovii*, *Anolis sagrei*, *Coleonyx elegans,* and *Goniurosaurus lichtenfelderi* share the greatest identity with one another compared to published sequences from non-reptilian species. Additionally, the highest percent of sequence identity is found between species of the same suborder of Squamata (Iguania: *S. undulatus*, *S. jarrovii*, and *A. sagrei*; Gekkota: *C. elegans* and *G. lichtenfelderi*). Although these species belong to the same order, there are differences in the C domain, which is likely due to the fact that Iguania and Gekkota diverged approximately 240 million years ago (Jonniaux and Kumazawa, 2008). These data support previous findings that the most variation in the IGF-1 sequence is located in the C domain. Altogether, these lizard sequences provided the foundation for developing reagents and assays that were specific to our species of interest.

To study growth regulation at the molecular level in lizards, an understanding of how IGF-1 responds to a known regulator was necessary. Therefore, I characterized the

responses of the GH/IGF-1 axis to variation in food intake, which is known to have a profound and predictable impact on IGF-1 in other classes of vertebrates, were characterized. In *S. undulatus*, zero ration decreased hepatic IGF-1 message and plasma IGF-1, while re-feeding restored these parameters to the level of the full ration group. However, in *S. jarrovii*, 1/3 ration had no effect on hepatic IGF-1 message when compared to the full ration group. Food restriction (1/3 ration) in *S. jarrovii* did not impose the intended nutritional stress, as supported by plasma corticosterone, and should not have been expected to decrease hepatic IGF-1 message. However, an early and transient response in hepatic IGF-1 message may have occurred but went undetected since samples were only collected at the conclusion of the experiment. Altogether, results demonstrate that IGF-1 in *Sceloporus* lizards expresses similar patterns of responsiveness to food intake as observed in other vertebrates. However, a nutritional threshold affects the association of hepatic IGF-1 message and growth regulation such that low food intake, which might commonly be observed under natural conditions, is not enough to turn off IGF-1 transcription – food deprivation is required.

To determine how T affects IGF-1 in female-larger species, circulating levels of T were manipulated by surgical castration with and without T replacement. In a female-larger species, *S. undulatus*, T-treated lizards had lower expression of hepatic IGF-I message, but T did not significantly affect plasma IGF-I. This result has not previously been reported, and the consistent effect in previous experiments has been that T caused an increase in expression of hepatic IGF-I message. However, data in the literature are biased because they have predominantly focused on male-larger species where T promotes growth. The effect of T on hepatic IGF-1 message in adults does not appear to

be mediated by a reduction in food intake since feeding rate was similar among treatment groups. Despite the fact that T did not reduce plasma IGF-1, T did reduce growth of juveniles. In juvenile females, and to a lesser extent in juvenile males, the effect of T on growth may have been mediated by a reduction in food intake. However, evidence from nutritional manipulation in *Sceloporus* lizards suggests that a decrease in food intake, short of starvation, may be required to affect hepatic IGF-1 message. Regardless of the mechanism, these data on growth responses in light of no change in plasma IGF-1 are consistent with the idea that growth can be varied substantially with changes in food intake, T, and potentially other factors even while IGF-1 remains above the threshold level required to support growth. We attribute these novel findings to the use of a female-larger species for this kind of study since previous experiments have consistently used male-larger species, regardless of the diversity of species of fishes and mammals that have been investigated**.** Furthermore, the notion that T causes males to grow faster than females by enhancing the production of IGF-1, which may be true in male-larger species in which T stimulates growth, is overly simplistic. Our results indicate that in female-larger species, in which T inhibits growth, the effect of T seems to be to disrupt the causal effects of IGF-1 on growth. Furthermore, data from *S. undulatus* suggest that the GH/IGF-1 axis plays a role in decreased growth rates in response to T. These results are complicated and further experimental work is needed to elucidate the mechanism.

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CURRICULUM VITA

Christine A. Duncan

Education

1999-2003	B.S. in Biology The Pennsylvania State University
2005-2011	Ph.D. in Endocrinology and Animal Biosciences Rutgers, The State University of New Jersey

Professional Appointments

2003-2005	Research Technician University of Pittsburgh, Department of Biological Sciences Principal Investigator: Dr. Jeffrey D. Hildebrand
2005-present	Teaching/Graduate Assistant Rutgers, The State University of New Jersey Advisor: Dr. Henry B. John-Alder

Publications

Duncan, C. A. and H. B. John-Alder. 2009. Food Restriction Inhibits Growth Rate but Not expression of Hepatic IGF-I Message in Yarrow's Spiny Lizard, *Sceloporus jarrovii*. *Integr. Comp. Biol.* 49 (suppl 1): e223.

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