The Molecular Underpinning of Life-History Evolution:
Roles of the IIS network and the Building of a Reptilian Model

by

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Abstract

The Insulin and Insulin-like Signaling (IIS) network regulates cellular processes including pre- and post-natal growth, cellular development, wound healing, reproduction, and longevity. Despite their importance on the physiology of vertebrates, the study of the specific functions of the top regulators of the IIS network — insulin-like growth factors (IGFs) and IGF binding proteins (IGFBPs) has been mostly limited to a few model organisms, namely lab-rodents. My dissertation aims to build a foundation for the development of a reptilian model to study IIS in the context of early life growth and reproduction. Towards this aim, the chapters of my dissertation (1) demonstrate that the expression patterns of IGF1 and IGF2 seen in lab-rodents are atypical relative to the typical patterns across amniotic clades, including humans; (2) characterize the gene expression of IGFs and IGFBPs across tissues and developmental stages in a model reptile, the brown anole lizard (*Anolis sagrei*); (3) discover that forced investment in tail regeneration results in increased investment in reproduction in the brown anole; and (4) demonstrate how a CURE focused on novel IIS research can be an effective teaching tool in the undergraduate biology classroom.

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List of Abbreviations

CURE	Course-based Undergraduate Research Experience
CI	Confidence Intervals
CQ	Quantitation Cycle
cDNA	Complementary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
Dpa	Days Post-autotomy
EEF2	Eukaryotic Translation Elongation Factor 2
GPA	Grade Point Average
IACUC	Institutional Animal Care and Use Committee
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IGFR	Insulin-like Growth Factor Receptor
IIS	Insulin and Insulin-like Signaling Network
mRNA	Messenger Ribonucleic Acid
RNA	Ribonucleic Acid
RNAseq	Ribonucleic Acid Sequencing
rRNA	Ribosomal Ribonucleic Acid
PCA	Principle Component Analysis
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
SQ	Starting Quantity
SVL	Snout-vent Length

INTRODUCTION

Bridging the Gap Between Genotype and Phenotype

Whether we are introducing the concepts of genetics, life-history theory, evolution, or a number of other biological fields, one of the first concepts taught is the Central Dogma of Biology. The association of "DNA to RNA to Proteins" is essential in phenotypic display. The relationship between genotypes and phenotypes are, at a fundamental level, regulated by the molecular mechanisms within the central dogma. Understanding the mechanistic function of the genome within and across species has become a central question within molecular biology (Lappalainen, 2015), and variation at the molecular level often dictates mechanistic hypotheses to explain the observations made at the organismal level.

Genetics has previously been centered around two primary questions: (1) How is trait variation mediated by genetics, and (2) How does the genomic blueprint result in living organisms expressing those traits (Lappalainen, 2015)? Fields such as molecular biology, cell biology, quantitative genetics, and molecular genetics each seek to answer these questions in distinct ways, and therefore our understanding of trait genetics and trait expression is disjointed. However, there is a widely accepted need to bridge the study of conventional, functional, and molecular genetics. In combination, these two previously mentioned questions lead to one central matter of increasing importance according to Lappalainen (2015), "What are the functional effects of genetic variation?" The study of the origin and contribution of variation to traits, such as disease and normal physiology, has developed independently within fields such as evolutionary biology, ecology, biomedical sciences, and population genetics. For this reason, a multidisciplinary

exploration of the molecular networks underlying traits of interest can significantly strengthen our understanding of the observations made in evolutionary, biomedical, and ecological studies.

Functional genetics and functional genomics are two fields that aim to address this much larger interdisciplinary question, determining how the components of a molecular system function together to produce a specific trait under a variety of conditions (Gardel, 2015). Functional genomics is an inherently interdisciplinary field, breaching genetics, chemistry, cell biology, physiology, and evolution. Functional genetics and genomics use molecular mechanisms as a tool, but move beyond them in an attempt to understand the functional effects of genetic variance (Gardel, 2015).

Functional genomics examines the genetic detailing of a system to understand the expression and variation of traits. As the Insulin and Insulin-like Signaling (IIS) network has pleiotropic effects (ex. Reproduction, Cell division, Embryonic Development, Early Life Growth, Longevity, Senescence) and is conserved across many species (Papatheodorou et al., 2014; Schwartz & Bronikowski, 2016), it is an ideal mechanism tail gain a better understanding of how genetics and the environment work together to explain variation observed within species as well as phenotypes and life history strategies between species.

Even though the IIS network is conserved across vertebrates, the patterns of expression can vary dramatically between species with varying life history traits (Dantzer & Swanson, 2012), making it an ideal candidate for exploring a molecular network across species. These comparative analyses allow us to, as suggested, move beyond the molecular mechanism alone (Gardel, 2015) to understand the larger physiological and evolutionary impacts of the network.

The Insulin Signaling Network

What we know: Human and Rodent Models

The insulin and insulin-like signaling (IIS) network is a complex mechanistic system with a plethora of downstream activations, affecting nearly all physiological processes. The insulin-like growth factor (IGF) portion of the IIS network is comprised of peptide hormones, cell membrane receptors, and circulating binding proteins (Denley et al., 2005) (**Fig**. **0.1**). The three main peptide hormones of the IGF system are IGF1 and IGF2 and Insulin (INS). On the cell surface, there are IGF1 receptors (IGF1R), IGF2 receptors (IGF2R), and insulin receptor (IR). IGF proteins have been shown to bind directly to the IGF1 receptor and the insulin receptor (both IR-A and IR-B) with high affinity (Denley et al., 2005). This binding of the IGF proteins to the receptor activates a signaling cascade that regulates many biological processes associated with cellular growth and cell division.

IGF1 and IGF2 proteins come in two forms. Before processing, both proteins are comprised of 5 domains: B, C, A, D, and E (N to C terminus). During processing the E peptide is cleaved, leaving what we refer to as the mature peptide. The C domain is the binding domain of the proteins to the receptors (Denley et al., 2005). In IGF1, IGF2, and insulin, there is over 50% homology across the B and A domain. It has previously been implicated that, in mammals, IGF1 is most essential during postnatal growth and IGF2 is essential in pre-natal growth (Zhu et al., 2017). However, this knowledge comes from work completed in lab-rodents, and due to its exclusive expression during embryonic development in lab mice and rats, we know very little about the function of IGF2 in other species (Zhu et al., 2017).

In mammals, six insulin-like growth factor binding proteins (IGFBPs) bind to IGF1 and IGF2 with high affinity. Together, these proteins both positively and negatively regulate the binding of IGF1and IGF2 to IGF receptors. This allows the binding proteins to increase the half-life of IGF proteins as much as tenfold and deliver the IGF proteins to tissues across the body (Daza et al., 2011; Denley et al., 2005). At any given time, as much as 99% of all IGF1 and IGF2 in circulation is bound to an IGFBP (Denley et al., 2005). In general, the IGFBPs are made up of four exons. The first is the IGFBP domain, which is conserved at a rate between 31% and 68% across the

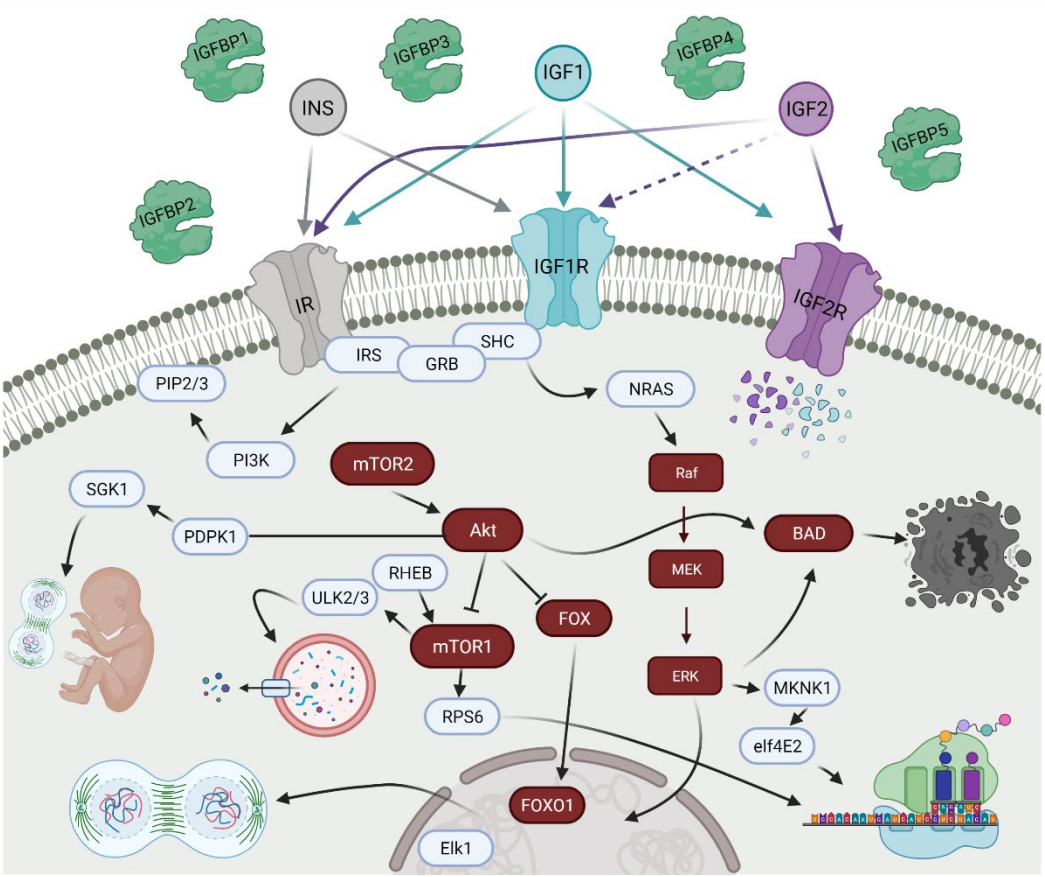

Figure 0.1: ***Extracellular and core intracellular components of the IIS network***. The IIS binding proteins (BP1-BP5) are shown in purple. The three hormones (INS, IGF1, and IGF2 are shown alongside each cellular receptor. Arrows indicate binding capability. Dashed arrows represent decreased binding efficiency, as seen in mammalian species. Modified from: Schwartz et al. (2016).

binding proteins (Daza et al., 2011). The second exon is highly variable between the different binding proteins as well as across species. The last two exons code for the thyroglobulin type-1 domain and is conserved at a rate between 31% and 59% across the proteins (Daza et al., 2011).

In addition to considerable conservation in the IGFs, insulin, and binding proteins, there is a substantial amount of homology between the IGF1R and the IR leading to interactions between the two proteins such that they can from heterodimers in the cell membrane. All three protein hormones can bind to the IGF1R with different levels of affinity. IR-B is the isoform most commonly associated with metabolic processes in humans and does not have a high affinity for binding the IGF proteins. However, IR-A, a second isoform has been shown to bind with a high affinity to IGF2, leading to activation of processes similar to those induced by IGF1R activation (Denley et al., 2005). The IGF2R is responsible for the modulation of IGF2 availability, and its main responsibility in the IIS network is to degrade and sequester IGF2 from activating other available receptors (Denley et al., 2005); However, unlike the IGF1R, the IGF2R is only known to bind IGF2 with high affinity, occurring exclusively in placental mammals (Denley, Clairmont & Czech, 1989; Yandell et al., 1999; Zhou et al., 1995).

When IGFs bind to their receptors, a conformational change activates the tyrosine kinase domain, leading to the phosphorylation of tyrosine residues (Al-Salam & Irwin, 2017). The phosphorylation in the beta subunits of the receptor allows for interactions with the IGF proteins, leading to downstream signaling (Al-Salam & Irwin, 2017). The binding of IGF hormones to the cellular receptors control biological processes such as growth, cellular proliferation, survival, aging, tissue formation, metabolism, and reproduction (Denley et al., 2005), and disruptions in these signaling

cascades can lead to severe physiological disorders in animals, including humans (for examples, see: Ashpole et al., 2017; Baxter, 2014; Boughanem et al., 2019; Chao & D'Amore, 2008; Fisher et al., 2005; Higashi et al., 2019; Koutsaki et al., 2011; Méio et al., 2009; Perks & Holly, 2003; Robinson et al., 2000; Thai et al., 2015).

What we don't know: Not everything can be studied in mice.

While we have gained a great deal of knowledge through correlation studies in humans (Garrone et al., 2002; Gourmelen et al., 1984) in addition to experimental students in rodents (ex. Cerro, JA et al., 1993; Clark et al., 2006; Rosen, 2007; Schuller et al., 1993; Shalamanova et al., 2008; Wolf et al., 1998; Yakar & Adamo, 2012; Yue et al., 2014) and a select few avian-based studies (Kocamis et al., 1998; Lodjak et al., 2017), a significant portion of IIS function remains unexplored. The IIS expression patterns observed in rodents, namely the lack of post-natal IGF2 expression (Yue et al., 2014), has led to spotlight focus on IGF1. Very little is known about the function of IGF2 beyond embryonic development across the vertebrate clade, and the roles of all IIS signaling components are largely unknown in reptiles.

Recent research by my lab group examining the IIS network in reptiles (McGaugh et al., 2015) has drawn attention to squamate species as a potential model for IIS function. The IIS network in reptiles has been shown to be under selection within the squamate clade, and a transcriptomic analysis of the network has shown that post-natal IGF2 expression is common across species, much like is seen in humans (McGaugh et al., 2015; Reding et al., 2016). However, this is dissimilar to studies completed in the lab-rodent (Smith et al., 2019; Yakar & Adamo, 2012; Yue et al., 2014), which is the most commonly used model in the study of the IIS network. The adoption of a new

reptilian IIS model species may lead to additional research avenues not available in frequently used mammalian systems.

Focal Study Species: *Anolis sagrei*

One squamate species shown to expressed both IGF1 and IGF2 throughout their lifespan and the focal species through this dissertation is the brown anole (*Anolis sagrei*). The brown anole is part of a large genus (361 recognized species) of lizards called the *Anolis* lizards. Brown anoles offer a host of benefits as a study organism. For example, they are an invasive species in Florida, that is easy to collect and maintain in a lab. Additionally, they have continuous vitellogenesis reproductive patterns (Goldberg et al., 2002; Losos, 2011; Norval et al., 2012), and possess the ability to completely regenerate tissue (Bateman & Fleming, 2011; Hoefer & Robinson, n.d.; Kaiser & Mushinsky, 1994; Kuo et al., 2015; Losos, 2011) - both traits are of interest to biomedical, ecological, and life-history fields. These characteristics are discussed in detail below.

Species Expansion

The brown anole (*Anolis sagrei*) is an invasive lizard species to the United States, radiating from multiple populations in Cuba and the Bahamas within the last 150 years (Kolbe et al., 2004). While the original invasion was limited to southern Florida, populations are now found as far west as Texas and as far north as Georgia (Fetters & Mcglothlin, 2017). Anoles are grouped into ecomorphs as sets of habitat specialists that vary in limb and toe length, girdle size, number of digital lamellae, and skull dimensions (Losos, 2011; Sanger et al., 2008), and the brown anole is classified as a "trunk-ground" (Huey et al., 1983; Losos, 2011) species fauna (**Fig. 0.2**) and has successfully colonized oceanic islands and the southern United States. The focal *Anolis* species

range from 4 to 8cm in length, weighing between 2 and 6 grams, with tail lengths as much as four times their body length (Losos, 2011). Due to the invasion of Florida, *Anolis sagrei* is readily available and has proven to reproduce well in captivity.

Reproduction

Brown anoles are sexually dimorphic (Cox et al., 2017; Losos, 2011; Reedy et al., 2016), with males presenting larger than females. In a population studied in the Bahamas, males were 32% larger in snout vent length, and 150% larger by mass (Reedy et al., 2016). Additionally, anoles possess a dewlap, or brightly colored structure on the front of their neck essential to communication, aggression, and mate attraction. Males have a more pronounced dewlap, tend to show increased aggression, and dorsal scale patterns can sometimes be used to visually distinguish sex.

Unlike most lizards that lay multi-egg clutches, anoles lay one egg every 7-10 days on average (can be as dramatic as 2-25 days depending on environment). Females develop eggs in alternating ovaries through the breeding season, utilizing multiple paternity and sperm storage as a means of fertilization for periods of up to seven months (Calsbeek et al., 2007; Fox, 1963).

Embryonic development in *Anolis sagrei* has been explored in great detail by Sanger *et.al* (2008), making it a model species for evolutionary developmental biology. Embryonic development has been laid out into 19 distinct stages (Sanger et al., 2008), with development time being highly dependent on incubation temperature.

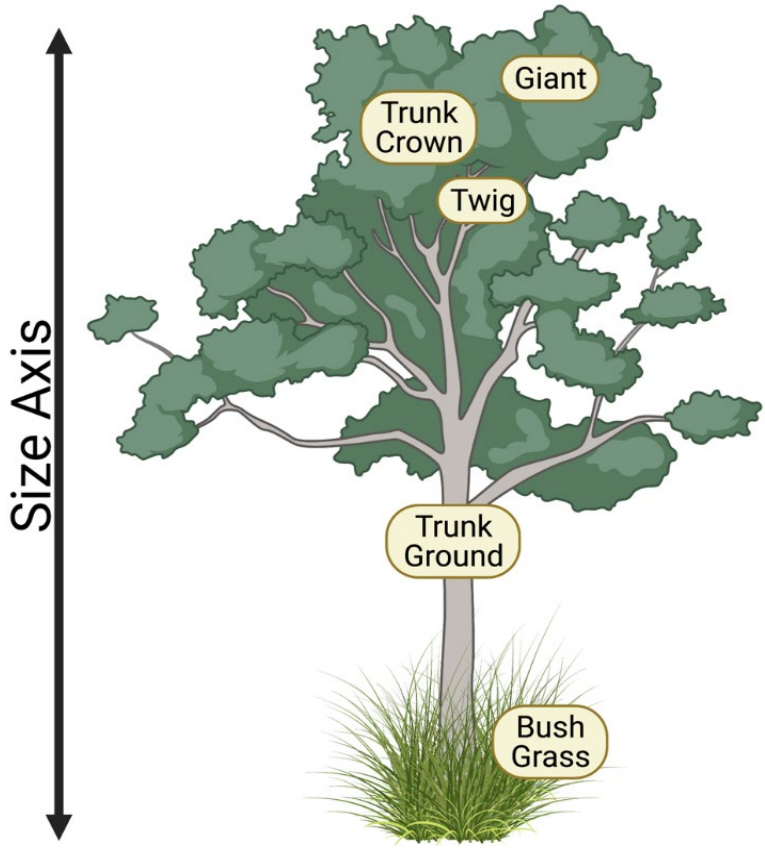

Figure 0.2: ***Anolis Ecomorphs***. The adaptive radiation of the anoles is shown above, displaying decreased body size with decreased elevation. Modified from Huey, 1983.

Post-natal Development and Lifespan

Following incubation, the hatchlings resemble adults but are approximately 20-30 mm in length, and weigh between 0.15 and 0.25 grams. By six months of age, animals begin to show signs of sexual dimorphism, and by approximately 9 months of age the juveniles are considered sexually mature and males begin displaying behavioral changes, such as increased aggression (Losos, 2011). While the lifespan of brown anoles is dependent on many factors such as predation, reproductive effort, body size, and habitat, the life expectancy for a hatchling in the wild is between

0.9 and 1.9 years for most species (Losos, 2011). However, lifespan has been shown to increase to as much as 5 years in captivity (Cox and Reedy, personal communications).

Description of Dissertation Chapters

My dissertation aims examine the IIS network in a reptilian system through a functional genomic lens to further understanding of how molecular mechanisms regulate life history traits. Towards this aim, the four data chapters of my dissertation (1) quantify the gene expression patterns of IGF1 and IGF2 across species in order to determine if the patterns seen in lab-rodents are atypical relative to those seen across the amniotic clade, including humans; (2) build the foundation for a reptilian IIS model by characterizing the gene expression of IGFs and IGFBPs across tissues and developmental stages in the brown anole lizard (*Anolis sagrei*); (3) examine the functional effects of forced investment in tail regeneration on reproduction in the brown alone; and (4) demonstrate how a course-based undergraduate research experience focused on novel IIS research can be an effective teaching tool in the undergraduate biology classroom.

SUMMARY OF CHAPTERS

Chapter 1: We need to talk, about IGF2.

A historical bias exists in the study of the vertebrate Insulin and Insulin-like Signaling (IIS) network. Insulin-like growth factor 1 (IGF1) and 2 (IGF2) are the key hormones regulating the IIS network through binding the Insulin-like Growth Factor 1 Receptor. Humans express both IGF1 and IGF2 as juveniles and adults. Rodent models for biomedical research have provided the wealth of information we currently have on this network; but they lack postnatal *IGF2* gene expression. This has led to the physiological effects of *IGF2* and its regulation of the IIS network during juvenile and adult stages to be largely ignored in biomedicine. This bias has translated to research in functional ecology, where IGF2 has also been understudied, likely due to the assumption that rodent-like IGF expression patterns exist across vertebrate species. To test this assumption, we quantify the relative liver gene expression of *IGF1* and *IGF2* across amniote lineages using two approaches: (1) analysis of adult liver RNAseq data from 82 amniote species from NCBI, and (2) qPCR on liver cDNA at embryonic, juvenile and adult stages of six species. Here, we present a cross species comparison that clearly demonstrates that *IGF2* is expressed postnatally in nearly all other amniotes tested, contradicting accepted patterns from laboratory rodent models. Additionally, we found that *IGF2* is expressed across embryonic, juvenile, and adult mammals, reptiles, and birds - often at higher relative expression compared to *IGF1*. Additionally, we find evidence of sex-biased adult expression in some species, and that outbred mouse strains lack IGF2 expression consistent with the lab-selected strains across two families. Our results demonstrate that postnatal expression of IGF2 is typical for amniotes, illustrating the need to pivot away from the null hypothesis defined by the laboratory rodents. Further, this study highlights a need for future studies examining the

roles of *IGF2,* alongside *IGF1,* in mediating variation in growth patterns and other life-history traits.

Chapter 2: Gene Expression of the IGF Hormones and IGF Binding Proteins Across Time and Tissues in a Model Reptile.

The Insulin and Insulin-like Signaling (IIS) network regulates cellular processes including pre- and post-natal growth, cellular development, wound healing, reproduction, and longevity. Despite their importance on the physiology of vertebrates, the study of the specific functions of the top regulators of the IIS network — insulin-like growth factors (IGFs) and IGF binding proteins (IGFBPs) has been mostly limited to a few model organisms. To expand our understanding of this network, we performed quantitative gene expression of IGF hormones in liver and qualitative expression of IGFBPs across tissues and developmental stages in a model reptile, the brown anole lizard (*Anolis sagrei*). We found that lizards express IGF2 across all life stages (pre-oviposition embryos to adulthood) and at a higher level than IGF1, which is opposite to patterns seen in lab rodents but similar to those seen in humans and other vertebrate models. IGFBP expression was ubiquitous across tissues (brain, gonad, heart, liver, skeletal muscle, tail, and regenerating tail) in adults, apart from IGFBP5 which was variable. These findings provide an essential foundation for further developing the anole lizard as a physiological and biomedical reptile model, as well as expanding our understanding of the function of the IIS network across species.

Chapter 3: Tails of Reproduction: Regeneration leads to increased reproductive investment.

Tradeoffs between life-history traits are due to limited resources or constraints in the regulation of genetic and physiological networks. Tail autotomy, with subsequent regeneration, is a common anti-predation mechanism in lizards and is predicted to trade-off tradeoff with life-

history traits, such as reproduction. We utilize the brown anole lizard with its unusual reproductive pattern, of single-egg clutches every 7-10 days, to test for a tradeoff in reproductive investment over eight weeks of tail regeneration on a limited diet. In contrast to predictions, we found that investing in tissue regeneration had a positive effect on reproduction in terms of egg size (11.7% relative to controls) and hatchling size (11.5% relative to controls), and no effect on egg number or survival, with the increase in reproduction starting at peak regeneration. We discuss mechanistic hypotheses that the process of regeneration may cause increased energetic efficiency or utilized shared physiological pathways with reproductive investment.

Chapter 4: Addressing the unique qualities of upper-level biology CUREs through the integration of skill-building.

Early exposure to course-based undergraduate research experiences (CUREs) in introductory biology courses can promote positive student outcomes such as increased confidence, critical thinking, and views of applicability in lower-level courses, but it is unknown if these same impacts are achieved by upper-level courses. Upper-level courses differ from introductory courses in several ways, and one difference that could impact these positive student outcomes is the importance of balancing structure with independence in upper-level CUREs where students typically have more autonomy and greater complexity in their research projects. Here we compare and discuss two formats of upper-level biology CUREs (Guided and Autonomous) that vary along a continuum between structure and independence. We share our experiences teaching an upper-level CURE in two different formats and contrast those formats through student reported perceptions of confidence, professional applicability, and CURE format. Results indicate that the Guided Format (i.e., a more even balance between structure and independence) led to more positive impacts on student outcomes than the Autonomous Format

(less structure and increased independence). We review the benefits and drawbacks to each approach while considering the unique elements of upper-level courses relative to lower-level courses. We conclude with a discussion of how implementing structured skill-building can assist instructors in adapting CUREs to their courses.

Chapter 1: We Need to Talk… About IGF2.

Beatty, AE, Rubin AM, Wada H, Heidinger B, Hood W, & Schwartz TS. *In Preparation.* We need to talk, about IGF2. To be submitted to *Functional Ecology* 7/2021.

Background

"*Mulla had lost his ring in the living room. He searched for it for a while, but since he could not find it, he went out into the yard and began to look there. His wife, who saw what he was doing, asked: "Mulla, you lost your ring in the room, why are you looking for it in the yard?" Mulla stroked his beard and said: "The room is too dark and I can't see very well. I came out to the courtyard to look for my ring because there is much more light out here* (Farzad, 1989)*"*

~ Mulla Nasreddin circa ….

The fable above describes a phenomenon defined as the "street-lamp" effect (Freedman, 2010), where there is a tendency to search for answers where it is easy to look, which may not always be the correct place to search. Once a discovery in science lights a street-lamp, it often defines the focus of the research community, and inadvertently discourages researchers from searching outside the pool of light, initiating biases in our research perspective. Here we illuminate such a bias that has arisen in the study of molecular mechanisms regulating life-history traits and their trade-offs.

The Insulin and Insulin-like Signaling (IIS) network has been well established in laboratory model organisms to regulate life history traits from embryonic development to aging (Allan et al., 2001; Anisimov & Bartke, 2013; Ashpole et al., 2015, 2016; Carter et al., 2002; Gubbi et al., 2018; McMurtry, 1998; Papaconstantinou, 2009; Richards et al., 2005; Schoenle et al., 1985; Soares et al., 1985; Stewart & Rotwein, 1996; Yakar & Adamo, 2012). This network is activated by the

paralogous hormones insulin, Insulin-like Growth Factor 1 (IGF1) and 2 (IGF2) that circulate in the blood and bind the Insulin-like Growth Factor 1 Receptor (IGF1R) and the Insulin Receptors, both IRA and IRB (Denley et al., 2005). The binding of the IGF hormones to cellular receptors stimulates signaling through the IIS network to promote biological processes such as growth, cellular proliferation, tissue formation, and reproduction (Constancia et al., 2002; Denley et al., 2005; Kocamis et al., 1998; Schoenle et al., 1985; Stewart & Rotwein, 1996; Yakar & Adamo, 2012). Decreased signaling through the IIS network is associated with stress resistance and increased longevity (Ashpole et al., 2017; Austad & Bartke, 2016; Greer et al., 2011; Tazearslan et al., 2011; Yakar & Adamo, 2012). IGFs were first studied in the laboratory rodent models, *Mus musculus* and *Rattus norvegicus*, (Rinderknecht & Humbel, 1976; Salmon & Daughaday, 1957), where *IGF1* was established to be highly expressed postnatally (Fagerberg et al., 2014; Yue et al., 2014). Thereafter, IGF1 has been studied extensively in the context of postnatal growth, maturation, body size, in the context of aging, and as a mediator of life history trade-offs (Clark et al., 2006; Elis et al., 2010; Lewin et al., 2017; Ohlsson et al., 2000; Tazearslan et al., 2011; Yakar & Adamo, 2012). In laboratory rodent models, it was found that *IGF2* is highly expressed during embryonic development, but down-regulated (i.e. turned-off) shortly after birth (Brown et al., 1986; Soares et al., 1985; Yue et al., 2014). In contrast to these rodents, *IGF2* is highly expressed postnatally in humans (Fagerberg et al., 2014), and plasma IGF2 protein is found to be 10-fold that of IGF1 in adult humans (Fowke et al., 2010). Thus, it is reasonable to suspect that IGF2 may also play an important role in influencing growth and other life-history traits after birth, but research in this area has been largely neglected.

While the rate of IGF1 and IGF2 publication was consistent from their discovery in 1978 through 1984, IGF1 experimentation increased rapidly between 1984 and 2020, far outpacing studies on IGF2 that remained fairly constant from the early 1990's through 2020, resulting in experimental studies on IGF2 comprising only 29.9% of all publications on IGFs **(Fig. 1; solid lines, unshaded)**.

This is likely a consequence of laboratory rodent models lacking expression of IGF2 postnatally. Furthermore, the research on *IGF2* has been largely focused on embryonic development (De Souza et al., 1995; White et al., 2018), the evolution of the mammalian placenta (Constancia et al., 2002), and *IGF2* misregulation in the development of cancer (Chao & D'Amore, 2008; Yu et al., 2017), leaving the roles of IGF2 in regulation of postnatal growth, reproduction, senescence, and potential trade-offs in these life-history traits under-explored (**Fig. 1, dashed lines**).

The studies on laboratory rodent models have lit a "street-lamp" defining IGF1 as **the** IGF hormone regulating IIS function postnatally. Traditionally, biomedical research forges the path for molecular research in functional and evolutionary ecology; and in this case, this established path has impacted the study of IGF2 in our field. While the levels of publication are significantly lower in species outside of rodents and humans, the proportion of studies on IGF1 remains consistently high relative to IGF2 **(Fig. 1; shaded areas)**. Recently, it has been documented that *IGF2* is expressed postnatally in reptile species (Beatty AE & Schwartz TS, 2020; Cox et al., 2017; McGaugh et al., 2015; Reding et al., 2016). In light of these results in the context of the bias against studies on IGF2, here we ask, "In amniote vertebrates, is the postnatal expression of *IGF2* the exception, or the norm?"

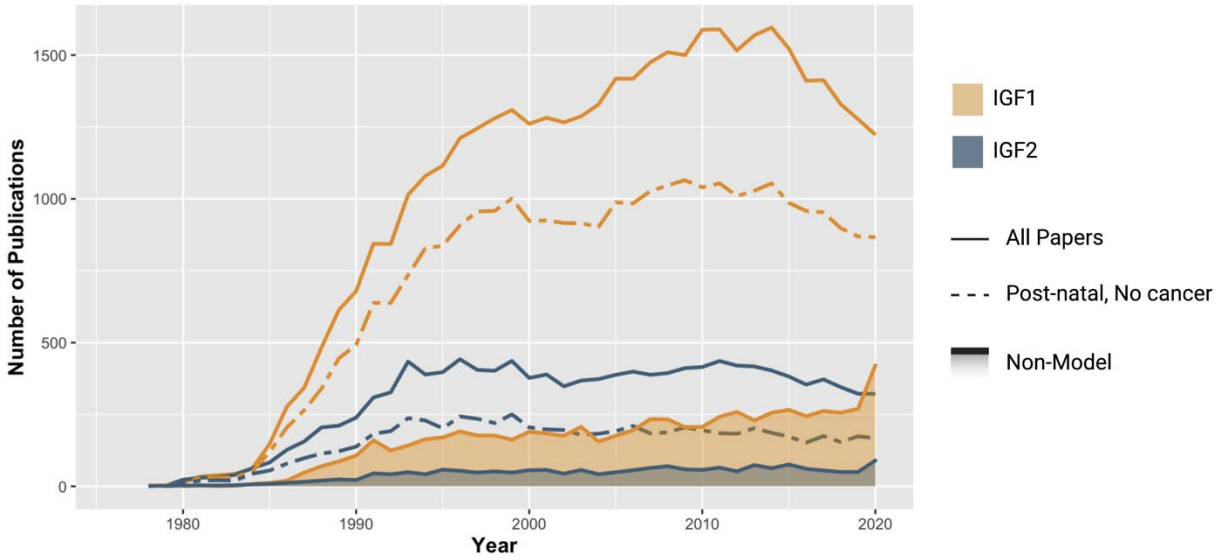

Figure 1.1**: **Research Bias on the study of IGF1 relative to IGF2. This graph shows the number of publications on either IGF1 or IGF2 each year. Results from a PubMed search using MESH terms. Solid lines represent the total number of IGF1 and IGF2 publications that were experimental in nature (MESH search queries: Insulin Like Growth Factor 2 NOT Review **and** Insulin Like Growth Factor 1 NOT Review). Dashed lines represent the proportion of papers at post-natal stages, excluding those performed in cancer research (MESH search query: Insulin Like Growth Factor 1 NOT Review NOT Developmental NOT Embryonic NOT Cancer **and** Insulin Like Growth Factor 2 NOT Review NOT Developmental NOT Embryonic NOT Cancer). Shaded curves represent the proportion of papers that were in non-biomedical model or humans (MESH search query: Insulin Like Growth Factor 2 NOT Review NOT human NOT Mouse NOT Rat **and** Insulin Like Growth Factor 1 NOT Review NOT human NOT Mouse NOT Rat). Graphical distribution of publications across years produced with R software (version 4.0.3, R Core Team) using ggplot2. Data and code provided in Supplemental GitHub.

To address this question, we examined postnatal gene expression of *IGF2* in mammals, birds and reptiles, by (1) mining liver transcriptome data to determine relative *IGF1* and *IGF2* gene expression patterns across 82 species from the amniote phylogeny, and (2) quantifying liver gene expression of both *IGF1* and *IGF2* across life stages in six reptile, bird, and mammalian species. Our aim is to further detail *IGF1* and *IGF2* gene expression patterns across the lifespan in other species outside of laboratory rodent models and to determine the overall prevalence of *IGF2* postnatal expression across the amniote phylogeny.

Methods

Liver RNAseq Survey

To evaluate the prevalence of postnatal *IGF2* expression across amniotes, we searched the NCBI Short Read Archive (SRA) database to identify RNAseq samples in amniotes that met the following search terms: adult OR juvenile, liver, RNAseq, Illumina. For each species we selected up to four individuals that represented the control conditions if they were from an experiment. When possible, we took two male and two female samples. For mice we used eight strains of *Mus musculu*s, including both inbred and outbred strains. SRA run files were downloaded using SRAtools (Wheeler et al., 2006) and cleaned using Trimmomatic (Bolger et al., 2014). For each major clade, an *IGF1* and *IGF2* reference transcript of the coding sequence (CDS) from a focal species was downloaded from ENSEMBLE, NCBI, or DRYAD (**Table 1**; Reference sequences and Code available in Supplemental Github). All the species from a clade were mapped to the same focal reference sequences (**Table 1**) using HiSat2 (Kim et al., 2019). Reads uniquely mapped to the reference transcripts were counted using Samtools (Li et al., 2009), and then normalized by size (kb) of the reference sequence and by number of cleaned reads in the SRA run (RPKM). Runs that had low numbers of reads resulting in no mapping to either IGF1 or IGF2 were removed from the study, resulting in a final sample size of 245 SRA Runs representing 82 species (Supplemental Github; **Table 1**).

For visual comparison we present the level of *IGF1* and *IGF2* as a ratio. When multiple samples were present within a species, the ratio of IGF1 to IGF2 was averaged across the individuals to

obtain a single proportion for each species. Relative expression of *IGF1* and *IGF2* can be compared within a sample, but we do not attempt to make statistical comparisons across samples as they are from different experiments, sequencing platforms, ages, etc. The sample sizes (n = 1 to 4 within species) are powered for detection and to provide a general idea of relative expression levels between *IGF1* and *IGF2*, but not for statistical testing of differences across species.

Gene expression across life stages using quantitative PCR

Because our focus was to survey the relative expression of *IGF* genes across life stages, rather than to statistically compare expression patterns between species, we utilized liver tissue from select species and ages that had been snap-frozen and stored in -80 °C from previously conducted experiments (**Table 2**). Quantitative gene expression analysis was completed on two birds (Zebra Finch and House Sparrow), two lizards (Brown Anole and Eastern Fence Lizard), and two rodents (House Mouse and Deer Mouse) across a series of life stages (embryo, juvenile, adult), using n=4 samples per group (**Table 2**). If these tissue samples were part of an experimental study, only control samples were used in this analysis. Total RNA was isolated from the liver samples using Illustra RNAspin Kit (Cytiva; 25-0500-70) including a DNAse digestion on a column membrane. Total RNA was quantified with the Nanodrop 2000 (ThermoFisher). Reverse transcription was conducted on 1000 ng of total RNA using qScript XLT cDNA Supermix (QuantBio; 95161-100). We quantified the expression of *IGF1* and *IGF2* using quantitative PCR (qPCR). For each species, a relative standard curve was created using a pool of cDNA over four 5-fold dilutions (1:1,1:5, 1:25, 1:125). Species-specific qPCR primers (**Table 2**) were designed to amplify a 100-150 bp product. PCR efficiency of primers were validated using the standard curve. The standard curve

Table 1.1. Summary of liver RNAseq samples downloaded from the Short Read Archive Database on NCBI used for mapping reads to IGF1 and IGF2 transcripts (CDS) from reference species in the same group. See Supplemental File 1 for full details on the samples used.

Category	Group	Number of species	Number of Runs	Source of Reference for Mapping
Mammals	Afrosoricide	1	2	Lesser Hedgehog tenrec: Transcript: IGF1-201 ENSETET00000018550.1 Lesser Hedgehog tenrec: NCBI IGF2 XM_004717217.1
Mammals	Carnivora	4	16	American Black Bear: Transcript: IGF1-201 ENSUAMT00000023612.1 American Black Bear: Transcript: IGF2-201 ENSUAMT00000030099.1
Mammals	Cetartiodactyla	4	16	Cow: Transcript: IGF1-201 ENSBTAT00000014713.6 Cow: Transcript: IGF2-201 ENSBTAT00000085576.1
Mammals	Chiroptera	8	24	Large Flying Fox: Transcript: IGF1-201 ENSPVAT00000005391.1 Large Flying Fox: Transcript: IGF2-201 ENSPVAT00000012242.1
Mammals	Eulipotyphla	3	6	Western European Hedgehog: NCBI XM_016190326.1 Western European Hedgehog: NCBI XM_007535866.2
Mammals	Lagomorpha	1	4	Rabbit: Transcript: IGF1-204 ENSOCUT00000053590.1 Rabbit: IGF2 NCBI NM_001171406.1
Mammals	Perissodactyla	1	4	Horse: Transcript: IGF1-201 ENSECAT00000055976.1 Horse: Transcript: IGF2-201 ENSECAT00000078762.1
Mammals	Pholidota	1	4	Malayan Pangolin: NCBI XM_017681363.1 Malayan Pangolin: NCBI XM_017669259.1
Mammals	Primate	4	17	Macaqua Mulatta: Transcript: IGF1-201 ENSMMUT00000065439.2 Macaqua Mulatta: Transcript: IGF2-201 ENSMMUT00000106459.1
Mammals	Rodentia, Mouse/Rat	3 (3 inbred strains and 5 outbreed strains of *Mus musculus*)	80	Mouse C57BL6: Transcript: Igf2-209 ENSMUST00000178921.1 Mouse C57BL6: Transcript: Igf1-204 ENSMUST00000121161.7 Norway Rat: Transcript: Igf1-201 ENSRNOT00000005995.5 Norway Rat: Transcript: Igf2-201 ENSRNOT00000050760.3
Mammals	Scandentia	1	2	Chinese Tree Shrew: NCBI IGF1 XM_006141400.3 Chinese Tree Shrew: NCBI IGF2 XM_014590814.1

Mammals	Xenarthra	1	2	Nine Banded Armadillo: Transcript: IGF1-201 ENSDNOT00000052460.1 Nine Banded Armadillo: NCBI XM_023585640.1
Marsupial	Dasyuridae	1	2	Gray Short-Tailed Opossum: NCBI IGF1 XM_007503333.2 Gray Short-Tailed Opossum: NCBI IGF2 DQ519591.1
Marsupial	Didelphidae	3	4	
Marsupial	Diprotodontia	1	3	
Marsupial	Peramelidae	2	2	
Monotreme	Platypus	1	1	Platypus: NCBI IGF1 XM_016227945.3 Platypus: NCBI IGF2 NM_001242705.1
Reptiles	Crocodilian	1		Chinese Alligator: Genome assembly GCA_000455745.1 IGF1 and IGF2 CDS alignments from McGaugh et al. https://doi.org/10.5061/dryad.vn872
Reptiles	Lizard	10	11	Green Anole: IGF1 ENSACAT00000041750.1 Green Anole: IGF2 ENSACAT00000044638.1 IGF1 and IGF2 CDS alignments from McGaugh et al. https://doi.org/10.5061/dryad
Reptiles	Serpentes	6	7	Western Terrestrial Garter Snake. IGF1 and IGF2 CDS alignments from McGaugh et al. https://doi.org/10.5061/dryad
Reptiles	Testudines	7	10	Painted Turtle: IGF1 ENSCPBT00000001991.1 Painted Turtle: IGF2 ENSCPBT00000011479.1 IGF1 and IGF2 CDS alignments from McGaugh et al. https://doi.org/10.5061/dryad
Reptiles	Aves	18	28	Zebra Finch: IGF1 ENSTGUT00000043214.1 Zebra Finch: IGF2 ENSTGUT00000009721.2 IGF1 and IGF2 CDS alignments from McGaugh et al. https://doi.org/10.5061/dryad
Total		82	245	

was run in triplicate along with the respective species samples using 3uL of cDNA at a primer specific dilution (see **Table 2**) in a 20 μL reaction using PerfeCTa SYBR Green SuperMix (QuantBio; 95054-050) with 0.25 μM of each primer. Reactions were run on BioRad 96FX thermal cycler using the cycle: 95 °C for 2 min, and then 40 cycles of 95 °C for 20 sec and 60 °C for 20 sec, followed by a melt curve from 60 °C to 95 °C in increments of 0.5 °C for 5 sec to test for off-target amplification. The specificity of each primer set was verified by single peaks in the melt-curves. Within a gene we calculate relative gene expression using the Ct value relative to the species-specific, gene-specific standard curve, and multiplied by the cDNA dilution factor. Expression levels can be compared between genes, and across ages within a gene and species, but not across species. Our sample sizes (n = 2 to 4 within species/age group) are powered for detection and general idea of expression level. While statistical analysis was performed to assess relative levels of *IGF1* and *IGF2* expression at each timepoint, samples were not statistically analyzed longitudinally due to a lack of statistical power.

Data Curation and Statistical Analyses

CFX Maestro Software (Bio-Rad) was used to convert CQ values to copy number of *IGF1* and *IGF2* for each sample, adjusting expression values based on the PCR efficiency of each primer pair as determined by the standard curve. All statistical analyses were performed using copy number as a measure of gene expression, and all analyses were completed using R software (version 4.0.3, R Core Team). All statistical code, data curation, and raw data output are provided in the Supplementary Github Repository.

To test for differences in relative gene expression of *IGF1* and *IGF2* at each life stage across species, data were subset by species and subsequently by life stage (embryonic, juvenile and adult) and analyzed separately. A linear mixed-effect model (Pinheiro, Jose et al., 2018) was used to analyze the relative differences between copy number of the genes (*IGF1*, *IGF2*). Individual was included as a random effect to account for sample triplicates during qPCR analysis. Sex was included as an independent variable at the adult life stage and as an interaction term when included as an independent variable at the adult life stage and as an interaction term when appropriate (at least two of each sex were available for analysis). When there was a significant interaction between gene and sex, the two sexes were then separated for analysis.

Results

Postnatal Liver RNAseq Survey

The RNAseq runs available in the SRA database provided reasonable coverage of species across the major clades in Mammalia and Reptilia, although some of the smaller clades are only represented by a single species and in some cases a single RNAseq run (**Table 1, Fig. 2**). Across the 82 amniote species for which we were able to download and map the liver postnatal RNAseq data, we found that only four species did not have detectable (less than 0.01%) postnatal *IGF2* expression; two were laboratory-rodents, the House Mouse (*Mus musculus*) and the Brown Rat (*Rattus novegicus*), and the remaining two were the European Hedgehog (*Erinaceus europaeus*), and the Asian House Shrew (*Suncus murinus*) (**Fig. 2**). Strikingly consistent across all the three inbred and five outbred mouse strains, there was no postnatal expression of *IGF2* detected (**Fig.**

Table 1.2. Summary of species, samples, and qPCR parameters used for relative qPCR analysis.

Group	Species	Age - Sex (M:F)	Primers	cDNA Dilution	Annealing Temp (C)	qPCR Efficiency
Aves	Zebra Finch (*Taeniopygia guttata*)	Embryonic - 4 Unk Juvenile - 4 Unk Adult - (2M:2F)	**IGF1(F):** GTG CTG AGC TGG TTG ATG C **IGF1(R):** TAT TCC CTT GTG GTG TAA GCG **IGF2(F):** GGA GCT GGT GGA CAC GCT GC **IGF2(R):** CAG CAC TCC TCC ACG ATC CC	1 : 3 1 : 3	60°C 60° C	E= 110.0% R^2= 0.996 E= 110.3% R^2= 0.998
	House Sparrow (*Passer domesticus*)	Juvenile - (4M:4F) Adult - (1M:3F)	**IGF1(F):** GTG CTG AGC TGG TTG ATG C **IGF1(R):** TAT TCC CTT GTG GTG TAA GCG **IGF2(F):** GGA GCT GGT GGA CAC GCT GC **IGF2(R):** CAG CAC TCC TCC ACG ATC CC	1 : 3 1 : 3	60° C 60° C	E= 100.1% R^2= 0.990 E= 116.7% R^2= 0.985
Squamates	Eastern Fence Lizard (*Sceloporus undulatus*)	Juvenile - 4 Unk Adult - 4M	**IGF1(F):** ACG ATC TGT ACG TGC TCA GC **IGF1(R):** GAG TGC TTT GGG GAT TGG GA **IGF2(F):** TGC CAT CGA TAT CTG TGG GC **IGF2(R):** TCA GAA ACC CTC TCA CCC CA	Undil. 1 : 3	60° C 60° C	E= 101.6% R^2= 0.982 E= 103.0% R^2= 0.987
	Brown Anole (*Anolis sagrei*)	Embryonic - 4 Unk Juvenile - (2M:2F) Adult - (2M:2F)	**IGF1(F):** GGA GGC AAT CGA CGT TCA GT **IGF1(R):** ACG GAT CGT GCG GTT TTA TCT **IGF2(F):** CTG TGG GCA GAA ACA GAG GA **IGF2(R):** TGA TTT TGC ACA GTA GGT TTC CAA	Undil. 1 : 5	65° C 60° C	E= 119.5% R^2= 0.993 E= 99.4% R^2= 0.997
Mammal	House Mouse (*Mus Musculus*) Deer Mouse (*Peromyscus maniculatus*)	Embryonic[$] - 4 Unk Adult* - (2M:2F) Juvenile[#] - 4 Unk Adult[#] - (2M:2F)	**IGF1(F):** GGG GCT TTT ACT TCA ACA AGC **IGF1(R):** CAG TCT CCT CAG ATC ACA GC **IGF2(F):** GAG GGG AGC TTG TTG ACA C **IGF2(R):** AGC ACT CTT CCA CGA TGC	1 : 3 1 : 3	65° C 65° C	E= 106.2% R^2=0.996 E= 101.8% R^2= 0.997

[$]C57BL/6J liver from late-stage fetuses, [#] obtained from the Peromyscus Genetic Stock Center, University of South Carolina, Columbia, SC and then maintained at Auburn University, *obtained from NIH-NIA Aged Rodent Tissue Bank, C57BL/6 liver tissue collected at 12mn of age

3a). We found three species (two birds and one turtle) that had no detectable *IGF1* expression, and an additional eight species from across mammals and reptiles that had very low *IGF1* expression (< 2% of total *IGF1* expression, **Fig. 2**). It is noteworthy that because the reads were being mapped to a reference from another species, if the nucleotide sequences for *IGF1* or *IGF2* were quickly evolving in that clade (e.g. *IGF1* in Squamates, McGaugh et al., 2015), it may decrease the number of reads that were able to map and thus underestimate the expression abundance. Across the amniote phylogeny there is a lot of variation in the relative levels of *IGF1* and *IGF2*, but consistently we see *IGF2* expressed postnatally across both mammal and reptile clades, in striking contrast to the pattern seen in the laboratory rodents.

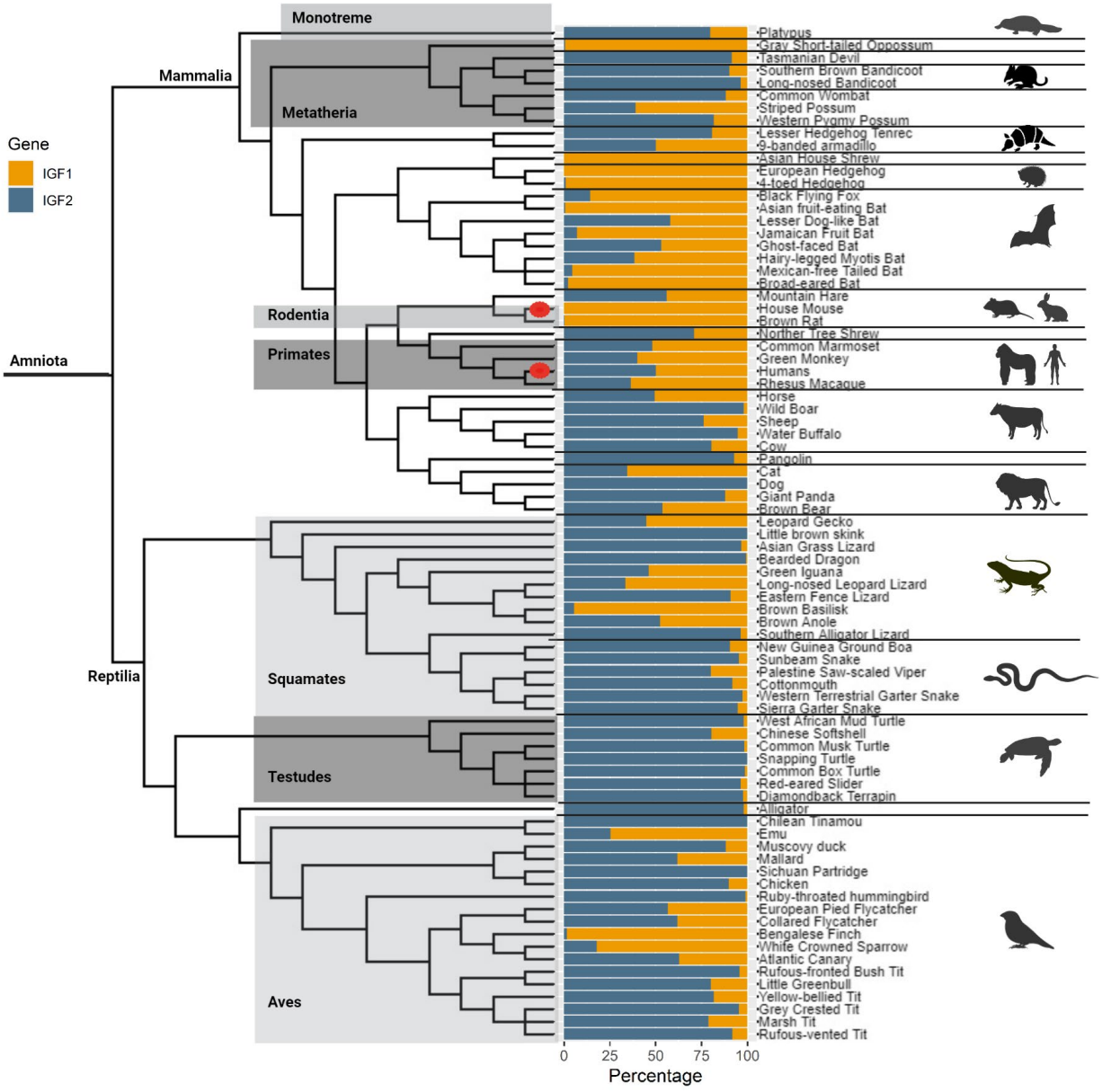

Figure 1.2: Relative IGF1 and IGF2 expression Across Amniotes. The phylogenetic tree represents relationships among the amniote species used in this transcriptomic analysis. Each species is represented by 1-4 liver RNAseq samples downloaded from NCBI (Supplemental File 1 for full details). The branch lengths do not represent evolutionary distance. The horizontal blue/orange bar represents the relative proportions of *IGF1* (orange) and *IGF2* (blue) gene expression for each species, averaged across all the individuals of that species if we had more than one sample. Orders of interest are labeled and outlined with gray shaded boxes. Red dots indicate house mouse and human values.

Quantitative reverse transcriptase PCR

Quantitative gene expression analysis was completed on two birds (Zebra Finch and House Sparrow), two lizards (Brown Anole and Eastern Fence Lizard), and two rodents (House Mouse and Deer Mouse) across a series of life stages (**Table 2**). The patterns seen within adulthood during the RNAseq survey were verified in each of our qPCR analyses, and we expand on those findings statistically, along with comparisons at the juvenile and embryonic stages.

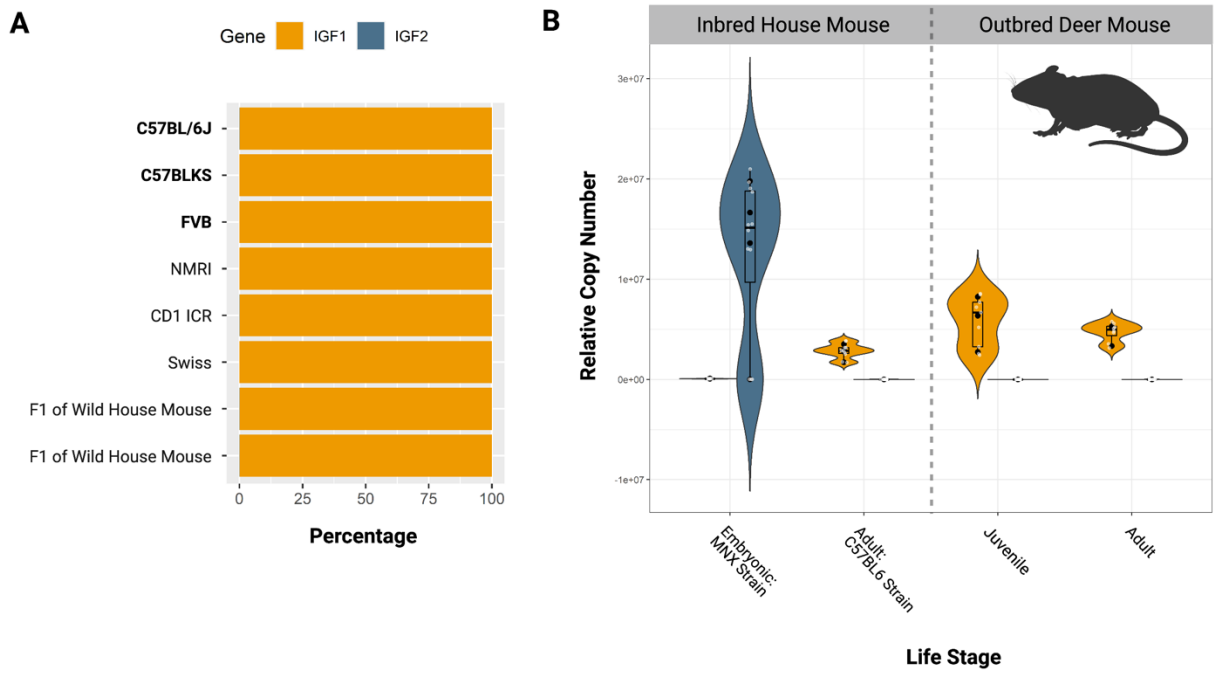

Figure 1.3: Relative *IGF1* and *IGF2* in Inbred and Outbred Mice. **(A)** Utilizing publicly available RNAseq data, relative levels of *IGF1* (orange) and *IGF2* (blue) expression were calculated for all accessible strains of the laboratory house mouse. Inbred strains are represented in bold text, while outbred strains are shown in unformatted text. **(B)** Quantitative PCR analysis was performed on embryonic and adult laboratory inbred house mouse liver as well as juvenile and adult outbred deer mouse samples. *IGF1* (orange) and *IGF2* (blue) expression is depicted in relative copy number. Triplicate qPCR runs are represented by individual white datapoints, while averages for individuals are represented by a single black datapoint.

Birds

As expected, based on the RNAseq survey, both the Zebra Finch and the House Sparrow expressed both *IGF1* and *IGF2* in adulthood. Within the House Sparrow, there was no statistically significant difference in relative expression between the two genes at adulthood (Estimate = 50,766.3 ± 97,480.5, p=0.612), or the juvenile stage (Estimate= -346,245.4 ± 255,461.0, p=0.184) (**Fig. 4a; Table 3**). Adult House Sparrow samples were limited to 3 females and a single male, therefore no gene by sex comparison was performed.

During embryonic development in Zebra Finches *IGF2* was expressed at a significantly higher level than *IGF1* (Estimate= 1,391,307 ± 249,475, p<0.0001). In contrast, during the juvenile life stage, *IGF1* was the predominantly expressed gene (Estimate= -379,543.5 ± 119,870.6, p=0.005). While there was no significant difference in relative expression at the adult stage based on gene alone (Estimate= -1,602,247.0 ± 783851.0, p=0.056), there was a gene by sex interaction that neared significance (Estimate= 2,025,798.0 ± 1,108,533.0, p=0.083). Due to marginal significance with our limited sample size and the moderate effect size, we analyzed each sex separately and found that *IGF2* was expressed at a significantly higher level than *IGF1* in male finches only (Estimate= 423,551.0 ± 138,612.4, p=0.014) (**Fig. 4a; Table 3**).

Lizards

The Eastern Fence Lizard exhibited significantly higher expression of *IGF1* during the juvenile life stage (Estimate= -760,675.0 ± 210,606.5, p=0.002). By adulthood (male samples only), there

was no statistically detectable difference in relative *IGF1* and *IGF2* expression (Estimate= -9,152.3 ± 189,742.0, p=0.962) (**Fig. 4b; Table 3**).

In comparison, there was no statistical difference in relative expression of *IGF1* and *IGF2* at either the embryonic (Estimate= 1,047.3 ± 952.6, p=0.286) or the juvenile life stages (Estimate= -5,569.1 ± 4,501.1, p=0.233) in the Brown Anole. By adulthood, *IGF2* expression was significantly higher than *IGF1* (Estimate= 308,578.4 ± 90,239.0, p=0.003). However, much like the Zebra Finch samples, there was an interesting gene by sex interaction (Estimate= -365,100.8 ± 127,617.1, p=0.010). It was found that *IGF2* was expressed at a significantly higher level than *IGF1* in females (Estimate= 308,578.4 ± 11,854.4, p<0.001), while there was no statistically detectable difference within males (Estimate= -56,522.5 ± 127,065.4, p=0.667) (**Fig. 4b; Table 3**).

Rodents

Due to the patterns of IGF expression within inbred and outbred mouse strains observed in the RNAseq analysis, we chose to expand our qPCR analysis to both inbred and outbred rodents across life stages. In order to do so, we used samples from inbred House Mouse C57BL/6J strain embryonic laboratory rodents, inbred House Mouse C57BL6 adult laboratory rodents, outbred Deer Mouse juveniles, and outbred Deer Mouse adults. Similar to what has previously been reported in laboratory rodents, we found a lack of *IGF2* expression following embryonic development (Estimate= -3,684,151.0 ± 328,946.5, p < 0.001) and significantly higher *IGF2* expression during embryonic development in the inbred laboratory reared individuals (Estimate= 16,312,472.0 ± 847,373.0, p < 0.001). Interestingly, these patterns persisted in outbred Deer

Mouse, with *IGF2* expression being nearly undetectable at both the juvenile (Estimate= -5,648,981.0 ± 606,787.2, $p < 0.001$) and adult (Estimate= -3,684,151.0 ± 328,946.5, $p < 0.001$) timepoints (**Fig. 3b; Table 3**).

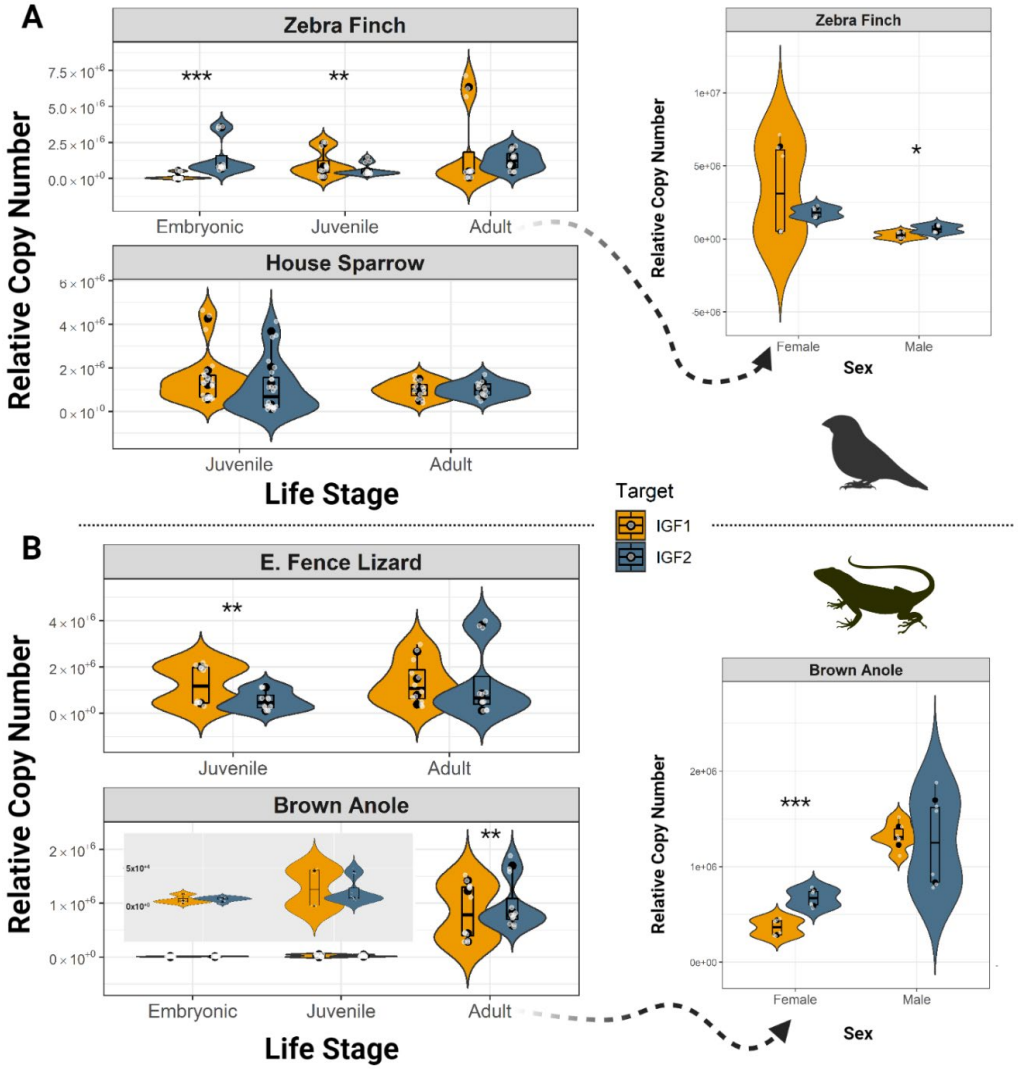

Figure 1.4: Relative expression of *IGF1* and *IGF2* across life stages. (A) Aves. Quantitative PCR analysis was completed on liver samples from the Zebra Finch at three life stages and the House Sparrow at two life stages. **(B) Squamates**. Quantitative PCR analysis was completed on liver samples from the Brown Anole at three life stages and the Eastern Fence Lizard at two life stages. To more clearly see the relative expression levels in the Brown Anole plot, an inset of the embryonic and juvenile life-stages is shown in grey. For all plots, *IGF1* (orange) and *IGF2* (blue) expression is depicted in relative copy number. Triplicate qPCR runs are represented by individual white datapoints, while averages for individuals are represented by a single black datapoint. When there was a significant interaction between sex and gene expression, the sexes were plotted separately. Significance is indicated with an asterisk (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 1.3. Statistical results from qPCR analysis. Comparisons evaluate relative expression of *IGF1* and *IGF2* at each life stage and between sexes when appropriate. A positive estimate indicates *IGF2* is expressed at a higher level, whereas a negative estimate indicates *IGF1* has relatively higher expression.

Species	Age	Sex Specific Comparison	Estimate ± SD	P-value
Zebra Finch	Embryonic	--	1,391,307 ± 249,475	**< 0.0001**
	Juvenile	--	-379,543.5 ± 119,870.6	**0.005**
	Adult	--	-1,602,247.0 ± 783851.0	0.056
		Gene: Sex Interaction	2,025,798.0 ± 1,108,533.0	0.084
		Male	423,551.0 ± 138,612.4	**0.014**
		Female	-1,602,247.0 ± 1,098,905.0	0.179
House Sparrow	Juvenile	--	-346,245.4 ± 255,461.0	0.184
	Adult	--	50,766.3 ± 97,480.5	0.612
E. Fence Lizard	Juvenile	--	-760,675.0 ± 210,606.5	**0.002**
	Adult	--	-9,152.3 ± 189,742.0	0.962
Brown Anole	Embryonic	--	1,047.3 ± 952.6	0.286
	Juvenile	--	-5,569.1 ± 4,501.1	0.233
	Adult	--	308,578.4 ± 90,239.0	**0.003**
		Gene: Sex Interaction	-365,100.8 ± 127,617.1	**0.010**
		Male	-56,522.5 ± 127,065.4	0.667
		Female	308,578.4 ± 11,854.4	**< 0.001**
Mouse	Embryonic (House Mouse)	--	16,312,472.0 ± 847,373.0	**< 0.001**
	Juvenile (Deer Mouse)	--	-5,648,981.0 ± 606,787.2	**< 0.001**
	Adult (House and Deer Mouse)	--	-3,684,151.0 ± 328,946.5	**< 0.001**

Discussion

Both IGF1 and IGF2 bind IGF1R to activate the IIS network. Lack of IGF2 expression in rodents after birth has led to a research bias towards IGF1 in amniotic postnatal life, and limited the interpretation and understanding of the functional impacts of the IIS network. Here, we demonstrate that IGF2 is indeed expressed postnatally across the mammalian and reptilian clades, and provide suggestive evidence that age-related changes in IGF1 and IGF2 are sex-specific in some species.

Through the transcriptomic analysis on 82 species, we found *IGF2* postnatal expression to be the "norm" across the amniote phylogeny, being detected in 95% of the species. In fact, most species (56 of the 82 total species) expressed *IGF2* at a level of 50% or greater of total IGF expression. These data confirm that the lack of IGF2 expression after birth seen in rodents, and perhaps a few other mammalian groups, are an exception. When examining the expression of the *IGF* hormones across the lifespan via quantitative gene expression analysis, we again confirmed that *IGF2* was expressed in both bird and lizard species at all life stages–from embryonic development to adulthood–and often at an equal or higher level than *IGF1*. While existing work examining *IGF2* expression and *IGF2* in circulation is limited, this is consistent with previous studies performed in both Aves and Squamates. For example, in the Wild Turkey (*M. gallopavo*), hepatic *IGF2* expression decreased significantly at the time of hatching relative to embryonic expression, but by three weeks post-hatching, the expression levels had risen to levels statistically similar to those of late embryonic development (Richards et al., 2005). Another study in turkeys found that circulating *IGF1* and *IGF2* levels were similar three weeks post-hatching with *IGF2* remaining detectable at high levels in circulation through 20 weeks post-hatching, showing that not only is *IGF2* expressed, but it is also being translated to protein and can be detected in circulation (McMurtry, 1998). Similarly, in Brown Anoles *IGF2* was expressed at the embryonic, juvenile and adult stages (Beatty AE & Schwartz TS, 2020), with males expressing both *IGF1* and *IGF2* at a higher level than females in adulthood (Cox et al., 2017). Further, a survey of 18 squamate juvenile or liver transcriptomes (also included in this study) found *IGF2* to be expressed in every species (McGaugh et al., 2015). The results presented herein further support and extend these previous findings.

Our transcriptome survey and qPCR results show unique patterns in the *IGF1*:*IGF2* expression ratios across clades and between sexes. It is worth noting that our sample sizes within a group are small and should be used to generate hypotheses for future in-depth experiments rather than generalizable conclusions beyond the nearly ubiquitous postnatally expression of *IGF2* across non-rodent species. Despite our limited sample size and high levels of individual variation, using two different methods, the data clearly demonstrate that *IGF1* and *IGF2* are both expressed across the lifespan in majority of species (all clades), and *IGF2* is often expressed at a higher level than *IGF1* postnatally. Interestingly, there were clear sex by gene interactions in Zebra Finch and Brown Anole adults. In each case, only one sex showed significant differences in relative *IGF1* and *IGF2* expression. In the Zebra Finch, the males expressed *IGF2* at a higher level than *IGF1*, while in the Brown Anole, it was the females that displayed this relationship. Additionally, in female Zebra Finches (no statistical difference in relative expression), *IGF1* displayed extreme variation in expression levels, while male *IGF2* expression was highly variable in the Brown Anole. While these findings are intriguing and may be biologically significant, with the limited sample size available in this study, the relationships should be explored further in the future.

Interestingly, this research also expands on the depth of the limitations of mouse models in IIS research. Both the RNAseq and the qPCR results demonstrate that the lack of *IGF2* expression seen in laboratory mice is not due to generations of inbreeding in an artificial environment. If IGF2 were expressed in outbred House Mouse or Deer Mouse, this would allow for the use of the extensive resources for the laboratory mouse such as existing knockout strains, antibodies, and quantification methods to study IGF2 in other rodents. However, as we dug deeper into mouse expression patterns, we found that neither the 8 strains with publicly available RNAseq data, nor

the post-natal samples collected from inbred laboratory house mice or outbred Deer Mice examined through quantitative gene expression displayed detectable *IGF2* expression. While the lack of mouse postnatal IGF2 expression is similar to what has been shown previously (Smith, CM et al., 2019; Yue et al., 2014), our results further illustrate that when it comes to understanding the functional effect of the IIS network and the interactions between the *IGF* hormones as they compete for cellular receptors and binding proteins, rodent models may be the exception to the normality of post-natal *IGF2* expression, regardless of inbreeding status or evolutionary clade (Muridae vs. Cricetidae family).

The results presented here demonstrate the street-lamp effect that has occurred in the study of the hormone regulators of the IIS network. The scientific community at large has known about the co-expression of *IGF1* and *IGF2* throughout the life in humans (Fagerberg et al., 2014; Pontén et al., 2008; Sussenbach et al., 1992; Uhlen et al., 2015; Zapf et al., 1981) and other species (McGaugh et al., 2015; Wolf et al., 1998) (**Fig 1**). The limitations of biomedical rodent models has focused our attention on IGF1 while our level understanding of IGF2 remains clouded. In consideration of the complexity of such a prolific physiological network, both evolutionarily and systematically, the concentrated focus of IGF1 rather than both hormones has the potential to strongly impact our understanding of its functional and ecological impact within the field. Importantly, both of these hormones can bind, and compete for binding, to the IGF1R to regulate signaling to promote growth and reproduction, thereby if only measuring one of these hormones we are only getting half of the story. Additionally, from a technical perspective IGF1 and IGF2 are highly similar hormones that are likely to compete for antibodies that would lead to cross-talk when trying to quantify either one (Denley et al., 2005; Zhao et al., 2011). If the level of cross-talk is not assessed for each IGF

antibody in a particular species it is unclear to what degree the measures represent quantities of IGF1, IGF2, or both.

Much research is still needed to understand the functions of each IGF hormone. Importantly, these hormones are pleiotropic and their functions may change with sex, age, energetic status, stress, and reproductive state, requiring controlled experiments in order to elucidate their individual functions. Functional experiments manipulating levels of IGFs through processes such as supplemental injections, CRISPR, and cell culture experimentation can be utilized to understand functions and consequences on phenotypes, physiology, and fitness as well as how those relationships evolve across species groups. In order to properly conduct these experiments, large technical advances are also necessary. Assays that can reliably detect IGF1 and IGF2 independently in order to accurately measure these hormones with minimal cross-talk are essential in studying their relationships in response to biotic and abiotic variables across species. But the first step is awareness that the bias is present; it is then up to researcher's to look beyond the current pool of light. We hope the results presented here, showing near ubiquitous expression of IGF2 across the amniote clade, encourages the functional ecology community to start talking about, and studying, IGF2.

Endnote

Additional materials related to this manuscript may be found at [https://github.com/aeb0084/IGFs-Across-Amniotes.git].

Chapter 2: Gene Expression of the IGF Hormones and IGF Binding Proteins Across Time and Tissues in a Model Reptile

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Background

The endocrine system mediates whole organism physiological responses through a variety of molecular pathways. This includes the Insulin and Insulin-like Signaling (IIS) network that is a complex mechanistic network with pleiotropic downstream effects. The regulation of this pathway is crucial to guiding processes such as growth and development, aging, tissue regeneration, stress resistance, and reproduction (Denley et al., 2005; Hutchins et al., 2014; Jones & Clemmons, 1995; Stewart & Rotwein, 1996). For this reason, the IIS network and the hormones regulating it are of interest to many disciplines ranging from biomedical physiology to life history evolution.

While a great deal of research has been conducted on the IIS network, the vast majority of this work has been focused on mammalian, specifically rodent, models. Despite the IIS network's importance, outside of the lab rodent models, there is relatively little information describing how the IIS network regulates physiology in other organisms. While the IIS network has been studied to some degree in every major vertebrate clade, reptiles – the group sister to mammals – has been considerably understudied in terms of the function of the IIS network (72, but see 33, 50, 61, 79, 80). This is despite their importance for biomedical research. Because of their many unique physiological adaptations and their evolutionary proximity to mammals, reptilian species have become models for developmental biology (Du et al., 2010; Gredler et al., 2014; Rhen & Schroeder, 2010; Sanger et al., 2008; Shine, 1999), evolution (Kolbe et al., 2012; Losos, 2011;

Reynolds et al., 2020; Stuart et al., 2014; Van Dyke et al., 2014), physiology (Fetters & Mcglothlin, 2017; Shine & Guillette, 1988), aging (Bradley Shaffer et al., 2013; Bronikowski & Promislow, 2005; Congdon et al., 2003; Hoekstra et al., 2020; McGaugh et al., 2015; Robert & Bronikowski, 2010; Warner et al., 2016), functional genomics (Castoe et al., 2011; Cox et al., 2017; Passow et al., 2019; Rasys et al., 2019), and tissue regeneration (Alibardi, 2014; Andrew et al., 2017; Hutchins et al., 2014; Hutchins & Kusumi, 2016; Perry et al., 2019).

Across vertebrates, three main hormones circulate in the bloodstream and regulate the IIS network: insulin, and the two insulin-like growth factors (IGFs), IGF1 and IGF2. These hormones compete for access to the cellular receptors. Within the IIS network, there are three cellular receptors capable of binding IGF1 and IGF2, the Insulin-receptor (IR), the IGF1-receptor (IGF1R) and the IGF2-receptor (IGF2R) (Denley et al., 2005). The function of the IGF2R in reptiles is unknown (Schwartz & Bronikowski, 2016), while in mammals it is primarily responsible for sequestering IGF2 from cellular activation (Denley et al., 2005). However, both IGF1 and IGF2 compete for binding of the IGF1R and IR at varying affinities to initiate a cellular cascade, resulting in the regulation of the physiological processes mentioned above (Denley et al., 2005).

Research on lab mice and rats has consistently shown that IGF1 can be quantified during all stages of life, whereas IGF2 is only expressed during embryonic development (Constancia et al., 2002; Wolf et al., 1998; Yakar & Adamo, 2012; Yue et al., 2014). In contrast, humans express IGF2 throughout their lifecycle (Fagerberg et al., 2014). In addition to humans, many other vertebrates are also known to express IGF2 post-natally. In a transcriptomic analysis surveying reptilian species, McGaugh *et al*. (2015) identified the American alligator, 11 squamates, and 3 turtles that

exhibited post-natal IGF2 expression (McGaugh et al., 2015). Additionally, widely recognized representative species from the avian, amphibian and fish groups were found to express IGF2 during adulthood in multiple tissues (Rotwein, 2018). While there is very little information available on the biological action of IGF1 and IGF2 in reptiles, bioinformatic analyses on the molecular evolution of the IIS network in reptiles indicate functionality of the IGFs and their cellular receptors (McGaugh et al., 2015). Correlational studies in reptiles have demonstrated positive relationships between IGF and adult body size (Sparkman et al., 2009), offspring number (Sparkman et al., 2009), and reproductive state (Sparkman et al., 2010) in garter snakes, IGF1 and reproductive state and egg yolk composition in the American alligator (Guillette et al., 1996), and IGF1 and energy balance in *Sceloporus* lizards (Duncan et al., 2015). Manipulative studies have been conducting in aves showing that administration of IGF1 during embryonic development in the broiler chicken (Kocamis et al., 1998) and juvenile development in wild passerines (Lodjak et al., 2017) leads to increased growth. Yet, no manipulative studies have focused on the roles of IGF2.

The findings from these narrowly focused studies raise concerns about the selective use of rodent models for studying IGFs in applied biomedical research, and emphasizes the question of the function of IGF2 in adulthood, particularly in the complex and competitive conditions of hormones, binding proteins, and receptors that comprise the IIS network (Austad & Bartke, 2016; Milman et al., 2016; Rotwein, 2018). Therefore, there is a clear need for more comparative research across species to understand the function and complexities of this fundamental signaling network.

As with IGF1 and IGF2, there has been minimal research on insulin-like growth factor binding proteins (IGFBPs) within the reptilian clade, including fundamental details on the timing and location of their expression. In mammals, a superfamily of IGFBPs (including IGFBP1-10) have been described (Hwa et al., 1999), but the function of the IGFs is modulated primarily by six high affinity IGFBPs (Boughanem et al., 2019; Denley et al., 2005; Hwa et al., 1999; Jones & Clemmons, 1995). However, in reptiles bioinformatic predictions suggest that IGFBP6 has lost its functionality in binding IGF hormones (McGaugh et al., 2015). Collectively, IGFBPs positively and negatively regulate the availability of IGF1 and IGF2 to cellular receptors. As such, at any given time, as much as 99% of all circulating IGF1 are bound to an IGFBP (Frystyk, 2004). Being bound to an IGFBP increases the half-life of IGF proteins up to tenfold, and are thought to deliver IGF proteins to tissues across the body (Clemmons, 2001; Firth & Baxter, 2002). Because IGFBPs are responsible for the preservation and delivery of the IGF hormones, understanding their abundance and function is essential in understanding mechanistic patterns of the IIS top regulators as a whole. Xenopus, chicken, bovine, and fish models have been studied in genomic and physiological contexts, showing extensive variation in the number and function of binding proteins across clades (Garcia de la Serrana & Macqueen, 2018; Ghanipoor-Samami et al., 2018; Haramoto et al., 2014; Shimizu & Dickhoff, 2017). The IGFBPs have been studied most extensively in a biomedical context in rodent species in reference to conditions such as abnormal fetal development (Elhddad & Lashen, 2013; Méio et al., 2009) and cancer (Baxter, 2014; Firth & Baxter, 2002; Perks & Holly, 2003; Slater et al., 2019; Thai et al., 2015). In contrast, research exploring the availability and function of the independent IGFBPs under normal physiological conditions is sparse, with the majority of studies conducted as Genome-wide or RNAseq analysis of tissue-

specific expression (Fagerberg et al., 2014; Smith, CM et al., 2019; Yue et al., 2014). However, to our knowledge, no studies have examined the function of the IGFBPs in reptiles.

A previous transcriptomic analysis across reptiles (McGaugh et al., 2015) identified the IIS network genes from a variety of squamate species, providing sequence information across the clade and elevating reptilian species as an exciting avenue for IGF research. Harnessing this opportunity, we can now begin to fill the deficiency of the fundamental knowledge necessary to study the function of the IIS network in depth in reptile systems, for example, the function of IGF2 in reptiles as well as competitive relationships with IGF1, IGFBPs, or IGF receptors (Schwartz & Bronikowski, 2016).

We focus on the brown anole lizard (*Anolis sagrei*), an invasive species to the United States that has radiated from multiple populations in Cuba and the Bahamas within the last 150 years (Fetters & Mcglothlin, 2017). Due to the invasion of Florida, the brown anole is readily available at high densities, has proven to reproduce reliably in the lab for long breeding seasons (Lee et al., 1989), displays a relatively short lifespan (median of 2 years in the field and 4 years in captivity) (A. Reedy Pers. Comm.), lays single egg clutches through the breeding season (Fetters & Mcglothlin, 2017), and has the ability to regenerate tail tissue similar to another well-studied anole species, *A. carolinensis*, which has been the focus of regeneration and wound healing biomedical studies (Bateman & Fleming, 2011; Hutchins et al., 2014; Hutchins & Kusumi, 2016; Jacyniak et al., 2017; Kuo et al., 2015). The embryonic development of the brown anole has been characterized in great detail (Sanger et al., 2008), and recently, CRISPR-CAS9 has been developed to work in this species allowing for functional genomic studies to be conducted

(Rasys et al., 2019). For these reasons, the brown anole lizard continues to be developed to be a model reptile for ecological, physiological, genomic, and biomedical research.

In the present study, we conducted an in-depth analysis of the gene expression of IGF1, IGF2, and the five functional IGFBP genes across seven life stages ranging from pre-oviposition to adulthood, and six tissue types in the brown anole lizard. To our knowledge, this is the first comprehensive study of IIS network expression across time and tissues in a reptilian model species. We contrast our gene expression patterns in the brown anole lizard to what is documented in other species, with a focus on the lab mouse (the most commonly used model species in IIS research) and humans. The data collected in this study is the foundation for further developing the anole as a model species in biomedicine and physiological genomics.

Methods

Sample Collections

Adult

In 2017, adult brown anoles were collected from Palm Coast, Florida and maintained a breeding colony for embryonic and juvenile tissue collections that summer and fall (Collected under IACUC 2017-3027). Adult lizards (30 individuals per sex) were euthanized in October 2017 by decapitation and brain, heart, kidney, liver, gonads, skeletal hindleg muscle, tail, and regenerating tail tissues were flash-frozen in liquid nitrogen and stored at -80°C. No tissues used in analysis displayed any visually detectable abnormalities. For every tissue type a subset of individuals from each sex (n=8) were used in the analysis. Non-regenerative tail tissue (1cm of tissue at base of tail) used in RNA extraction was obtained from individuals that possessed a tail indicative of no

previous regenerative events. Regenerative tail tissue is easily discerned from original tail in appearance. Regenerative tissue used in analysis was collected 1cm below the regenerative breakpoint.

Juvenile

We collected eggs from adult females three times per week during the months of August and September of 2017. Eggs were placed in a 6cm petri dish half filled with vermiculite (100g vermiculite to 112g water) and sealed with parafilm. Eggs were incubated at 27°C in a Percival incubator (Model: I36NL) until hatching ~ 30 days later. Eggs were randomly assigned to one of three timepoints for dissection: Day 0, Week 4, or Week 8. Within 24 hours of hatching, the first n=4 male and first n=4 female hatchlings were euthanized by decapitation. Additional hatchlings were raised on fruit flies and crickets 14:10 LD cycle with reptile UV lights until 4 or 8 weeks of age when n=4 females and n=4 males were euthanized at each age and organs harvested in September 2018. All tissue samples were flash frozen in liquid nitrogen and stored at the -80°C, the liver was used for gene expression.

Embryonic

Embryonic stages for the brown anole range from 1 to 19 (Sanger et al., 2008), with stage 19 representing hatching (Sanger et al., 2008). We sampled three embryonic stages: 2/3, 6/7, and 16/17. These stages were chosen to quantify expression at landmarks in development. Stage 2/3 embryos are still developing inside the mother, pre-oviposition, and are characterized by discernable limb buds that are not yet emerging, enlargement of the mesencephalon and diencephalon, and an optic cup. Stage 6/7 embryos (~6 days post-oviposition) are characterized

by initial medial digitization of limb paddles, an unpigmented eye with retinal pigmentation, the emergence of the eye-lid at the ventral margin and a visible medial nasal process, pointing caudally. Stage 16/17 embryos are preparing to emerge from the egg (~20 days post-oviposition) and thus are fully formed with fully developed scales with slight pigmentation and mesencephalon that have been reduced or have begun reduction to protuberances. In order to obtain pre-oviposition early embryonic samples at stages, 2/3 of development (n=4), we collected eggs by dissecting gravid females (one egg per female). Eggs for Stage 6/7 and 16/17 embryos were haphazardly and continually assigned for dissection based on their length of incubation until we found four embryos at each of these precise stages. Eggs were incubated as described above for either 4 or 20 days at 27°C to target stages 6/7 or 16/17 respectively. On day 4 or 20, the embryos (n=4 per stage) were dissected from the egg, separating the shell and yolk from the embryo. Developmental stage was estimated using the standards indicated in Sanger *et al*. (2008), and whole embryos were flash frozen in liquid nitrogen and stored at the -80°C. These timepoints were chosen to examine a range of developmental stages, each representing a different developmental environment and possible gene expression patterns. From this point forward, these embryonic stages will be referred to as early-, mid-, and late-embryonic development. Whole embryos were used for gene expression.

RNA Extraction and cDNA Synthesis

Adult tissue (brain, gonad, heart, liver, skeletal muscle, tail, and regenerating tail) and whole embryo total RNA was isolated with the Illustra RNAspin Isolation Kit following the manufacturer's protocol (GE, Cat. No. 25-0500-70). Samples were lysed in RNAspin Lysis buffer (GE, Cat. No. 25-0500-70) with 5mm stainless steel beads (Qiagen Cat. No. 69989) using the Tissuelyser II (Qiagen) at 30Hz for 3 minutes. From the juvenile livers, total RNA was extracted

using the RNeasy Plus Micro Extraction Kit (Qiagen, Cat. No. 74034). A DNA digestion was performed on the column to ensure no DNA carry-over. RNA concentration was quantified using a high sensitivity QuantiFluor RNA System Kit (Promega, Cat. No. E3310) on a Cytation 3 plate reader (BioTek) in triplicate. RNA extractions were electrophoresed on a 1% agarose gel for 30 minutes at 100V. All samples used in cDNA syntheses were validated for RNA quality by the clear visualization of two rRNA bands (Sambrook et al., 1989). Detailed protocols for extraction and validation can be found in the **Supplemental Data Repository**.

Sample concentrations were standardized by the use of 100 ng of total RNA for cDNA synthesis. Following manufacturer protocols, total RNA (100 ng) was used in cDNA synthesis reactions to create single stranded DNA using qScript XLT cDNA SuperMix (QuantaBio, Cat. No. 95161-500), which utilizes both titrated concentrations of random hexamer and oligo(dT) primers. The cDNA samples were diluted 1:100 for qPCR analysis and 1:2 for presence-absence PCR.

IGF Quantitative Expression Across Life Stages

Absolute Standard Curve Preparation

EEF2 (Eukaryotic Translation Elongation Factor 2) was chosen as a reference gene for normalization as it has been shown to display consistent expression across tissues and age in mice (Eissa et al., 2016, 2017). IGF1, IGF2, and EEF2 primer pairs and fluorescent probes were designed with Geneious (11.0.5) using a brown anole liver transcriptome (McGaugh et al., 2015) (**Table 2.1**). An absolute standard was created for each of these three genes. Primer pairs were used to amplify each gene region using IBI Taq and the PCR product was cloned into the Expresso® Rhamnose Cloning and Expression System (Lucigen, Cat. No. 49011). Clones were

grown in LB medium containing 50mg/mL of Kanamycin for 8 hours. Overnight cultures (3mL) were centrifugation at 3260G for 10 minutes and the plasmid DNA was isolated using a plasmid prep (Qiagen MiniPrep Kit, Cat. No. 27104). Isolated plasmids were linearized in CutSmart Buffer with restriction enzyme ApaI (New England Biolabs, Cat. No. R0114S) following manufacturer recommendations. Linearized plasmid DNA concentration was quantified using the High Sensitivity Qubit Kit on a Qubit 3.0 (ThermoFisher, Cat. No. Q32854).

For each gene, copy number was determined from Plasmid DNA concentration and size of the plasmid (Staroscik, 2004). Each plasmid was diluted in sterile water to a final concentration of 100,000,000 copies/µL and the plasmids for each gene were pooled in equal proportions. A serial dilution was then performed on the pool resulting in a 7-point absolute standard curve decreasing 10-fold from 3x10^7 to 3x10^1 copies. Lambda DNA (NEB, Cat. No., N3011S) was prepared at a concentration of 310 ng/µL and added to sterile water prior to dilution in order to normalize total nucleic acid concentration in each standard. The seventh standard was dropped from analysis due to the high variance associated with low copy number. PCR efficiency based on the standard curve for IGF1 was determined to be 103.7% (r^2=1.0), IGF2 was 105.3% (r^2=1.0), and EEF2 was 107.0% (r^2= 1.0) (**Supplemental Fig. 2.1**).

Table 2.1: qPCR primers and probes used in assays of IIS and normalizing genes. Italicized portion of IGF primers indicate adapters added to primer sequence for cloning of product for use as qPCR standard curve.

Gene	Amplicon Length (bp)	Primer Name	Primer Sequence (5'-3')
EEF2	94	EEF2_Asag_qPCR_F	GAC ACC CGG AAA GAT GAG CA
		EEF2_Asag_qPCR_R	TGA AGG CCA AGT CGT TCT CC
		EEF2_Probe_Cy5Tao	/5Cy5/CGC TGC ATC /TAO/ACC ATC AAG TCC ACG G/3IAbRQSp/
IGF1	121	IGF1 BA_Partial_ 241 F	GGA GGC AAT CGA CGT TCA GT
		IGF1 BA_Partial_362 R	ACG GAT CGT GCG GTT TTA TCT
		IGF1_Asag_Probe_FamZen	/56-FAM/AGC TGT GAC /ZEN/CTG ACG CGA CTG GA/3IABkFQ/
IGF2	100	IGF2 BA_Partial_140 F	CTG TGG GCA GAA ACA GAG GA
		IGF2 BA_Partial_240 R	TGA TTT TGC ACA GTA GGT TTC CAA
		IGF2_Asag_Probe_HexZen	/5HEX/TGT GGA GGA /ZEN/GTG CTG CTT CCG GA/3IABkFQ/
IGFBP1	100	IGFBP1_Asag_qPCR_F	GAA CCA GAA GAC ATA CCT ACC G
		IGFBP1_Asag_qPCR_R	AAG TGG CGG ATT TCT CTT GG
IGFBP2	99	IGFBP2_Asag_qPCR_F	AGT GAA TGG CCA ACG AGG
		IGFBP2_Asag_qPCR_R	AGA TGG CAT TCA GGA TCT CC
IGFBP3	100	IGFBP3_Asag_qPCR_F	TGG TCC TTG TCG AAG AGA AA
		IGFBP3_Asag_qPCR_R	ACC CCT TTT GTC ACA ATT TGG
IGFBP4	97	IGFBP4_Asag_qPCR_F	CCA TGA CAG AAA ATG CCT CC
		IGFBP4_Asag_qPCR_R	CCC GGA TAG GTA GGA TCC C
IGFBP5	100	IGFBP5_Asag_qPCR_F	TTC TCG TGG CCG AAA ACG
		IGFBP5_Asag_qPCR_R	AAA GTT GTG ACA CTG GAG G
IGF1	232	Brown Anole IGF1 UP	*CAC CGC GAT CGC C*TC AGA GAC ACT TTG TGG TGC
		Brown Anole IGF1 DOWN	*GAT GGT TTA AAC* TCG TGC GGT TTT ATC TCG TTT AG
IGF2	229	Brown Anole IGF2 UP	*CCA AGC GAT CGC C*TC ACA TGG CCC TGC TGA AAC T
		Brown Anole IGF2 DOWN	*AAG CGT TTA AAC* TTC TGA TTT GAC TGA TTT TGC ACA

Multiplex Quantitative PCR (qPCR).

Real time qPCR was performed on whole embryonic samples (n=4) at each early-, mid-, and late-embryonic developmental time point, and liver tissue (n=4 female, n=4 male) at each Day 0, Week 4, Week 8, and Adult timepoints (**Table 2.2**). The absolute standards were run on each qPCR plate, along with a set of samples randomized to plate locations across two 96 well plates (ThermoFisher

Scientific, Cat. No. AB3496). All reactions were performed in triplicate in a total volume of 20µL with final concentrations of 1X PrimeTime Gene Expression MasterMix (IDT, Cat. No. 1055772), 0.3 µM of each EEF2, IGF1 and IGF2 forward and reverse primers, and 0.2 µM of EEF2, IGF1, and IGF2 PrimeTime qPCR fluorescent probes (IDT), and 5µL of cDNA at a 1:100 dilution. Reactions were run on the BioRad CFX96 qPCR thermal cycler, following an inactivation at 95°C for 3 minutes, and amplification consisting of denaturation at 95°C for 15 seconds, annealing/extension at 60°C for 1 minute, repeated for 45 cycles. Water replaced cDNA as no template control. Imaging was completed after each extension using the Cy5 (red), FAM (blue), and HEX (green) fluorophore channels.

Table 2.2: Sample sizes, developmental stages, and tissue type used in each experiment.

Experiment	**Tissue Types**	**Stage**	**Sample Size per stage and sex**	**Total Sample Size**
Quantitative PCR	Whole embryo	Development (early, mid, and late)	4	12
	Liver	Hatching (Day 0)	8	8
		Week 4	4 ♂ and 4 ♀	8
		Week 8	4 ♂ and 4 ♀	8
		Adult	4 ♂ and 4 ♀	8
Presence-Absence	Whole Embryo	Development (Early, mid, late)	4	12
	Brain, Gonad, Heart, Liver, Skeletal Muscle, Tail, and Regenerating Tail	Adult	4 ♂ and 4 ♀	56

Statistical Analysis and Quality Control

CFX Maestro Software (BioRad) was used to calculate copy number for each sample from CQ values using the standard curve and qPCR efficiency values. All statistics were performed used copy number as the measure of gene expression. All analyses were performed using R software (version 3.5.1, R Core Team), the code for analyses is provided in the **Supplementary Data Repository.**

Data for EEF2, IGF1, and IGF2 indicated patterns of heteroscedasticity and non-normality. To correct for those parameters, the package bestNormalize (Peterson, Ryan A. & Cavanaugh, Joseph E., 2019) was used to identify an appropriate transformation model. Ordered Quantile transformation was indicated as the proper method, and all data was transformed. Transformation of data eliminated heteroscedasticity and non-normal patterns, meeting all assumptions for linear mixed model analysis.

To test for differences among life stages for each gene, data was first subset by gene: IGF1, IGF2, and EEF2. Expression across life stages was analyzed separately for IGF1 and IGF2. Additionally, we ran the embryonic samples (early-, mid-, and late-embryonic development) that used the whole embryo in a separate model from the postnatal samples (weeks 0, 4, 8 and adulthood) that used liver specifically. A linear mixed effect model (Pinheiro, Jose et al., 2018) was run using mRNA copy number as the response variable, and life-stage as the independent variable. Sex was included as an independent variable and interaction term in the postnatal analysis. It was found that the effect of sex was not significant ($F_{(1,30)} = 0.0056$; $p = 0.9407$). An additional F-drop test was performed comparing a model with sex as a fixed effect, and with sex dropped from the model. It

was determined that sex did not improve the statistical model ($p = 0.8689$), and it was therefore removed. It should be noted that body size was not included in the statistical model, as it would be highly confounded with age. While all measurements were endpoint, individual was added as a random effect variable, to account for each sample being run in triplicate during qPCR analysis and prevent pseudoreplication of data. Tukey based Post-Hoc analysis functions were used to obtain pairwise significance between life stages, which adjusts p-values for multiple comparisons. During these analyses it was found that EEF2 varied significantly across life stages (**Supplemental Fig. 2.2;** $F_{(6, 36)} = 4.081$; $p = 0.0032$), making it inappropriate for use as a normalizing gene and thereby it was not used to normalize data in any analyses. Rather, we rely on the normalization of amount of RNA used at the cDNA synthesis stage. RNA quantification was performed in triplicate, a standardized amount of RNA was used in each cDNA synthesis step, and samples were randomized during RNA isolation, cDNA synthesis, qPCR steps. These considerations would ensure that any technical error (that a house keeping gene would be used to normalize), would be spread across treatments and thus any significant results we obtain would be considered conservative.

To test the relative gene expression between IGF1 and IGF2 at each life stage, data was subset by life stage and statistically analyzed at each of these seven timepoints separately. A linear mixed effect model was used to analyze relative differences between copy number of the genes (IGF1, IGF2). Individual was included as a random effect variable to account for sample triplicates during qPCR analysis. Sex was included as an independent variable for the hatchling and adult time points, but the effect was non-significant.

Presence-Absence Survey of IGFBP and IGF Gene Expression

PCR Amplification in Brown Anole Across Life-stages and Tissues

We surveyed for the presence of gene expression of five IGFBPs (IGFBP1 through IGFBP5) in embryos, juvenile livers, and six adult tissues (**Table 2.2**). Primer pairs for IGFBP1 through IGFBP5 were designed using the green anole (*Anolis carolinensis*) reference genome (Alföldi et al., 2011), using the predicted transcripts from NCBI. Primer pairs were designed to produce a PCR product between 100bp to 250bp in length and were located within an exon so we could verify their ability to amplify DNA if the cDNA reactions did not amplify, thus indicating no expression. DNA was used as a positive control, and water replaced cDNA as the no template controls. All samples were amplified in 25µL reactions with final concentrations of 1X IBI Taq Mastermix (IBI Scientific, Cat No: IB43101), 0.15µM of each forward and reverse primers, and 1µL of cDNA at a 1:2 dilution. Amplifications (presence/absence) were verified on 2.5% agarose gels with 1µg/mL GelGreen (Biotium; Cat No: 41004). PCR product (10µL) was mixed with 2µL of 6X Loading Dye (NEB, Cat. No. B7024S) and run at 120V for 1 hour. Samples were classified as positive for expression if a band was visible in blue light. If no band was visible, it was considered negative (no expression). For all samples where no expression was detected, the PCRs were repeated for verification along with genomic DNA as a control and a no template control. See **Supplemental Data Repository** for detailed protocols.

PCR product from one sample for each gene was sequenced to verify amplification of the target gene. PCR product was treated with ExoSapIT (ThermoFisher Scientific; Cat No: 78202) and sent to Eurofin Genomics for sequencing in the forward and reverse directions using gene specific primers (**Table 2.1**). By this time, we had obtained the sequences of the IGFBP transcripts from

the brown anole. The resulting PCR product sequences were aligned to brown anole reference transcripts in Geneious (Version 11.1.4) and verified they were the correct targets. Resulting sequences are provided in **Supplemental Data Repository**.

Results

IGF1 and IGF2 Hormone Quantitative Gene Expression Analysis

Contrasting Gene Expression Across Lifespan for Each Hormone

From the whole embryos, IGF1 expression was below the detectable limit at early- and mid-stage development and increased by late-embryonic stage. To further verify the undetectable expression levels of IGF1 at early and mid-development, undiluted cDNA was used in qPCR amplification. Even when the samples were analyzed in this manner, the average copy number was determined to be 159 at early-embryonic development and 31 copies during mid-embryonic development, both of which were off the standard curve as we dropped the 30 copy standard due to the highly variable amplification. In comparison, there was a significant increase of 2,924 copies detected by late-development ($F_{(2,8)} = 11.339$; $p = 0.0046$). In contrast, IGF2 was expressed at every embryonic stage and expression did not change significantly across embryonic stages (**Fig. 2.1, Table 2.3;** $F_{(2,8)} = 1.182$; $p = 0.3550$).

Among juvenile life stages, there was no statistically significant difference in IGF1 liver gene expression between Day 0, Week 4, and Week 8 stages (**Fig. 2.1, Table 2.3;** $F_{(2,21)} = 0.928$; $p = 0.4109$). However, IGF2 liver gene expression did vary significantly across juvenile stages with expression at Day 0 presenting significantly higher than at Week 4 and Week 8 timepoints (**Fig. 2.1, Table 2.3;** $F_{(2,21)} = 13.046$; $p = 0.0002$). Both IGF1 and IGF2 liver gene expression in the

adult life stage is significantly higher than all juvenile life stages ($F_{(3,28)} = 17.759$; $p < 0.0001$, and $F_{(3,28)} = 37.668$; $p < 0.0001$ respectively; See **Table 2.3** for pairwise comparisons).

Table 2.3: Summary of statistical results for qPCR data analysis. Linear mixed effect model and pairwise comparisons within each life stage are displayed with their corresponding Beta value, SE, and p-value (bolded if less than 0.05). Overall model is the original p-value produced by linear model, while Tukey post-hoc comparisons are displayed as adjusted p-values.

Statistical Test Group		Comparison	Beta	Std. Error	p-value
Relative IGF1 and IGF2 Levels	Embryonic	Early-Emb. Dev. (N=4)	1.3638	0.1358	**< 0.0001**
		Mid-Emb. Dev. (N=4)	1.1973	0.1239	**< 0.0001**
		Late-Emb. Dev. (N=4)	0.7402	0.2178	**0.0068**
	Juvenile	Day 0 (N=8)	1.7246	0.1526	**< 0.0001**
		Week 4 (N=8)	1.1425	0.0674	**< 0.0001**
		Week 8 (N=8)	0.5592	0.0650	**< 0.0001**
	Adult	Adult (N=8)	0.9497	0.0554	**< 0.0001**
IGF Expression Across Embryonic Developmental Timepoints	IGF1	**Overall Model**	---	---	0.0046
		Early - Mid	-0.848	0.451	0.2093
		Mid - Late	1.253	0.416	**0.0400**
		Early - Late	2.101	0.451	**0.0041**
	IGF2	**Overall Model**	---	---	0.3550
		Early - Mid	0.188	0.242	0.7273
		Mid - Late	-0.214	0.261	0.7032
		Early - Late	-0.402	0.261	0.3258
IGF Expression Across Juvenile Developmental Timepoints	IGF1	**Overall Model**	---	---	0.4109
		Day 0 - Week 4	-0.131	0.279	0.8869
		Week 4 - Week 8	-0.244	0.279	0.6627
		Day 0 - Week 8	-0.374	0.279	0.3885
	IGF2	**Overall Model**	---	---	**0.0002**
		Day 0 - Week 4	0.430	0.148	**0.0217**
		Week 4 - Week 8	0.321	0.148	0.0987
		Day 0 - Week 8	0.752	0.148	**0.0001**
IGF Expression in Adulthood relative to Juvenile Timepoints	IGF1	**Overall Model**	---	---	**< 0.0001**
		Adult - Day 0	1.685	0.260	**< 0.0001**
		Adult - Week 4	1.554	0.260	**< 0.0001**
		Adult - Week 8	1.331	0.260	**0.0001**
	IGF2	**Overall Model**	---	---	**< 0.0001**
		Adult - Day 0	0.912	0.166	**< 0.0001**
		Adult - Week 4	1.343	0.166	**< 0.0001**
		Adult - Week 8	1.664	0.166	**< 0.0001**

Relative Levels of IGF1 and IGF2 Gene Expression at each Time Point

Across all timepoints, IGF2 expression was significantly higher than that of IGF1 ($p < 0.006$ at all life stages) (**Fig. 2.1; Table 2.3**). Interestingly, there were substantial spikes in IGF2 expression at the Day 0 and adult timepoints. Following the Day 0 timepoint (hatching) there was a 2.24-fold decrease in expression by Week 4, followed by another 2.23-fold decrease by Week 8. Between Week 8 and adulthood, there was a 38.12-fold increase in IGF2 expression. Additionally, there

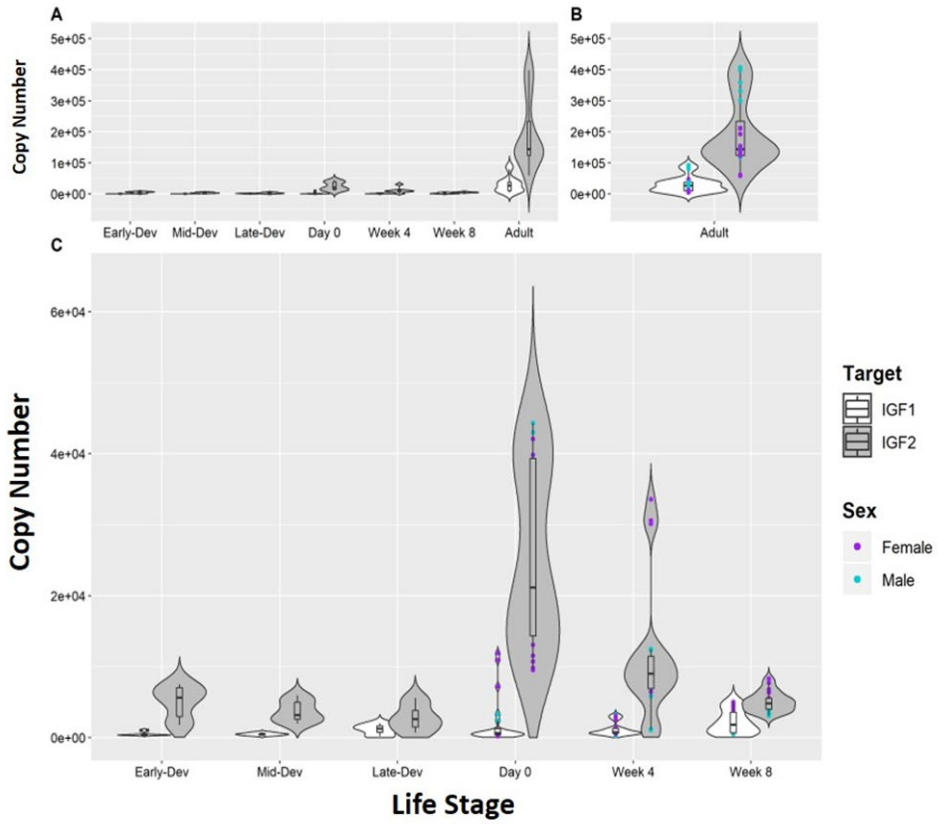

Figure 2.1: Quantitative Gene Expression of IGF1 and IGF2 Across Life Stages plotted by Sex. Expression of IGF1 and IGF2 was quantified from liver cDNA across life stages. Data was back transformed for visualization, and represented as raw copy number values. IGF1 expression is depicted with a white violin plot, while IGF2 expression is shown in grey. Post-hatching data is coded by sex, with females represented with a purple data point, and males represented with a blue data point. A) Overview of all life stages, demonstrating both IGF1 and IGF2 expression is significantly higher at the adult stage than any other life stage. B) Adult samples. IGF2 expression was significantly higher than IGF1. Additionally, there are non-significant patterns of males expressing both IGF1 and IGF2 at a higher level than females. C) Embryonic and juvenile development stages. IGF2 is expressed at a significantly higher level than IGF1 at all life stages, and IGF1 expression becomes detectable at the late-developmental stage.

was significantly more variation in IGF2 expression at each timepoint when compared to IGF1 ($p < 0.006$ in each case).

We found no statistical differences based on sex at any timepoint when included as a main effect ($F_{(1,30)} = 0.0056$; $p = 0.9407$) or interaction term ($F_{(1,138)} = 0.5259$; $p = 0.4696$); however, this study was not powered to detect sex differences.

Presence-Absence Survey of IGFBP and IGF Gene Expression

Expression in Brown Anole Across Life-stages and Tissues

All five IGFBPs were expressed at each embryonic stage (**Fig. 2.2A, B; Supplemental Fig. 2.3**). In adults, the expression of each binding protein was detected in every tissue in at least one individual, and in many cases, all individuals (**Fig. 2.2C; Supplemental Fig. 2.3**). IGFBP1 through IGFBP4 were expressed in over 95% of all tissues across individuals, with all the variation among individuals occurring in muscle tissue **(Fig 2.2D. Supplemental Fig. 2.3).** However, IGFBP5 displayed the highest levels of individual variation. IGFBP5 was expressed in brain, gonad, and heart tissue consistently, but variation ranging from 50-100% was seen across tissues (i.e. liver, muscle, tail and regenerating tail tissues) and individuals (**Fig. 2.2D; Supplemental Fig. 2.3**). Images of the electrophoresis agarose gels used for qualitative categorization of expression can be found in the **Supplemental Data Repository**.

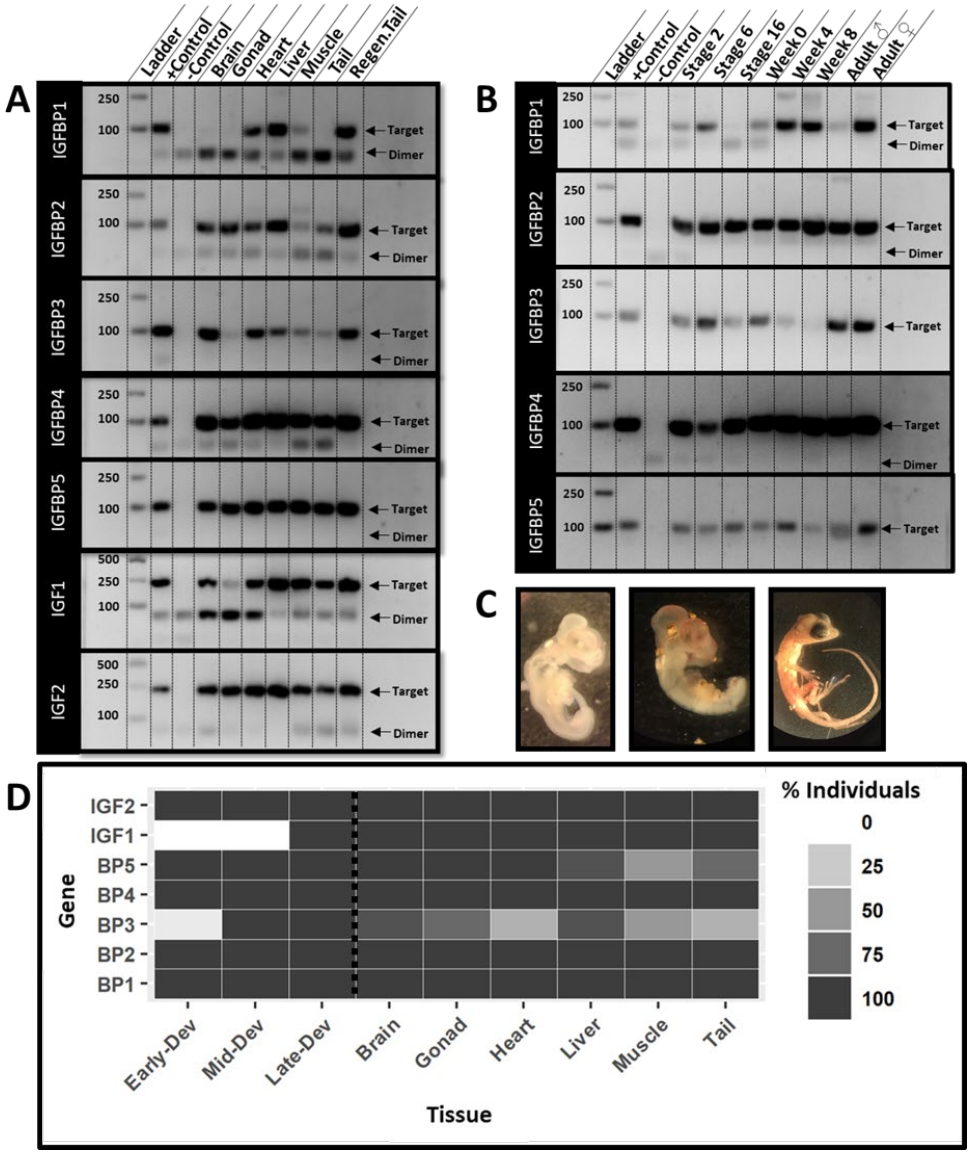

Figure 2.2: Summary of results for qualitative PCR of IGFBPs expression across tissues and life stages. Presence-absence PCR was performed on cDNA from each developmental stage and adult tissue (brain, ovary/testes, heart, liver, skeletal muscle, tail, and regenerating tail). Sample sizes were n=4 for embryos, n=8 for juvenile livers, and n=8 for adult tissues (see Table 2 for details). (A) Representative gel electrophoresis images of IGFBP and hormone transcript amplifications in presence-absence PCR across adult tissue types. The first lane is the DNA standard with the 100bp, 250bp, and 500bp markers labeled for reference. +Control is a DNA sample, and the NTC is a No Template Control. The target band and the band representing excess primer-dimer are labeled with arrows on the right side of the gel image. (B) Representative gel electrophoresis image of IGFBP transcript amplifications in presence-absence PCR from whole embryos, and the liver for juvenile stages, with adult liver from a male and female. (C) Representative images of early-, mid-, and late-developmental stage embryos used in PCR and qPCR analysis. (D) Summary of gene expression of the IGFBPs, IGF1, and IGF2 across tissues across all samples (N=20). The full table of individual results for each gene is provided in Supplemental Figure 3. Consistent with the results from the quantitative PCR, expression of IGF1 was not detected in early- or mid-embryonic development, while expression of each IGFBP and IGF2 was detected in all individuals at each stage of development. In adult tissues, IGF1, IGF2, IGFBP1-4 were expressed in 95-100% of all individuals. IGFBP5 varied in liver, muscle, and tail tissues.

Discussion

Considering the widespread physiological effects of the IIS network, the paucity of information on the function of this network in non-traditional model organisms makes this study vital to ongoing research in fields ranging from ecology to physiological genomics and endocrinology. To this point, this is the first comprehensive study examining the expression levels of the IIS top regulators across lifespan and tissues in any reptilian species. Unlike patterns seen in biomedical lab rodents, in the brown anole lizard, we found IGF2 was expressed throughout all life stages. This expression pattern more closely match those seen in other vertebrate species that have been studied thus far including multiple juvenile reptilian species (McGaugh et al., 2015), avian, amphibian, and fish species (Rotwein, 2018). As such, the brown anole is a promising comparative model for understanding the function of IGF2 in adulthood, as well as for understanding how IGF2 potentially competes with IGF1 for binding their shared cellular receptors at the different life stages.

While in the biomedical literature, IGF2 has been termed a pre-natal growth factor and IGF1 a post-natal growth factor — a generalization that came from the expression patterns seen in lab-rodents as IGF2 expression is downregulated after birth (Brown et al., 1986; Soares et al., 1985; Yue et al., 2014)— the findings of the present study and previous work in other vertebrates indicate that the expression patterns seen within lab rodent models are inconsistent with those commonly seen across vertebrate taxa. To explore these contrasts further, we use previously published data on gene expression of the IGFBPs and IGFs to qualitatively compare the patterns seen in mice and humans with the results of the anole PCR analysis. Yue *et al*. (2014) performed a transcriptomic analysis across laboratory mouse tissues. Similarly, Fagerberg *et al.* (2014)

Table 2.4: Qualitative comparison of adult gene expression from this lizard study to literature on humans and mice. For each gene, the "X" indicates detection of gene expression in that species and tissue. The lizard data is based on PCR amplification; expression of IGFBP1 through IGFBP5 were detected in each tissue type of the brown anole. The human and mouse data are from the literature and are provided for comparison. The transcriptomic mouse patterns from Yue et al. (2014) and the Alliance of Genome Resources and human data from Fagerberg et al. (2014) and The Human Protein Atlas (Pontén et al., 2008; Uhlen et al., 2015) indicate expression of IGFBP3 through IGFBP5 in all examined tissue types, but IGFBP1 in only liver tissue, and mice IGFBP2 in brain, gonad, and liver, but not heart. For the ligands, both IGF1 and IGF2 are present in all examined tissue types in the lizard and human, while only IGF1 is expressed in the adult mice.

Gene	Species	Brain	Gonad	Heart	Liver
IGFBP1	Lizard	**X**	**X**	**X**	**X**
	Mouse				**X**
	Human				**X**
IGFBP2	Lizard	**X**	**X**	**X**	**X**
	Mouse	**X**	**X**		**X**
	Human	**X**	**X**	**X**	**X**
IGFBP3	Lizard	**X**	**X**	**X**	**X**
	Mouse	**X**	**X**	**X**	**X**
	Human	**X**	**X**	**X**	**X**
IGFBP4	Lizard	**X**	**X**	**X**	**X**
	Mouse	**X**	**X**	**X**	**X**
	Human	**X**	**X**	**X**	**X**
IGFBP5	Lizard	**X**	**X**	**X**	**X**
	Mouse	**X**	**X**	**X**	**X**
	Human	**X**	**X**	**X**	**X**
IGF1	Lizard	**X**	**X**	**X**	**X**
	Mouse	**X**	**X**	**X**	**X**
	Human	**X**	**X**	**X**	**X**
IGF2	Lizard	**X**	**X**	**X**	**X**
	Mouse				

performed a comprehensive RNA-seq analysis across human organs and tissues to classify tissue-specific expression. Additionally, the Human Protein Atlas (Pontén et al., 2008; Uhlen et al., 2015) and the Alliance of Genomic Resources for the Mouse Gene Expression Database (Smith, CM et al., 2019) have compiled results from peer-reviewed RNA-seq analyses across developmental stages and tissues. Data from these transcriptomic analyses were used to determine the presence or absence of each IGFBP and IGF across adult human and mouse tissues including the brain, gonad, heart, and liver. We reference these results below in the context of our findings reported in this study on the brown anole lizard.

IGFBP1 is only expressed in whole liver tissue in both mice (Smith, CM et al., 2019; Yue et al., 2014), and humans (Fagerberg et al., 2014; Pontén et al., 2008; Uhlen et al., 2015). In the anoles, IGFBP1 gene expression was detected from each tissue type from all individuals (**Table 2.4**). IGFBP2 was not detectable in mouse whole heart tissues (Smith, CM et al., 2019; Yue et al., 2014); however, it was detected in human heart tissue (Fagerberg et al., 2014; Pontén et al., 2008; Uhlen et al., 2015) and 100% of the anole samples in this study. The presence of IGFBP3, IGFBP4, and IGFBP5 gene expression was consistently detected across all examined tissues from this study in the mouse (Yue et al., 2014), human (Fagerberg et al., 2014; Pontén et al., 2008; Uhlen et al., 2015), and lizard during adulthood (**Table 2.4**).

It is evident that there is much less variation in IGFBP expression across tissues in anoles than seen in mice and humans. In anoles, we found considerable variation of IGFBP5 expression across tissue types and will focus on the implications of this differentially expressed protein. In mice and humans, the differential expression of this particular binding proteins has been highly linked to

pathologies including fetal growth regulation, fetal limb development, metabolism, and most notably, cancer in mammals (Ghoussaini et al., 2014; Gleason et al., 2010; McQueeney & Dealy, 2001; Perks & Holly, 2003; Salih et al., 2004; Sureshbabu et al., 2012). However, many types cancer are thought to be rare in reptiles, occurring at much lower rates than those seen in humans (Hernandez-Divers & Garner, 2003; Lucke & Schlumberger, 1949; Natarajan et al., 2007). Interestingly, anoles display unique reproductive (Fetters & Mcglothlin, 2017) and regenerative life-history patterns (Hutchins et al., 2014) that maybe regulated and explained in part by the IIS network. The differential regulation of IGFBP5 seen in reptiles could be a potential area of interest in cancer associated and life-history fields.

In model mammalian species, IGFBPs during development have previously been thought of as possible maternal factors, with expression patterns shifting during pregnancy, often correlated with fetal growth deficiencies (Han et al., 1996; Koutsaki et al., 2011; Robinson et al., 2000). However, other studies have shown embryonic expression of the IGFBPs (Allan et al., 2001; Wood et al., 1992). Embryonic models in lab rats have shown that expression for each of the IGFBPs occurs as early as day 14 of development (Cerro, JA et al., 1993; Green et al., 1994; Schuller et al., 1993), while chickens expressed IGFBP2 3.5 days after a mating event (Schoen et al., 1995). The anole embryonic samples showed ubiquitous expression of all IGFBPs at early-, mid-, and late-stage development. The IGFBPs expression in the earliest stage suggests embryos are producing their own IGFBPs even before oviposition, and potentially utilizing each of the IGFBPs at a very early point in development. Identifying the independent primary functions of the IGFBPs in relation to how they regulate IGF hormone availability and action will be instrumental in understanding the regulation and activation of the IIS signaling network. Future investigation of the embryonic IGF

top regulators via *in situ* hybridization may shed light on the temporal and spatial regulation of these genes throughout development.

IGF1 was expressed in all tissue types in adult mice, humans, and anoles. We have also shown that IGF2 is expressed at a higher level than IGF1 during all life stages of the brown anole while in rodents, IGF2 is not expressed in adulthood in any analyzed tissues (Yue et al., 2014). This is inconsistent with the patterns displayed in humans (Fagerberg et al., 2014), and anole lizards, which show high post-natal expression levels of IGF2 in brain, gonad, heart and liver tissue (**Table 2.4**). This begs the question, "What role is the highly expressed IGF2 playing in juvenile and adult physiology?"

Finer examination of our quantitative expression data presents some interesting patterns that further the intrigue into the function of both IGF1 and IGF2 in physiology outside of biomedical rodents. First, across life stages, we found significant spikes in IGF expression at the adult time-point. Both IGF1 and IGF2 expression was significantly higher, 15.2 and 38.12-fold respectively, in adults relative to the juveniles (Week 8). The high expression levels and high variation in adults may be due to a number of factors, including age, reproductive efforts or stage of reproductive cycle in females. It should be noted that these lab animals were fed *ad lib* and expression at the juvenile and adult stages should be maximal as high food levels increase IGF production.

It is important to highlight that while we found no differences in IGF expression due to sex, this was likely due to limited sample size and previous studies have shown that adult anoles display differential expression between sexes. However, the adult expression patterns in this study are

consistent with those studies where significant differences were detectable. For example, a transcriptomic study in brown anoles demonstrated IGF1 and IGF2 were differentially expressed between sexes, with IGF2 being expressed at a significantly higher level in males relative to females by 12 months of age (Cox et al., 2017). Further studies with increased sample sizes are recommended to further evaluate the sex-specific patterns of gene expression in the IIS network.

Second, there was a similar spike in liver IGF expression at the Day 0 (day of hatching) timepoint. During this time, the juvenile anoles are utilizing yolk that had been internalized prior to hatching as their sole food source meaning the lab reared animals had yet to ingest prey or experience competition of any kind, so the increased expression cannot be explained by diet alone. The increased IGF2 expression at Day 0 could be due to a number of factors including a stress response to emerging from the egg and physiological changes associated with hatching.

Lastly, in the brown anole, IGF1 expression was negligible until late-embryonic development. This is inconsistent with developmental patterns studied in humans, where both IGF1 and IGF2 are thought to play a pivotal role in fetal development. In our study, embryonic expression of IGF1 increased 239% between early- and late- embryonic development. Human embryonic serum IGF1 levels during development displayed a similar pattern, increasing dramatically between early and late development (Hellström et al., 2016). While both human and mouse IGF1 expression levels increase over fetal development (Yakar & Adamo, 2012), the delay of activation of IGF1 expression in our samples until late-development indicates that embryo-derived IGF1 may not play a large role in early and mid- pre-natal development in reptiles. Research into the large variation

of IGF1 and IGF2 expression after maturation and a fine-scale analysis during late-stage development is needed to fully understand these patterns and their physiological consequences.

Conclusions

This study is a substantial building block for the brown anole lizard as a reptilian model for the study of IIS, filling an obvious void for comparisons across vertebrates. Reptiles are phylogenetically sister to mammals, and the brown anole lizard shows adult IGF2 expression patterns more similar to humans than rodent models. Through this analysis, it is clear that the anole lizard is a promising potential avenue for studying the effects of IGF2 through adulthood and its interactions with IGF1 and the IGFBPs in a biomedically, evolutionary, and ecologically relevant system. Combining the data here with future fine-scale embryonic analyses and sex-based expression data would provide the information needed for manipulative studies in squamates, furthering the use of the anole lizard as a model species in physiological and biomedical research fields.

Endnote

Additional materials related to this manuscript may be found at [https://github.com/aeb0084/IGF-Network-Expression-in-the-Brown-Anole.git].

Supplemental Figures

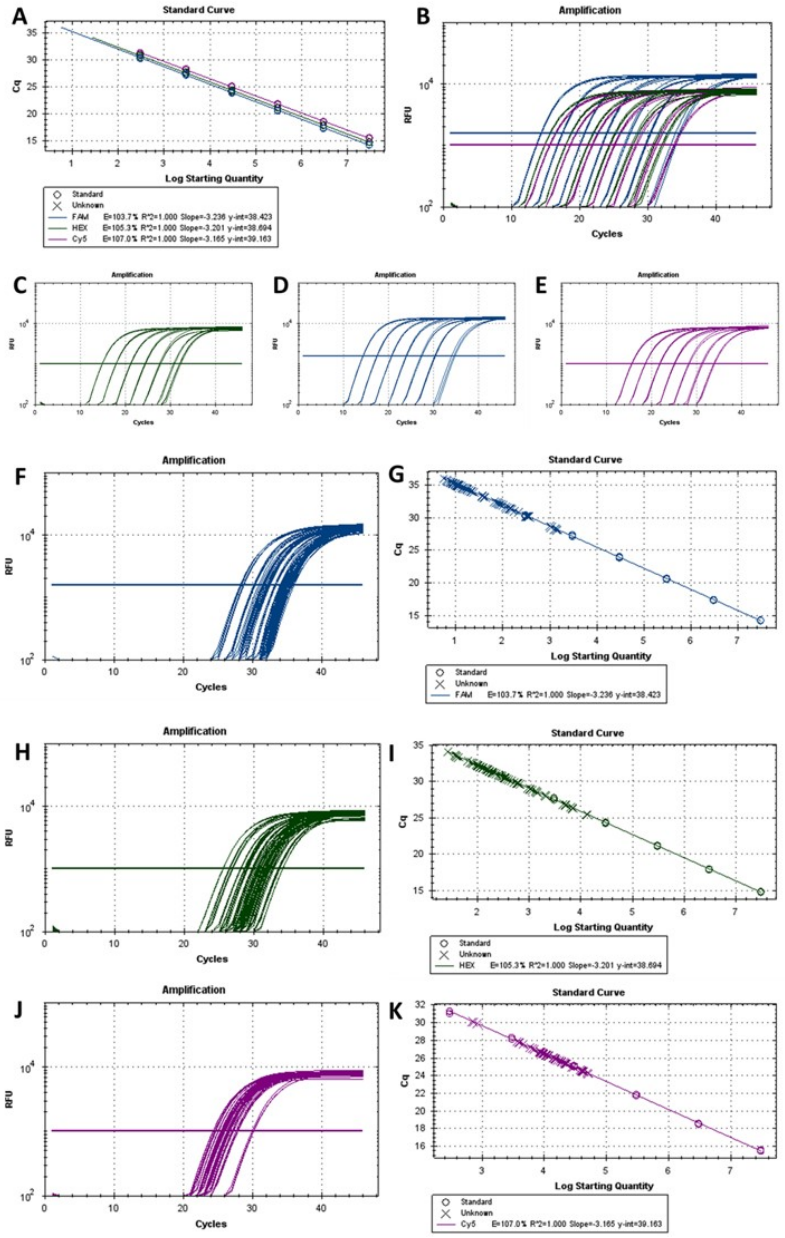

Figure S2.1: Raw data plots for qPCR anlaysis. (A-E) Absolute standard curves were analyzed for PCR efficiency for IGF1 (green; 103.7%; r2=1.0), IGF2 (blue; 105.3%; r2=1.0), and EEf2 (purple; 107.0%; r2=1.0). Amplification cycle and sample placement on the standard curve can also be seen for IGF1 (F & G), IGF2 (H & I), and EEF2 (J & K).

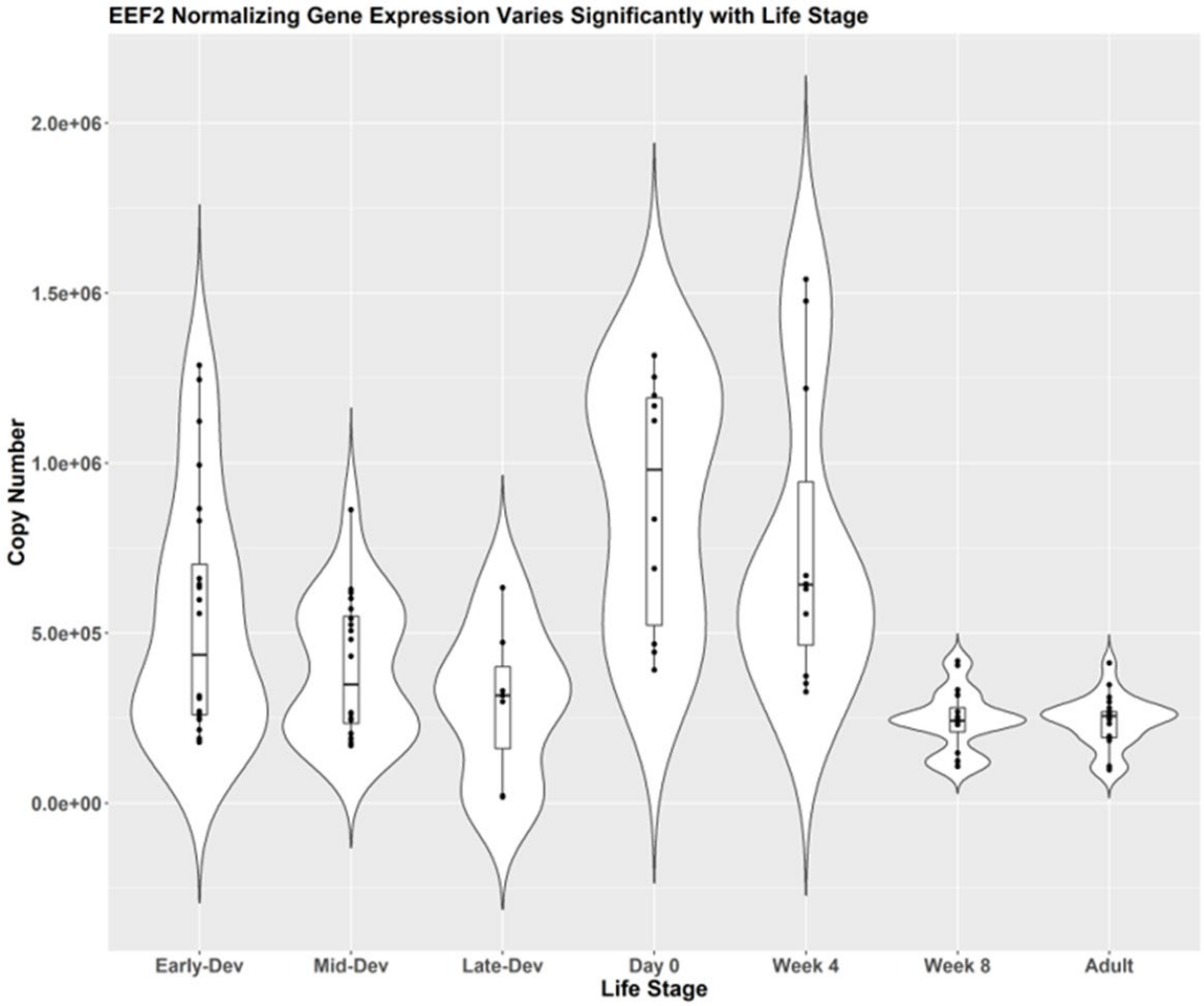

Figure S2.2: EEF2 Reference Gene Expression Across Life Stages. EEF2 expression varied significantly across life stages (p=0.0011; F= 4.785) and was not used to normalize IGF1 and IGF2 expression data.

		MALE					FEMALE			
Tissue	Gene	M1	M2	M3	M4		F1	F2	F3	F4
Brain	BP1	M61	M21	M69	M36		F51	F13	F60	F48
Gonad		M61	M21	M69	M36		F51	F44	F30	F48
Heart		M1	M46	M69	M73		F23	F46	F30	F28
Liver		M61	M21	M69	M36		F51	F13	F60	F48
Muscle		M11	M51	M39	M54		F54	F44	F30	F24
Regen Tail		M64		M39			F32		F30	
Tail		M36	M21	M69	M61		F51	F13	F32	F28
Brain	BP2	M61	M21	M69	M36		F51	F13	F60	F48
Gonad		M61	M21	M69	M36		F51	F44	F30	F48
Heart		M1	M46	M69	M73		F23	F46	F30	F28
Liver		M61	M21	M69	M36		F51	F13	F60	F48
Muscle		M11	M51	M39	M54		F54	F44	F30	F24
Regen Tail		M64		M39			F32		F30	
Tail		M36	M21	M69	M61		F51	F13	F32	F28
Brain	BP3	M61	M21	M69	M36		F51	F13	F60	F48
Gonad		M61	M21	M69	M36		F51	F44	F30	F48
Heart		M1	M46	M69	M73		F23	F46	F30	F28
Liver		M61	M21	M69	M36		F51	F13	F60	F48
Muscle		M11	M51	M39	M54		F54	F44	F30	F24
Regen Tail		M64		M39			F32		F30	
Tail		M36	M21	M69	M61		F51	F13	F32	F28
Brain	BP4	M61	M21	M69	M36		F51	F13	F60	F48
Gonad		M61	M21	M69	M36		F51	F44	F30	F48
Heart		M1	M46	M69	M73		F23	F46	F30	F28
Liver		M61	M21	M69	M36		F51	F13	F60	F48
Muscle		M11	M51	M39	M54		F54	F44	F30	F24
Regen Tail		M64		M39			F32		F30	
Tail		M36	M21	M69	M61		F51	F13	F32	F28
Brain	BP5	M61	M21	M69	M36		F51	F13	F60	F48
Gonad		M61	M21	M69	M36		F51	F44	F30	F48
Heart		M1	M46	M69	M73		F23	F46	F30	F28
Liver		M61	M21	M69	M36		F51	F13	F60	F48
Muscle		M11	M51	M39	M54		F54	F44	F30	F24
Regen Tail		M64		M39			F32		F30	
Tail		M36	M21	M69	M61		F51	F13	F32	F28
Brain	IGF1	M61	M21	M69	M36		F51	F13	F60	F48
Gonad		M61	M21	M69	M36		F51	F44	F30	F48
Heart		M1	M46	M69	M73		F23	F46	F30	F28
Liver		M61	M21	M69	M36		F51	F13	F60	F48
Muscle		M11	M51	M39	M54		F54	F44	F30	F24
Regen Tail		M64		M39			F32		F30	
Tail		M36	M21	M69	M61		F51	F13	F32	F28
Brain	IGF2	M61	M21	M69	M36		F51	F13	F60	F48
Gonad		M61	M21	M69	M36		F51	F44	F30	F48
Heart		M1	M46	M69	M73		F23	F46	F30	F28
Liver		M61	M21	M69	M36		F51	F13	F60	F48
Muscle		M11	M51	M39	M54		F54	F44	F30	F24
Regen Tail		M64		M39			F32		F30	
Tail		M36	M21	M69	M61		F51	F13	F32	F28

Figure S2.3: Summary of adult cross-tissue PCR gene amplifications. Each individual is categorized by tissue on the far left, followed by target gene which are separated by color blocks. Male and female ID (M1-4 and F1-4) are shown on the top and can be paired to gel electrophoresis images. The genes and tissues are then separated by individual. Individuals that amplified for each gene are colored, while verified negative samples are white.

Chapter 3: Tails of Reproduction: Regeneration leads to increased reproductive investment.

Beatty, AE, Mote, DM & Schwartz TS. (2021) Tails of Reproduction: Regeneration Leads to Increased Reproductive Investment. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology.* https://doi.org/10.1005/jez.2472.

Background

Life-history theory is used as an analytical framework to explain variation across species in life-history strategy through traits such as birth size, growth, age of maturity, reproductive patterns, and longevity (Stearns, 1992). Life-history tradeoffs—an increase in one trait leading to a decrease in another when competing activities require shared materials and energy within one organism (Stearns, 1992)—are thought to occur due to resource limitation or constraints in the regulation of genetic and physiological networks (Stearns, 1992). Tissue regeneration is studied in a wide variety of fields including evolutionary ecology and biomedicine. The energetic investment in regeneration of large tissues, such as limbs or tails, is predicted to affect other life history traits such as reproduction, growth, and survival (Althoff & Thompson, 1994; Bateman & Fleming, 2009; Chu et al., 2011; Constancia et al., 2002; Naya & Božinović, 2006; Rinkevich, 1996; Starostová et al., 2017). One relevant example, halting or reducing reproduction to invest in tail regeneration (Dial and Fitzpatrick, 1981; Rinkevich, 1996; Wilson and Booth, 1998; Chapple et al., 2002; Bernardo and Agosta, 2005) would impact both current and future fitness potential. The ability to autotomize a tail as an anti-predation mechanism is directly related to survival (Starostová et al., 2017). Tail regeneration can take one or more months, potentially a large proportion of the breeding season in some species. If regenerating a tail utilizes resources that would otherwise be used for reproduction, this could have a large impact on fitness.

While all organisms are capable of regenerating tissue, the degree to which regeneration may occur varies greatly across species (Tsonis, 2000). Amphibians and lizards are well known for their regenerative abilities (Gilbert et al., 2013; Starostová et al., 2017; Tsonis, 2000). As with much work attempting to quantify life-history tradeoffs, the impact of tail regeneration on life-history traits such as growth or reproduction has varied. In a review by Bateman and Fleming (2008), numerous species displayed reduced fecundity in response to tissue regeneration such as decreased litter size (Chapple et al., 2002), and decreased allocation of calories to reproduction (Dial & Fitzpatrick, 1981); in contrast, another study showed no negative impacts of tail regeneration on reproductive investment (S. F. Fox & McCoy, 2000). Many factors may contribute to this variation, but one yet to be considered is that the process of regeneration is not continuous. Rather, regeneration rate varies with time (Hutchins et al., 2014). All of the studies to date testing for a tradeoff between tail regeneration and reproduction have been conducted in species that lay clutches of eggs (or litters of live offspring). Thus, we have a gap in our understanding as to how different periods of the regeneration process may affect reproductive investment.

In this study we use the brown anole (*Anolis sagrei*), exploiting its reproductive characteristics to address how the temporal variation in the process of regeneration may differentially affect investment in reproduction. First, unlike many reptile species that lay clutches, the brown anoles lay one egg approximately every 7-10 days from March to October. The consistent vitellogenesis of eggs through an entire experimental period provides the ability to track how tail regeneration may affect reproductive output continuously, rather than its unpredictable effect on a single clutch due to the interaction between time of autotomy, peak investment into tail regeneration, and stage of vitellogenesis. Second, as income breeders, anoles do not store fat bodies as a crucial supply of

energy within their caudal region (in contrast to species such as fat tailed geckos), eliminating the confounding effect of abrupt energy-store loss due to tail autotomy. Lastly, they naturally autotomize their tails as an anti-predation mechanism and have the ability to completely regenerate the tail tissue (Gilbert et al., 2013; Hutchins et al., 2014).

In this study reproductive females on a resource-limited diet were randomized to Control or tail Autotomy groups. Investment in reproduction and tail regeneration were tracked for eight weeks (**Supp. Fig. 3.1**). Reproductive investment and regenerative investment were quantified in two-week intervals over the 8-week period. If there is a tradeoff between tail regeneration and reproductive investment, we would predict a reduction in reproductive investment in the autotomy group relative to the control, or a negative correlation between rate of regeneration and reproductive investment within the regeneration group. Importantly, continuous reproduction of the brown anole provides the ability to assess changes in reproductive investment across the collective eight weeks, as well as evaluate key periods during the regeneration process.

Methods

General Husbandry

Adult *A. sagrei* lizards (n = 48 females, n = 54 males) were collected from Palm Coast, Florida in April of 2018. The lizards were transported to Auburn University in Auburn, AL and acclimated to experimental conditions for three weeks (**Supp. Fig. 3.1**). Each lizard was housed individually in a cage (15.25”L x 11.5”W x 11.75” H) on a 12:12 hour light/dark cycle at ~29°C in a temperature-controlled room. Experimental conditions mimicked summer temperatures at the collection site, stimulating breeding and egg production. Each cage contained reptile cage carpet,

two bamboo perches, artificial greenery, and a nesting pot filled with a 1:1 ratio of soil and peat moss. Lizards were provided UV illumination with Arcadia D3 + 12% UVB T5 Bulbs (Reptile Basics).

Resource-limited Control Diet

At the beginning of this acclimation period, lizards were transferred to a resource limited diet of two adult crickets twice weekly, supplemented with Herptivite multivitamins (Rep-Cal) and Calcium with Vit.D (Rep-Cal). This led to the halt of reproduction in eleven of 40 females. For these individuals, food supply was increased to 5 crickets per week; however, they did not resume reproduction. In order to not compound tradeoffs due to diet restriction with those due to tail regeneration, individuals that stopped reproduction due to diet restriction were excluded from the study, adjusting the sample size to N=29. Verifying that experimental females were in a resource limited state, the transition to a limited diet for all experimental animals decreased the mean ($F_{1,45} = 3.70$; $p = 0.06$) and variance (ratio of variance = 4.96; $p = 0.002$) of maternal mass prior to treatment implementation (**Supp. Fig. 3.2A**). There were no differences in maternal mass ($F_{1,19} = 0.018$; $p = 0.89$) and snout-vent length (SVL) ($F_{1,19} = 1.54$; $p = 0.23$) between treatments at the time of tail autotomy (see experimental design below). Additionally, there was no difference in percent weight loss between treatments over the study period ($F_{1,76} = 0.15$; $p = 0.69$; **Supp. Fig. 3.2B**).

Experimental Design

We used females that had no evidence of prior tail loss or regeneration. Females were randomly assigned to one of two treatment groups, Control and Autotomy (n=14 and n=15 respectively;

Supp. Table 3.1). One female assigned to the Control group died of natural causes, decreasing the sample size to n=13 individuals at the start of the experiment. Females can store sperm for many months, but to ensure a supply of sperm through the experiment, males (N=10) were randomly assigned as a mate to a set of two or three females. Females were strategically assigned a shelf-location based on their mate, alternating Control and Autotomy females. During both the acclimation period and the experimental period, to ensure each female had a supply of sperm for egg fertilization, the mate was provided once every 14 days for 24 hours. Males were housed separately from females at all other times so they would not compete with the females for resources. Males were always removed before feeding.

Induction of Autotomy

For the Autotomy group, lizards were placed on a flat surface and forceps were used to apply even pressure 2cm below the base of the tail. While pressure was applied to the tail, the lizard was able to move freely. This process continued until the lizard utilized its natural breakpoints to release the tail. The entire process was completed in less than one minute. To mimic the stress of tail autotomy in the Control group, individuals were removed from their housing and forceps were used to apply pressure to the hind leg for a period of 30 seconds.

Measuring Regenerative Investment

Snout-vent length (SVL), tail length and mass measurements (to the nearest 0.01 grams) were taken immediately preceding and following autotomy. Every 14 days the length (mm) of newly regenerated tail was measured along with SVL and mass. Regeneration rate was calculated as the change in length over the two-week period.

Measuring Reproductive Investment

After tail autotomy, egg collection continued for 8 weeks. Nesting pots were checked three times weekly (Monday, Wednesday, and Friday). Eggs were weighed to the nearest 0.001 grams and incubated at 27°C in a 6-cm petri dish half filled with vermiculite (1:1.20 vermiculite to water ratio by mass) to hatching. Egg measures of maternal reproductive investment included: egg number, incubation duration, egg mass, and survival. Hatchling measures of maternal reproductive investment included: hatchling SVL, hatchling mass, and sex ratio over the eight-week experiment.

Statistical Analysis

The first seven days of egg production were excluded from all analyses, as eggs laid during that time may have been yolked and shelled prior to treatment implementation. Hatchling SVL and hatchling mass were highly correlated ($r = 0.511$; $p < 0.001$) and a Principal Component Analysis found that 75.53% of the variation was explained by the first principal component (**Supp. Fig. 3.3)**. The Principal Component including hatchling SVL and mass (PC1), on which loadings were equally strong at 0.7, was then termed "Hatchling Size" in statistical analyses (Part 3 of Supplemental Code File). For analysis of reproductive investment into egg production, egg mass was normalized by maternal mass to account for relative maternal investment to eggs and used as a dependent variable in egg mass analyses.

The effect of regeneration on reproductive investment was first analyzed across the eight-week time period for treatment differences using a linear mixed effect model in R (Pinheiro et al., 2020) on the average measures of reproductive investment for each female, with maternal ID as a random

effect variable to account for multiple eggs produced by a single female (Part 2 in Supplemental Code File).

Secondly, reproductive investment was then evaluated categorically between time periods defined as Low and High, referring to the rate of regeneration (Part 4 of Supplemental Code File). Peak tail regeneration rate occurred at week 4, then decreased dramatically at weeks 6 and 8 (**Fig. 3.1A**). Therefore, days 8-28 (weeks 1-4) were grouped as "High", while days 28+ were grouped as "Low". A linear mixed effect model was used to compare relative egg mass and hatchling size (PC1) response variables among High vs. Low in the Autotomy group, and in comparison to the Control group over the eight-week period. Finally, we tested for a correlation between regeneration rate, relative egg mass, and hatchling size throughout the experiment (Part 5 of Supplemental Code File). Maternal ID was included in all linear mixed effects models as a random effect variable to account for multiple eggs produced by a single female.

Statistical significance was based on $p < 0.05$ and confidence intervals that exclude zero. Tukey based Post-Hoc analysis functions were used to obtain pairwise significance, adjusting p-values for multiple comparisons.

Results and Discussion

We found the brown anole can regenerate its tail within eight weeks, with 76.6% of the length regenerated by the end of the fourth week. The average peak regeneration rate was 11.98 mm/two-weeks in the first 4 weeks post-autotomy. The last four weeks the regeneration rate dropped to an average of 3.19 mm/two-week, a significant decrease of regeneration rate. While tail regeneration

rate can vary across species (Arnold, 1984; Bateman & Fleming, 2009), our findings are similar to the green anole (*Anolis carolinensis*) where considerable outgrowth occurs within 15 days post-autotomy (dpa), and concludes around 60 dpa (Hutchins et al., 2014).

Across the cumulative eight-week experiment, we found no support for a tradeoff where regeneration negatively affected reproductive investment. Over this period, we found no statistical differences in egg number ($F_{1,141} = 2.95$, $p = 0.09$), incubation duration ($F_{1,62} = 1.86$, $p = 0.17$), egg mass ($F_{1,140} = 1.14$, $p = 0.28$), survival ($F_{1,113} = 0.04$, $p = 0.83$), hatchling mass ($F_{1,64} = 0.78$, $p = 0.37$), sex ratio ($F_{1,64} = 0.001$, $p = 0.09$) (**Supp. Fig. 3.4**). Surprisingly, the Autotomy group produced larger eggs in terms of relative egg mass ($F_{1,136} = 8.17$; $p = 0.004$) and larger hatchlings in terms of SVL ($F_{1,64} = 4.002$; $p = 0.049$) (**Fig. 1A; Supp. Fig. 3.4**). In further support of this lack of a tradeoff, while there was no significant correlation between regeneration rate and hatchling SVL, the regeneration rate was positively correlated with hatchling mass ($r = 0.310$; $p = 0.03$) (**Fig. 3.1B**). This indicates that during peak tail regeneration the females had increased investment into reproduction. It is worth noting that even though hatchling SVL was significantly higher in the Autotomy group than in the Control group, mass and SVL loaded equally on PC1 (composite measure of hatchling size) it is possible that an increase in water mass may drive part of this relationship and have downstream effects on adaptiveness of hatchlings (Fox & McCoy, 2000). Although there may be a tradeoff with unmeasured traits, these findings demonstrate an overall positive impact of regeneration on reproduction.

The increased relative egg mass and hatchling size over the experimental period **(Fig. 3.1A)** as well as the longitudinal shift in regenerative investment **(Fig. 3.1C)** led us to believe that there

may be an interaction between regeneration and reproduction over time. Due to an overall decrease in reproductive output while on a restricted diet, the total number of offspring at each measurement period limited our power to run longitudinal statistics. However, using the regenerative patterns observed, where regeneration rate in weeks 2 and 4 were significantly greater than the rate during weeks 6 and 8 ($F_{4,46} = 37.802$; $p < 0.001$), we were able to statistically test for this shift in reproductive investment in the Autotomy group by splitting the data into two categorical time periods based on the regeneration status of the mother at the time they were produced: High (weeks 1-4) and Low (weeks 4-8) rate of tail regeneration periods **(Fig. 3.1C**). No differences were found in either relative egg mass or hatchling size between the Autotomy group during the High regeneration period relative to the Control group. Relative to the High regeneration period, eggs produced by the Autotomy group during the Low period were 0.006 relative grams (±0.005; 95% CI) larger in terms of relative egg mass ($p = 0.015$), and hatchling size (PC1 of mass and SVL) was 1.264 units (±0.92; 95% CI) larger ($p = 0.005$) (**Fig. 3.1D**). This represents a 7.6% increase in relative egg mass, 17.2% increase in hatchling mass, and 4.0% increase in SVL during the Low relative to the High regeneration rate period.

Additionally, the individuals produced in the Low period by the Autotomy group were 0.01 grams (±0.005; 95% CI) larger in relative egg mass ($p = 0.013$) and hatchling size was 1.307 units larger (±0.917; 95% CI, $p = 0.004$) than the individuals produced by the Control group. This represents an 11.7% increase in relative egg mass, 11.5% increase in hatchling mass, and 4.7% increase in SVL during the Low period by the Autotomy group relative to the Control group (**Fig. 3.1D**). By week 4 the Autotomy animals were at peak investment in both tail regeneration and reproduction, and they maintained the increased reproductive investment through the rest of the experiment

while regeneration tapered off by week 8. More fine-scaled analyses may reveal this to be a biologically relevant tradeoff during early investment between regeneration and reproduction for that short time.

Collectively, these findings propose interesting hypotheses that anoles may not be capable of uncoupling the investment strategies between regeneration and reproduction, or that this is an adaptive response as larger hatchling size is often associated with higher survival (Warner & Lovern, 2014). While many studies have found a negative impact of regeneration on reproduction (Dial and Fitzpatrick, 1981; Rinkevich, 1996; Wilson and Booth, 1998; Chapple et al., 2002; Bernardo and Agosta, 2005), only one other study to our knowledge has demonstrated a positive effect of tail regeneration on reproduction. Fox and McCoy (2000) found in common side-blotched lizards that offspring from regenerating females were larger, while clutch size was unaffected. While the pattern of increased investment in reproduction is similar to this study, the use of a clutch-producing capital breeder occludes the interaction between these traits throughout the regeneration process. Our study using a continuous breeder demonstrates increased co-investment in reproduction during mid-regeneration, followed by maintained reproductive investment as regeneration tapers off.

By what mechanism could the increase in offspring size in response to regeneration be occurring? We propose two non-mutually exclusive hypotheses: cross-talk between regeneration and reproduction via shared molecular pathways, and increased energetic efficiency allowing the regenerating females to do more with their limited diet. Regeneration and reproduction are both

proliferative processes. The coordinated activation of growth stimulating networks are essential during tissue regeneration (Bosch et al., 2005; Brenner, 1998; Gauron et al., 2013; Nix et al., 2011; Rämet et al., 2002; Santabárbara-Ruiz et al., 2015; Waetzig et al., 2006; Zhang et al., 2016)

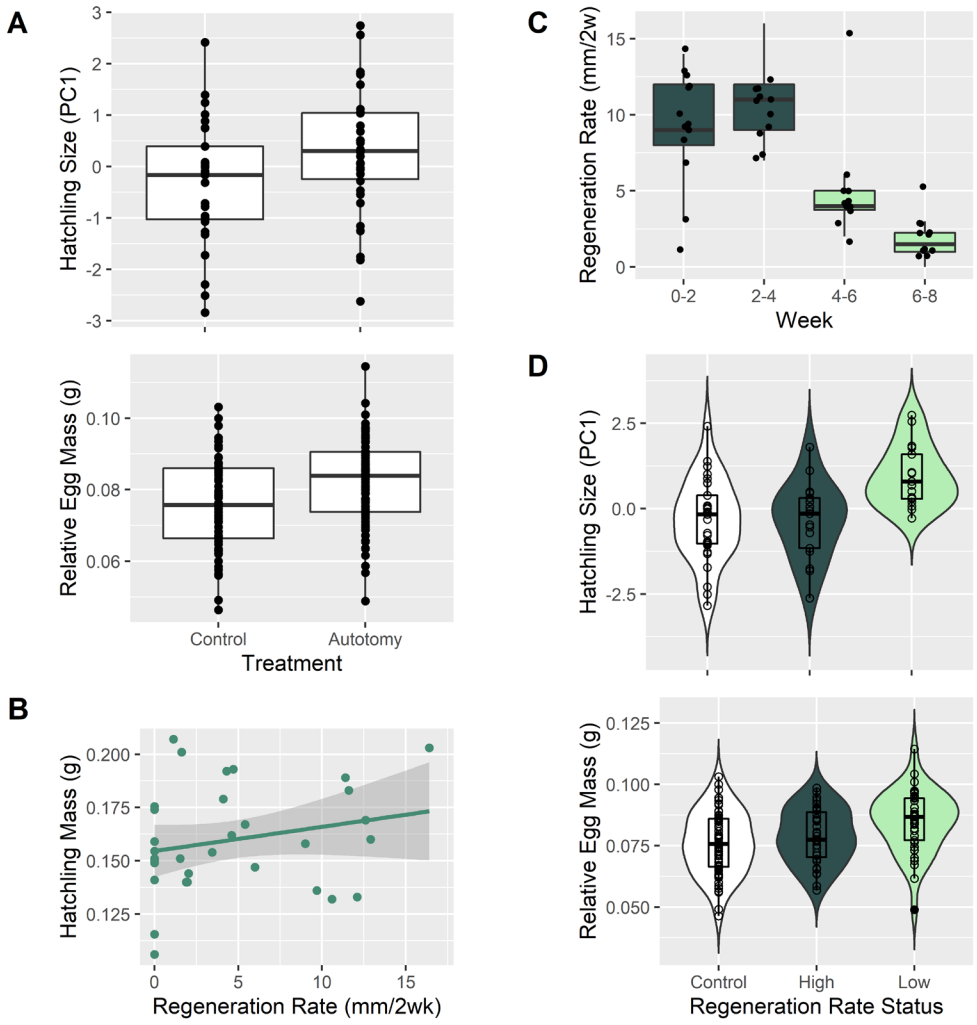

Figure 3.1: Effect of regeneration on reproduction. (A) Both hatchling size and relative egg mass were larger in autotomy group relative to control over the 8-week experiment ($p = 0.004$ and $p = 0.089$, respectively). (B) Regeneration rate was positively correlated with hatchling mass ($r = 0.310$; $p = 0.03$). (C) Regeneration rate varied over time ($p < 0.0001$); being highest at days 0-28 ("High" regeneration period) and decreased in days 29-60 ("Low" regeneration period). (D) Offspring from the "Low" period were larger relative to those from the "High" period in both relative egg mass ($p = 0.015$) and hatchling size ($p = 0.005$), and relative to those from the Control group ($p = 0.013$ and $p = 0.004$ respectively).

and are plausible mediators for pleiotropic effects on reproduction. It is well established that maternal physiology and circulating hormones during reproduction can alter offspring phenotypes (Dantzer et al., 2013; Groothuis & Schwabl, 2008). In oviparous species this can be done through maternal investment into the eggs; transfer of growth-mediating factors, hormones, or mRNAs to eggs (Ahi et al., 2018; Groothuis & Schwabl, 2008) may be an efficient way to increase offspring size with minimal energetic cost. It is plausible that regeneration-induced production and circulation of proliferative factors have this pleotropic effect on maternal investment into eggs.

A second mechanistic hypothesis for our observed results is that the regeneration process has caused the animals to become more energetically efficient. An increase in energetic efficiency in response to energetically demanding treatments has been documented across vertebrate clades. Acclimation to changes in energetic demands, including caloric restriction (Johnston et al., 2006; López-Lluch et al., 2006; Monternier et al., 2014, 2015), physical training (Ghiarone et al., 2019) and lactation (Mowry et al., 2017), can increase energetic efficiency. For instance, cold-acclimated ducklings on a 4-day fast led to an increase in metabolic efficiency when compared to their *ad lib* counterparts (Monternier et al., 2015). Additional work in the wild-derived house mouse showed that females at peak lactation had increase skeletal muscle mitochondrial efficiency (Mowry et al., 2017) when compared to non-reproductive females. The increase in energetic efficiency in response to the energetic demands can be persistent and alter reproductive investment. For example, following caloric restriction, mice reintroduced to a normal diet have an increase in litter mass relative to controls that never experienced caloric restriction (Johnston et al., 2006). Each of the above studies mention the sensitivity of timing, tissue selection, and environment on such relationships.

As income breeders with equivalent limited diets intake to their Control counterparts, the capacity for regenerating anoles in the Autotomy group to increase hatchling size and relative egg mass at and following peak tail regeneration suggests an increased efficiency of energy use. While regeneration studies on other species show contradictory effects on metabolic rate (Dial & Fitzpatrick, 1981; Fleming et al., 2009; Naya et al., 2007; Starostová et al., 2017), this study suggests that an increase in energetic efficiency with regeneration is plausible. It is clear that acclimation and response to changes on the energetic environment can have lasting effects on energy use and the regulation of the underlying molecular networks, but future experiments are needed to test these hypothesized mechanisms to explain how tail autotomy in anoles results in increased reproductive investment rather than the predicted life-history trade-off.

In conclusion, this study does not support the predicted negative trade-off between energetic investment between tail regeneration and reproductive investment. Rather our longitudinal data, via the continuous single egg clutches of the brown anole, suggests a more complex effect of tail regeneration on reproduction, demonstrating the importance of longitudinal analyses of the study of life-history tradeoffs.

Data Availability

All supplementary figures have been provided as a supplementary file. The data that support the findings of this study are openly available in GitHub at https://github.com/aeb0084/Tails-of-Reproduction.

Supplemental Information

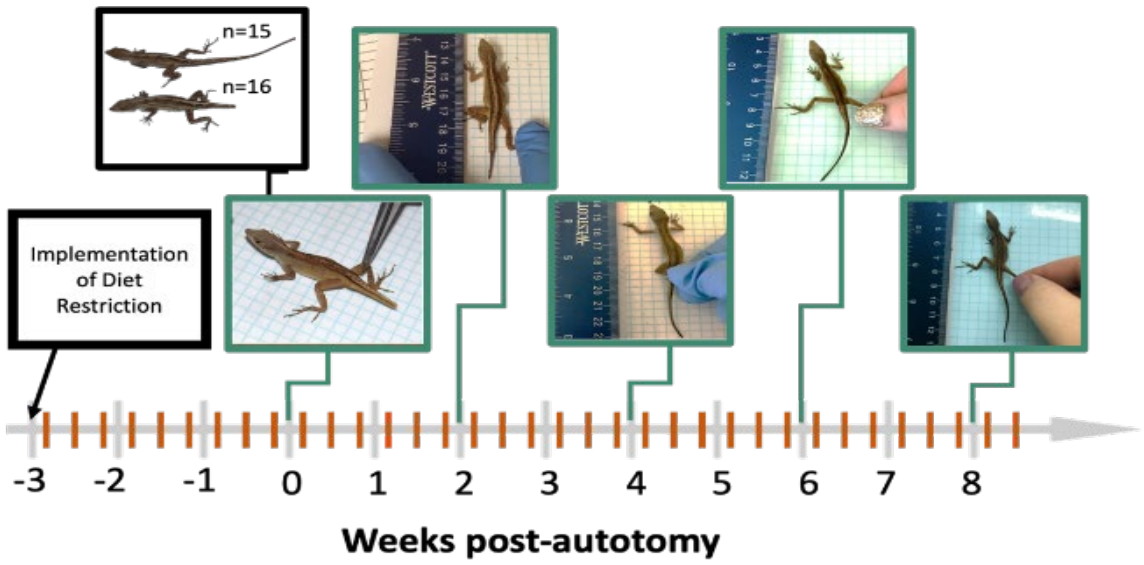

Figure S3.1: Timeline and Experimental Design. Three weeks before tail autotomy (week -3), female lizards were place on a limited resource diet (4 adult crickets/week). Animals acclimated to the diet for a period of three weeks. At week 0, all reproductive lizards were assigned to a treatment. Those in the autotomy group underwent tail loss, while the control group underwent a process to mimic the stress of tail loss while keeping tail intact. Regeneration was measured every two weeks for eight weeks (indicated by green boxes above with representative image of regenerated tail). Orange dashes represent egg collections used as offspring for measures of reproductive investment.

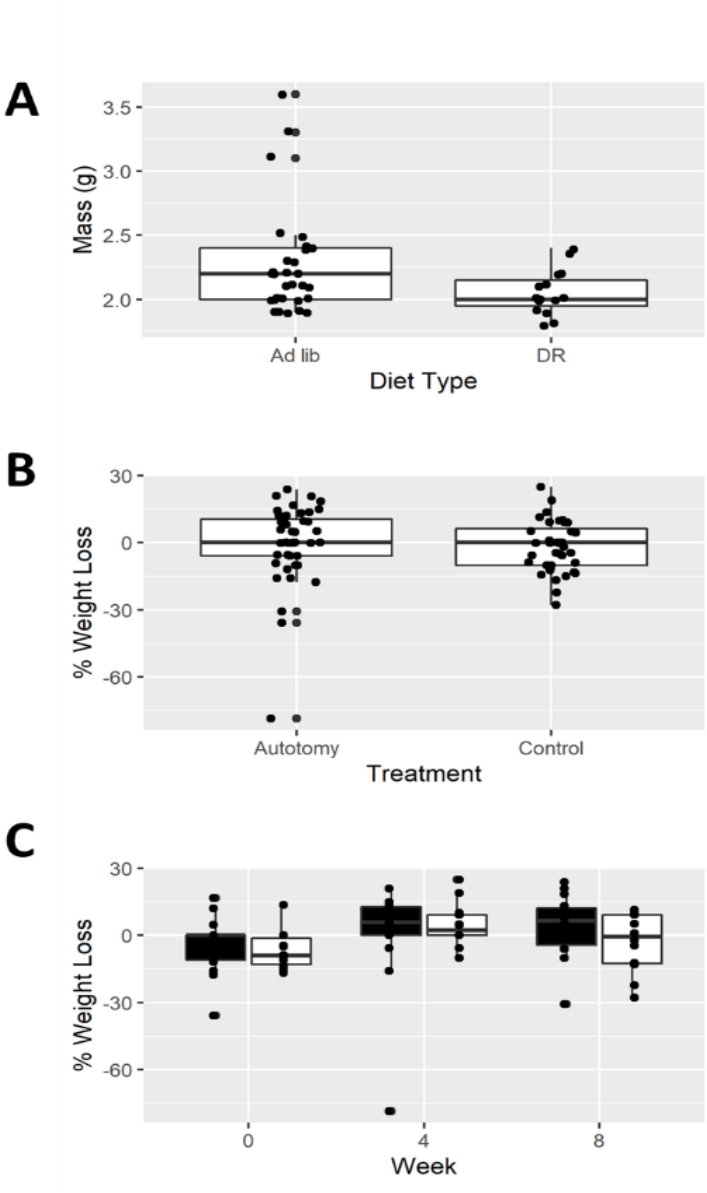

Figure S3.2: Effects of Diet Restriction Implementation. **(A)** Diet restriction implementation decreased the mean body mass of females prior to beginning the experiment ($p = 0.06$). Additionally, it significantly decreased the variance in body mass between experimental individuals ($p = 0.002$). **(B)** Over the experimental period, there was no difference in percent weight loss between the two treatments ($p = 0.66$). **(C)** Percent weight loss was collected at the beginning, midpoint, and end of the experiment. Once implementation of the treatment, there were not significant changes in mean body weight over time ($p = 0.09$).

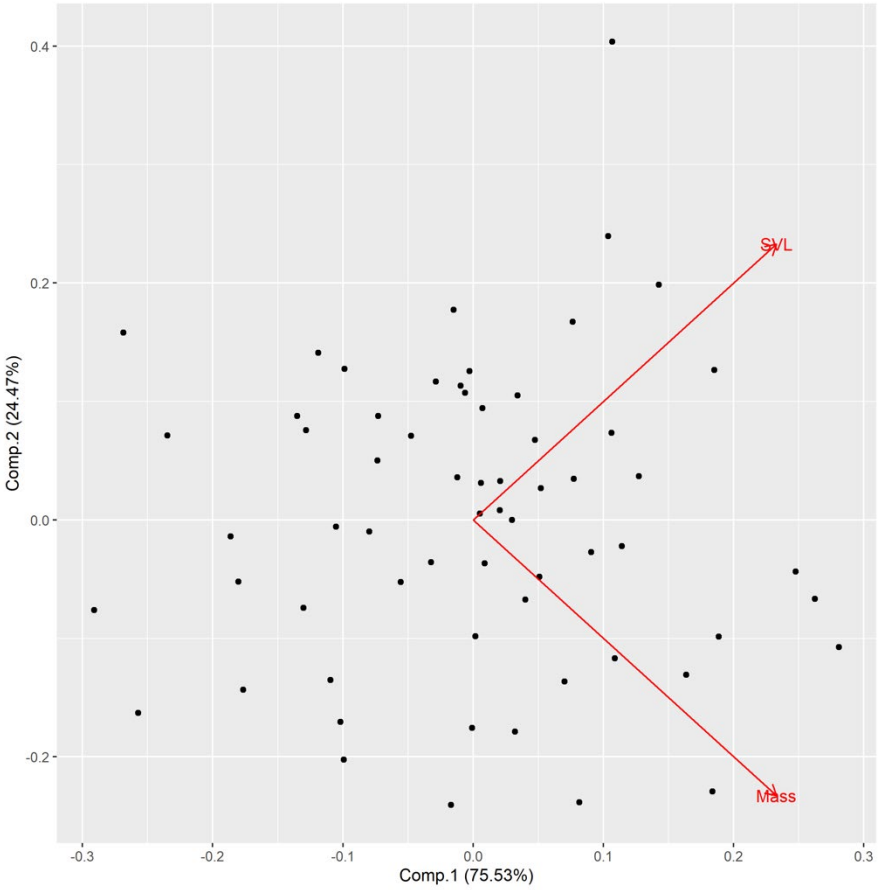

Figure S3.3: PCA Analysis of Hatchling Size. Due to the correlation between hatchling SVL and mass, a Principal Component Analysis was completed on the two variables. The first principal component explained 75.53% of the variation and was used as a measure of hatchling size in statistical analyses. Red arrows are relative to the loadings of PC1 mass and SVL variables.

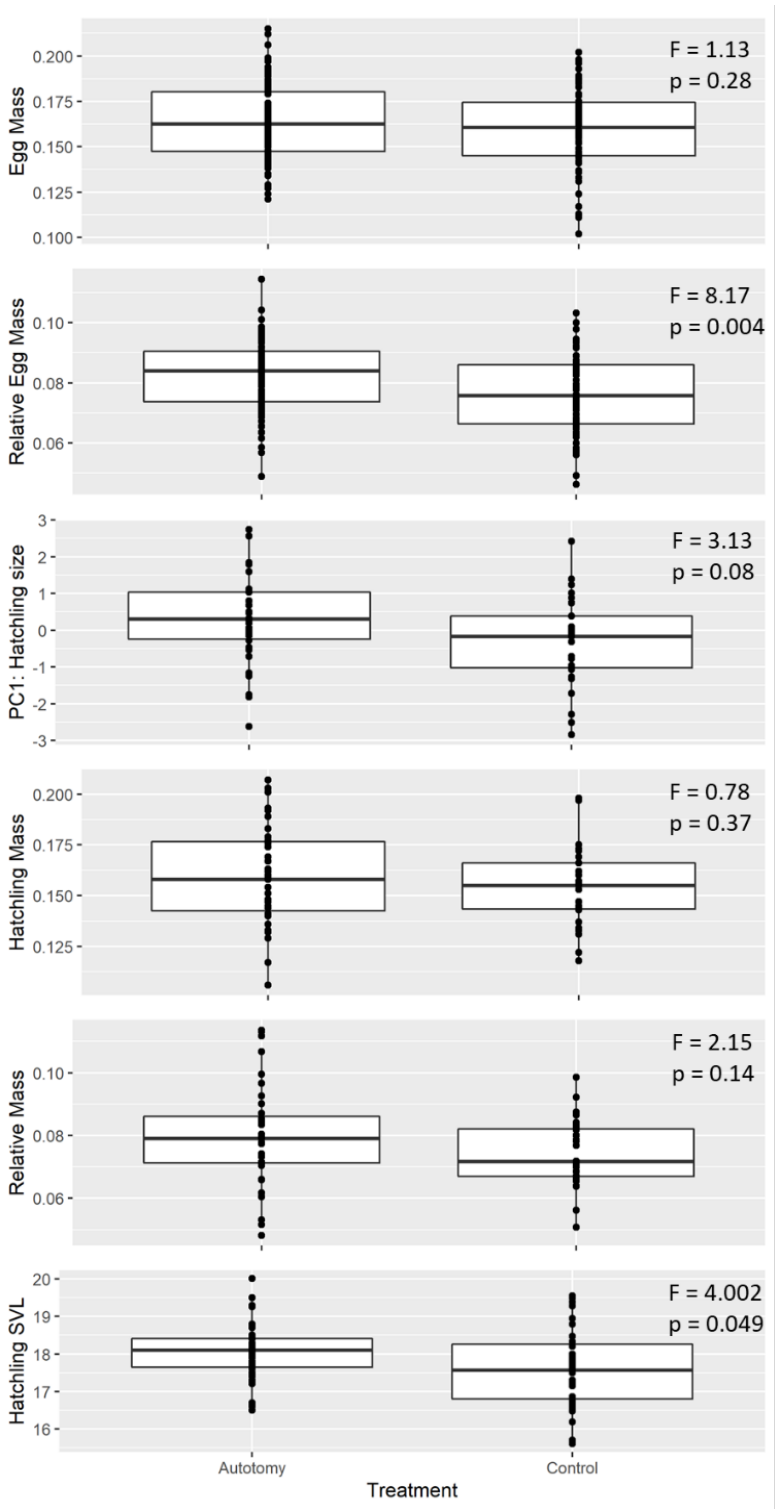

Figure S3.4: *Reproduction Investment in response to tail autotomy.* Autotomy group was either not different or had higher reproduction in response to tail regeneration relative to the Control group. Reproductive investment measures included egg mass, egg mass normalized by maternal mass, hatchling size (a combination of mass and SVL), hatchling mass, hatchling mass normalized by maternal mass, and hatchling SVL. While some measures show significant increase in reproductive investment in the Autotomy group relative to the Control group between treatments, no measures indicated a decrease in reproductive investment in response to tail regeneration.

Table S3.1: Description of Maternal and Offspring Sample Sizes. Females (N=29) were split evenly between two treatments, autotomy (n=15) and control (n=14). Following a natural death the samples size in the control group was n=13 individuals. The breeding column represents the number of females that reproduced during the experimental period. Days 1-7 were excluded from these values as eggs laid during that time may have been yolked and shelled prior to treatment implementation. Status represents the categorical assignment for statistical analyses. The number of eggs laid, and number of resulting hatchlings are displayed within status. Animals were sexed at hatchling, and the ratio of males to females is display in the last column. There were no significant differences in egg number ($p = 0.09$), survival to hatching ($p = 0.83$), or resulting sex ratio ($p = 0.09$).

Treatment	Breeding ♀	Regeneration Rate Status	Eggs	Hatchlings	Sex	
Autotomy	15	High	35	18	♀	9
					♂	9
		Low	41	17	♀	10
					♂	7
Control	13	High	27	11	♀	6
					♂	5
		Low	40	20	♀	10
					♂	10

Chapter 4: Addressing the unique qualities of upper-level biology CUREs through the integration of skill-building

Beatty, AE, Ballen CJ, Driessen EP, Schwartz TS, & Graze RM. (2021). Addressing the unique qualities of upper-level biology CUREs through the integration of skill-building. *Integrative and Comparative Biology*, icab006. https://doi.org/10.1093/icb/icab006

Background

Efforts to engage students in meaningful research experiences early in their undergraduate education have demonstrated a number of positive impacts for students majoring in science fields. One approach to student engagement in research, known broadly as Course-based Undergraduate Research Experiences (CUREs), includes the integration of *authentic* research experiences into the lecture/laboratory component of a course (Auchincloss et al., 2014; Brownell & Kloser, 2015). In this case, we use the term "authentic" to describe a CURE curriculum in which students address novel, applicable, and relevant research questions, benefiting faculty research programs, scientific progress, and student learning (Ballen et al., 2017). Following participation in a CURE, students reported increased interest levels, preference for authentic lab experiences, and the ability to "think like a scientist" (Brownell et al., 2012). Huntoon et al. (2001) demonstrated that participating in independent research increased students' intentions to pursue graduate school or a profession in a science field, particularly for underrepresented groups. Thus, the implementation of CUREs throughout undergraduate education also has the potential to promote historically underrepresented students in science, increasing diversity within the field.

These documented positive impacts of CUREs come from investigations of lower-level majors courses (i.e., first or second-year courses) and may not be entirely generalizable to upper-level courses, which are unique in several ways (**Table 4.1**). For example, upper-level courses tend

to be smaller, more focused, and composed of students who have experience taking undergraduate level science coursework, in comparison to introductory courses. While CUREs have been described as "*scalable* laboratory learning environments" that expose students to research at "an *early* point in their college careers" (Ballen et al., 2017), this characterization excludes upper-level courses that are inherently more challenging. Therefore, it is reasonable to assume the effectiveness of these experiences may vary based on the course level and associated complexity, but research on the impacts of CUREs on upper-level courses is lacking.

Given the increased course complexity and the nature of authentic course-based research experiences, instructors must consider the appropriate level of independence and autonomy for students in these courses. This is important because some students may not have previous research experience and/or may not be familiar with the new, more complex subject material. Instructors can address gaps in student preparation by providing structured, skill-building content before they encourage students to pursue more independent research within a CURE framework. While some skill-building in upper-level courses is critical for students to be able to apply research skills to address their scientific questions, generally skill-building lab activities follow a more linear path to a known outcome rather than encourage student autonomy, creativity, and curiosity. Thus, instructors must titrate the relative amounts of structure (i.e., skill-building) and independence (i.e., exploratory, with trial and error) to suit their students' learning needs. These two attributes represent trade-offs in a laboratory environment, detectable as negative

Table 4.1: Description of the qualities that differ between lower-level and upper-level classes. The potential implications related to conducting CUREs in upper-level courses are discussed with respect to course structure and student independence.

Qualities	Unique Aspects of Lower-level Courses	Unique Aspects of Upper-level Courses	Potential Implications for Upper-level CUREs
Balancing Student Experience with Course Complexity	1. Students have few basic, hands-on skills. 2. Students are expected to recall and apply relatively little information from previous courses (Zheng et al., 2008). 3. Projects are accessible for students at all skill levels (Auchincloss et al., 2014).	1. More likely to have had exposure to independent apprenticeships in research laboratories. 2. Students have developed a relatively advanced knowledge base. 3. Students are expected to apply skills developed in prerequisite coursework and incorporate complex protocols.	• Advanced skill sets increase the possibilities for potential CURE designs and complexity. • Instructors must be cognizant of a balance between providing student's independence and assisting them in recalling previously developed skills. • There is potential to build common themes across multiple levels with coordinated curriculum development.
Student Confidence	1. Participation in CURES at the introductory level has previously led to increased confidence levels (Thompson, 2016; Kloser, 2013, Harrison, 2011).	1. There is very little information available on student confidence reports in response to a CURE.	• If protocols are more complex and require advanced skills, students may perceive they are slow to progress through an experiment, or are not accomplishing their research objectives. • Confidence gains may be different for upper-level CUREs than for those previously reported in introductory CUREs.
Potential for Authenticity	1. Students have minimal existing skillsets and exposure to problem solving and scientific practices (Hoskinson et al., 2013).	1. Students have a well-developed incoming skillset and confidence gained in introductory courses. 2. Students are more likely to have previous exposure to hypothesis formation, methodology, and interpretation of scientific materials.	• A well-developed incoming skillset means that the instructor can commit less time to skill-building before students can address authentic research questions. • Existing skillsets and exposure potentially increases the depth and breadth of potential collaborative projects.
Professional Applicability	1. Students are less likely to have well developed plans in relation to careers in STEM. 2. Students are less likely to see the direct applicability of methodology to use in the 'real world' (Wieman, 2017).	1. Students are more likely to have well developed plans in relation to careers in STEM. 2. Students are less likely to alter future plans in response to participation in a CURE.	• Students may be more likely to see the applicability of the skills they are learning to the future plans that they have, if these correspond well to one another. • Students' may care more about their ability to complete an exercise that is similar to a common skill applied in their field, as they see its applicability.
Reduced Class Size	1. Larger class size, and sample size 2. Many lab sections 3. More likely to have primary instruction from Graduate Teaching Assistants and Undergraduate Teaching Assistants	1. Smaller class sizes, and sample sizes 2. Fewer lab sections 3. Increased one on one instruction with faculty members	• The instructor or primary researcher can work directly with students to achieve learning and research goals. • Small samples of students in upper-level CUREs make it difficult to generalize findings from research. • Research is currently biased toward introductory courses, but results at different stages of education are required to optimize CUR Es for all students.

correlations between the two, where increasing the level of structure decreases student independence.

In this perspective paper we share our experiences teaching an upper-level CURE in two different formats and contrast those formats using student survey data. As we are unaware of any studies examining the relative importance of course structure and student independence in the context of upper-level CUREs, we address the following research question: How does the delicate balance between structure (emphasis on distinct skill-building prior to research) and independence (emphasis on trial-and-error research experiences) impact student confidence and perceived applicability of the laboratory experience in an upper-level CURE? We addressed the unique learning environment in upper-level laboratory courses (**Table 4.1**) through the development of a series of skill-building activities over two years (N = 63 in four sections). Considering the unique elements of upper-level courses, we discuss our experience adapting a CURE to the needs of upper-level biology students as well as the benefits and drawbacks to increased structure versus increased independence. Our conclusions will assist instructors as they adapt the large body of literature on lower-level CUREs to their upper-level courses, and aid them in choosing the most appropriate formats for their classrooms based on unique student bodies and course curricula.

Methods

Course and Research Design

We implemented a CURE in a semester-long upper-level biology laboratory course taught twice weekly (110 minutes each) in two iterations that were 1 year apart. In each iteration, students were responsible for cloning and purifying a reptilian protein in a bacterial system as part of a larger

ongoing research project in a research laboratory on campus. At the beginning of the semester, the collaborating laboratory introduced the study system and the significance of the research project. Within the scope of the collaborative research project, students worked in pairs to develop specific research questions, providing opportunities for autonomy in their selection of a specific gene and how to alter their focal gene. This resulted in the creation of a variety of specific research questions that differed between pairs, but highly related parts of the overall research question. As is expected when implementing CUREs in the classroom, the specific research questions and methodological protocols differed slightly between semesters (**Supp. Fig 4.1.** contrasts the designs for each semester). However, the instructor, the topic of research, the collaborating laboratory, and the general methodology to conduct the research were the same across semesters.

In the first iteration, we taught the course in a Guided Format, structured with defined skill-building at the beginning of the semester followed by a more independent research portion. The skill-building introduced students to the methodology required to clone proteins by allowing students to practice in a traditional cookbook teaching format on a system known to work efficiently and consistently. We then asked students to apply those skills to their novel research project. This first iteration that included the skill-building portion is hereafter referred to as the "Guided Format". Due to student reports that the skill-building portion was unengaging, in the second iteration of the course we decided to test the effect of removing the skill-building and starting immediately from research focused lab activities where the students would learn the techniques through independent research experimentation as they needed to use them. This second iteration, which was taught in the absence of a skill-building portion, is hereafter referred to as the 'Autonomous Format'. This change inherently increased student independence and decreased

course structure. With this change, students had more independence in selecting the specific research questions and had to do more troubleshooting, similar to the experience of an undergraduate or first year graduate student working in an actual research lab (See **Table 4.2** for more details). In the 'Autonomous' Format, time dedicated to skill-building was replaced with a series of troubleshooting days near the end of the semester, providing students with an opportunity to repeat skills that may have failed during their independent research.

Table 4.2: Timelines between the Guided and Autonomous CURE Format iterations. In the Guided Format, the first six weeks of the course were used to develop the methodological skills necessary to complete the novel research projects. In the Autonomous Format, the students began the authentic research project immediately, following one day of review of basic lab skills. At the end of the semester, the students used the excess time to troubleshoot their projects.

Guided		**Autonomous**	
Week	**Exercises**	**Week**	**Exercises**
1-6	Skill-Building	1-2	Research Introduction and Design
7	Research Introduction and Design	3-10	Research Project Experimentation
8-14	Research Project Experimentation	11-13	Troubleshooting
15	Discussion	14-15	Discussion

Without incentives, we requested all students enrolled in the Guided (N=27) and Autonomous (N=36) Format participate in a pre- and post-course survey, resulting in participation rates of 89% and 86% respectively. We used anonymous identifiers to track individuals, and all data was

Table 4.3: CURE Survey Response Rates. Bolded survey questions represent a statistical different between the Guided and the Autonomous groups. Level of significance is represented with an asterisk (* <0.05, ** <0.01, ***<0.001). Note responses are post-survey scores, unadjusted for pre-survey responses.

Construct	Survey Question	Implementation	Strongly Disagree (1)	(2)	Neutral (3)	(4)	Strongly Agree (5)
Student Perceptions of the CURE	I was often excited to attend class and see the previous weeks result.	Guided	0%	8%	38%	**45%**	8%
		Autonomous	4%	25%	**37.5%**	25%	4%
	I believe students benefit from a class taught in the CURE format.*	Guided	4%	4%	8%	38%	**45%**
		Autonomous	4%	16%	16%	**38%**	21%
	The skills I gained were worth the time investment in comparison to a traditional lab course.	Guided	8%	4%	0%	25%	**58%**
		Autonomous	4%	8%	25%	**38%**	21%
	The CURE curriculum allowed instructors to become more engaged with students.**	Guided	0%	0%	13%	25%	**63%**
		Autonomous	4%	0%	33%	**42%**	16%
	I would recommend this course to another student.**	Guided	4%	4%	8%	29%	**54%**
		Autonomous	4%	13%	33%	**33%**	13%
	The CURE broadened my interest in research.	Guided	8%	8%	21%	29%	**33%**
		Autonomous	8%	**29%**	16%	25%	16%
	Participating in the CURE helped to prepare me for participating in a research lab.	Guided	4%	0%	25%	**38%**	33%
		Autonomous	4%	8%	25%	21%	**38%**
	I feel as though the CURE curriculum was more engaging than traditional teaching methods.	Guided	0%	8%	13%	25%	**54%**
		Autonomous	0%	13%	25%	**33%**	25%
	I participated in a project that will lead to scientific discovery.**	Guided	4%	8%	13%	**45%**	29%
		Autonomous	16%	8%	**42%**	21%	8%
	I feel as though the CURE curriculum will help me retain knowledge for a longer period of time.	Guided	4%	8%	13%	**38%**	29%
		Autonomous	4%	8%	21%	**50%**	13%
	The CURE required more time input than traditionally taught laboratory courses.*	Guided	4%	13%	4%	**50%**	30%
		Autonomous	0%	4%	21%	30%	**46%**
Confidence	I can perform an experiment without aid, when given a protocol.	Guided	0%	0%	13%	**50%**	38%
		Autonomous	0%	0%	8%	**50%**	42%
	I can design an experiment from beginning to end.	Guided	0%	17%	**38%**	34%	13%
		Autonomous	4%	13%	29%	**46%**	8%
	I am confident in my ability to keep a well-structured lab notebook properly detailing experiments.	Guided	0%	8%	8%	**46%**	38%
		Autonomous	0%	8%	8%	**42%**	42%
	The CURE curriculum made it easier to identify and address my weaknesses throughout the semester. **	Guided	8%	4%	8%	29%	**50%**
		Autonomous	4%	25%	**29%**	21%	17%
Applicability	**Research that I do in lab courses will lead to scientific discovery.*****	Guided	4%	8%	**42%**	38%	8%
		Autonomous	4%	4%	**58%**	30%	4%
	In laboratory classes, I gain skills that will be applied in my future career.	Guided	4%	0%	13%	38%	**46%**
		Autonomous	0%	8%	21%	25%	**46%**
	The practices taught in molecular biology courses are applicable in everyday life.	Guided	17%	**25%**	**25%**	21%	13%
		Autonomous	4%	8%	**42%**	29%	17%

deidentified. The survey questions covered general constructs including student perceptions of Confidence, Applicability, and CURE Format (**Table 4.3**). The survey instrument was a Likert-scale response system ranging from 1 (strongly disagree), to 5 (strongly agree), designed for this study by the lead author (AEB). The survey items were piloted by five individuals to ensure consistent interpretation. All handling of data and survey administration was approved by the Auburn University Institutional Review Board (Approval 18-314).

Student demographics were comparable between the two formats in terms of self-reported disciplines, previous research experience, and self-reported GPA (**Table 4.4**). The ratio of undergraduate to graduate students enrolled in each CURE was also comparable across formats

Table 4.4: Demographic information from both the Guided and Autonomous CURE Formats.

Gender	Guided (N=28)	Autonomous (N=35)	University Demography (Based on 2018 Enrollment)
Male	28.57 %	50%	50.7 %
Female	71.43 %	50%	49.3 %
Degree Type			
Bachelor	87.5 %	81.8%	84.54 %
Graduate	12.5 %	18.2%	15.46 %
Self-Reported Discipline			
Microbial, Cellular, and Molecular Biology	81.5%	78.8%	--
Agricultural Biology	3.7%	6.1%	--
Biomedical	7.4%	3.0%	--
Pre-professional	0	6.1%	--
Other	7.4%	6.1%	--
Self-Reported GPA			
2.0-2.4	8%	4%	--
2.5-2.9	16%	17%	--
3.0-3.4	36%	29%	--
3.5-4.0	52%	50%	--
Previous Research Experience			
No experience	4%	12%	--
< 1 year	15%	20%	--
1-2 years	44%	52%	--
3+ years	37%	16%	--

and was comparable with reported University-wide statistics from 2018. The distribution of men and women students did vary between the two course formats (71.43% women in Guided and 50% women in Autonomous).

We were unable to validate constructs through factor analyses (Knekta et al., 2019) due to limited statistical power given the course size; therefore, we loosely grouped questions that were similar into measures of students' perceptions of confidence, applicability, and CURE Format, as increased student reports of confidence and views of applicability to their professional aspirations are commonly reported benefits of CURE implementation at the introductory level. For the purposes of presenting the results and discussion, we analyzed individual survey items within those three aforementioned measures of perceptions (**Table 4.3**). We analyzed data using linear mixed models (Pinheiro, Jose et al., 2020), testing for reported gains (differences in pre- and post-survey reports) in the measures (i.e., confidence, applicability, and CURE Format) between iterations. When pre- and post- survey responses were co-analyzed, pre-survey responses were included as a random effect to control for incoming variation in student responses. We then utilized Tukey post-hoc analyses (Lenth, Russel, 2019) for pairwise comparisons of pre- and post- timepoints within years, and pairwise comparisons between calculated gains of post-surveys between formats. In each case, anonymous identifiers were used in the model to account for multiple repeated sampling. Select comparisons are discussed below.

Results

Guided Versus Autonomous

Applicability - Value of skills in everyday life and career

When comparing the two laboratory formats, our results showed that students in the Autonomous Format were more likely to identify the applicability of their skills to everyday life (Estimate = 1.062 ± 0.44; p = 0.017) and were more likely to express perceptions of contribution to scientific discovery (Estimate = 1.625 ± 0.44; p = 0.0003). However, they were not any more likely to identify the applicability of their skills to their future careers than students in the Guided Format (Estimate = -0.708 ± 0.44; p = 0.11; **Figure 4.1A**).

Confidence

Pairwise comparisons between the two formats revealed students in the Guided Format were more confident in their ability to identify their own weaknesses following participation in a CURE (Estimate = 0.86 ± 0.279; p = 0.021). Despite this, students reported being equally prepared in their ability to design an experiment (Estimate = -0.229 ± 0.282; p = 0.417), produce a comprehensive lab notebook (Estimate = 0.229 ± 0.282; p = 0.42), and perform an experiment using a protocol (Estimate = -0.188 ± 0.282; p = 0.507), regardless of format (**Figure 4.1B**).

CURE Format

We identified five survey items that showed significant differences between the Guided Format and Autonomous Format iterations of the course (**Figure 4.1C; Table 4.3**). Of these survey items, students consistently responded more positively in the Guided Format. For example, students in

the Guided Format reported they were more likely to recommend the course to another student (Estimate = 0.85 ± 0.317; p = 0.007), more likely to believe students benefit from the CURE format (Estimate = 0.60 ± 0.317; p = 0.058), and to report that CUREs are more likely to lead to scientific discovery than traditional lab courses (Estimate = 0.918 ± 0.317; p = 0.004). Student responses also indicated that instructor engagement was highest in the Guided Format (Estimate = 0.80 ± 0.317; p = 0.014). While both formats indicated that CUREs take more time than cookbook lab courses, students expressed this more strongly in the Guided Format (Estimate = 0.61 ± 0.320; p = 0.057) (**Figure 4.1C**). The combination of these findings indicate that students responded more positively to the Guided CURE Format.

Additionally, students in the Guided Format reported that the CURE was more likely to broaden their interest in research and reported increased excitement to attend class. Although the estimates were large, these findings were not statistically significant at our predefined cut-off of 0.05 (Estimate = 0.578 ± 0.317; p = 0.06 and Estimate = 0.54 ±0.317; p = 0.08 respectively), possibly due to our small sample size.

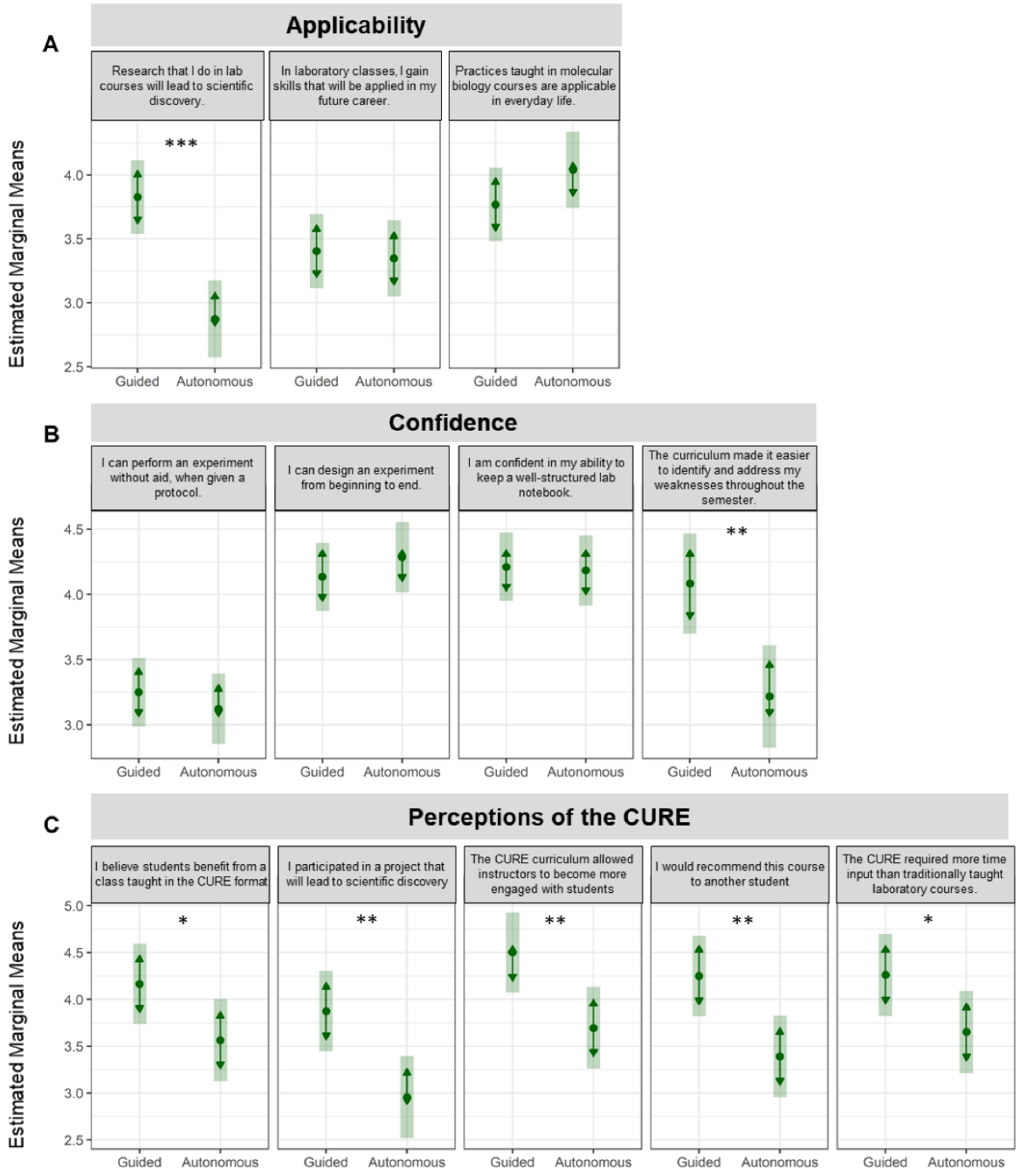

Figure 4.1: Student Post-survey Gains of Applicability, Confidence, and Perceptions of the CURE. Student measures of gains in applicability, confidence, and perceptions of the CURE were compared between the Guided and Autonomous Formats. Least squared means were plotted with 95% CI displayed by shaded regions, and arrows represent comparisons among the means, with overlapping arrows indicating non-significance based on Tukey p-value adjustments (Lenth et al. 2021). Statistical significance is noted by an asterisk (* <0.05, ** <0.01, ***<0.001). Only statistically significant measures were plotted for the CURE format. See table 2 for comprehensive post-survey response comparisons.

Discussion

The goal of this work was to reflect on the most salient elements of upper-level CUREs and identify how teaching approaches – specifically the inclusion of skill-building activities – may affect student perceptions of confidence, applicability, and CURE structure. Our main conclusion is that implementing upper-level CUREs that require advanced knowledge calls for a balance between structure *and* independence. And while we acknowledge that failure is an important part of the scientific process, too much failure can deter some students from scientific research and minimize their feelings of scientific discovery. Below we reflect on our experiences and our empirical results and make several suggestions for future implementations of upper-level CUREs.

1. Balancing Student Experience with Course Complexity

Increasing complexity may increase the potential for students to perceive failure, or a difference in an expected or desired result and the one that the student experiences. In this course, failure could occur at many stages. Students may fail at producing their final protein product or minor methodological failures could occur throughout the experiment. An emphasis on skill-building in the Guided Format filled knowledge gaps that were apparent in the Autonomous Format, allowing for complex protocols to run more smoothly (**Supp. Fig. 4.1**). The Autonomous Format, that de-emphasized skill-building, meant students did not have as many opportunities to practice skills that were required for a successful project, leading to more frequent methodological failures. Additionally, the increased freedom for hypothesis formation decreased the amount of project overlap among students; therefore, students were less able to engage in peer instruction and they could not ask their peers for additional shared materials if a step in their project failed.

The increased complexity of research projects in the Autonomous Format led to an increased workload for teaching assistants and instructors. However, students in the Autonomous Format indicated it required less time investment than student reports from the Guided Format; students in the Autonomous Format also reported less benefits of education through a CURE format (Estimate = -0.601 ± 0.317; p = 0.05). Consequently, they were less likely to recommend the course to another student (Estimate = -0.859 ± 0.317; p = 0.007). Without significant demonstratable benefits from the Autonomous Format over the Guided Format, and with increased workload for instructors and teaching assistants in the Autonomous Format, we conclude that a Guided Format, or a related approach, represent an effective balance of skill-building and independence.

2. Student Confidence

When we examined the individual survey items, we observed that students in the Autonomous Format reported a decreased confidence in their ability to identify and address weaknesses throughout the semester. This was surprising because this skill was especially important in the Autonomous Format, as students had to troubleshoot to complete experiments. Previous empirical research on lower-level biology students showed that a CURE, compared to a cookbook lab, resulted in gains in student confidence in biology majors' ability to execute biology-related laboratory tasks (Kloser et al., 2013).

We make sense of these results through one of the following possible explanations. First, committing time to skill-building may increase student confidence because as they performed the experiments, they knew more about what to expect and they knew that they could complete the task under controlled conditions. Alternatively, students in the Autonomous section had to

troubleshoot problems more. While troubleshooting leads to the development of communicative and metacognitive skills that are crucial components to the science process, these experiences may have felt like failure and an inability to complete laboratory tasks.

3. Potential for Autonomy

One of the leading recommendations for professors looking to implement CUREs in their classroom is to cultivate a classroom environment in which students can embrace uncertainty (Shortlidge et al., 2016). However, in the Autonomous Format of our upper-level course, this meant much of class time was spent troubleshooting methods for diverse research questions. This may have been in part because we did not provide them with enough foundational knowledge to address their research question, and because relative to the skill-building section, we did not provide as much guidance about what types of questions could productively be addressed. Conversely, the skill-building experiences in the Guided Format built a knowledge base for students, giving them the confidence to effectively strategize a troubleshooting plan that was meaningful in their independent projects, which overall led to increased productivity and less reliance on the instructors. Additionally, they had a better understanding of realistic research questions that they could address during the semester. It also led to more in-depth, meaningful discussions between instructors and students, rather than mostly troubleshooting inquiries.

4. Professional Applicability

As this CURE was implemented in an upper-level biology course, we expected students in both formats to report that they could apply the methods from the lab course to their everyday life and their future career. Yet, we observed a decrease in student perception of applicability in the Guided

Format in terms of scientific discovery and applicability in everyday life following participation in the CURE, whereas students in the Autonomous Format reported positive gains of scientific discovery and applicability to everyday life. However, students were more likely to view the applicability of skills to their future careers following participation in the Guided Format, although this finding was non-significant.

This was a surprising result, given that none of the groups in our Autonomous Format were able to yield a tangible product, compared to the 67% of groups in the Guided Format that were able to produce their chosen protein product. It is worth noting that a lack of protein as an end product does not necessarily indicate that students in the Autonomous Format did not gain technical research skills and perform skills effectively. In fact, students in both treatments had plenty of opportunities to learn technical skills that will be applicable in scientific professions. We recognize the importance of students' view of professional applicability, and discuss below how to adjust this balance in order to improve perceptions of applicability in a Guided Format.

As most students only have exposure to cookbook laboratory experiences in lower-level science courses, they are not exposed to the realities of struggle and failure that are common in science. Previous studies show that overcoming failure is essential in producing competent scientists (Andrews & Lemons, 2015; Laursen et al., 2010; Lopatto, 2007; Simpson & Maltese, 2017; Thiry et al., 2012). However, other work has shown that not all students perceive challenges associated with failure as a learning experience (Marra et al., 2012). For example, students who believe intelligence is a fixed, unchangeable trait are more likely to quit in response to challenges or setbacks (Henry et al., 2019). In our study, student mindset likely influenced students' decisions

to persevere when faced with challenges and adversity (Duckworth, 2016; Hochanadel & Finamore, 2015). While students in the Autonomous Format did not produce tangible products (the end product of the experimental workflow), they were given the opportunity to troubleshoot their methodologies, which has been shown in the past to positively impact views of failure and persistence (Henry et al., 2019). Due to time restrictions, students in the Guided Format were given the opportunity to discuss possible steps for troubleshooting, but were were not able to troubleshoot failed methodologies. Encouraging a growth mindset in students who encounter failure may be the difference between their viewing scientific failures as learning experiences rather than unconquerable barriers.

5. Reduced Class Size

Class size has been recognized as one of the most highly reported instructor barriers to CURE implementation (Shaffer et al., 2014; Spell et al., 2014). The class size per section for this course was a maximum of 15 students, at two sections per year. With the increasing complexity of upper-level courses, we believe that the personal interaction with instructors was essential for student success. Increased class size or additional lab sections would have made the advanced methodologies used in these CUREs unfeasible, reducing authenticity. With two sections, instructors and teaching assistants co-taught each session, allowing increased opportunities to engage with instructors. The positive impacts of these interactions were reflected by student survey responses through consistently high scores of student and instructor engagement. We recommend continued implementation of CUREs in upper-level biology courses of small class size, as we found them manageable for instructors, and they allowed for valuable personal student-instructor interactions.

Integration Moving Forward

Both the Guided and Autonomous course formats had distinct benefits and drawbacks. However, based on our results and experiences in the classroom, we recommend instructors front-load upper-level CUREs with skill-building exercises to maintain structure and consistency and encourage students to then apply their advanced skillset to develop and execute independent projects in their research experience. The extent of structure and skill-building required for students to carry out an independent project will vary depending on the project. In our course, the skill-building portion of the Guided Format required approximately half the semester. Depending on the level of independent project complexity, the skill-building to novel research ratio could be adjusted to the length necessary to fill pre-existing knowledge gaps. An alternative format adjusting the proportion of skill-building to novel research may also allow for maximization of the student and instructor benefits of skill-building, while increasing student gains of perceived applicability.

One limitation of this study is that all measures are based on student perceptions collected at one time point. Due to these constraints, as well as our small sample size, it is worth noting that these data are exploratory in nature and warrant further investigation. We were unable to collect information on long-term impacts or meaningful measures of learning gains. For example, students in the Autonomous Format gained trouble-shooting skills that may lead to measurable gains in scientific critical thinking. In the future, we plan to adapt the course to incorporate student reported benefits from each iteration, while also measuring learning gains using validated pre- and post-course concept inventories. While we believe the experiences of failure and troubleshooting are still essential in preparing students for careers in the fields of science, technology, engineering and mathematics, it is important to highlight small successes throughout the semester to build

student engagement and confidence. To accommodate these needs, we plan to introduce the research topic followed by a shortened skill-building section in the future. This will allow students to learn practical applications of the skill-building methodology and encourage the connection of learned skills and concepts to research applications. In turn, resulting confidence will increase student engagement during more independent research, and likely incorporate the views of professional applicability reported in the Autonomous Format. Another limitation of this research relates to observed differences in binary gender ratios across semesters, which in turn might impact student responses to survey questions. We acknowledge demographic characteristics such as gender impact student experiences in science, and future work will benefit from an explicit focus on how these laboratory experiences hinder or enhance learning for different subsets of students.

Historically, research on CURE formatted courses has not focused on upper-level students or analyzed upper-level performance in response to different laboratory experiences. However, there is tremendous potential to support this fledgling group of students through evidence-based approaches as they transition from upper-level coursework to post-undergraduate career development. We hope this report provides instructors with questions to ask during course development, knowledge of potential barriers to studying upper-level CUREs and methods to incorporate pedagogical research into their own inquiry-based teaching. Collaborative efforts to share results among institutions will be essential in making general recommendations of best practices for teaching CURES across different contexts – inclusive of upper-level CURE courses.

Supplementary Materials

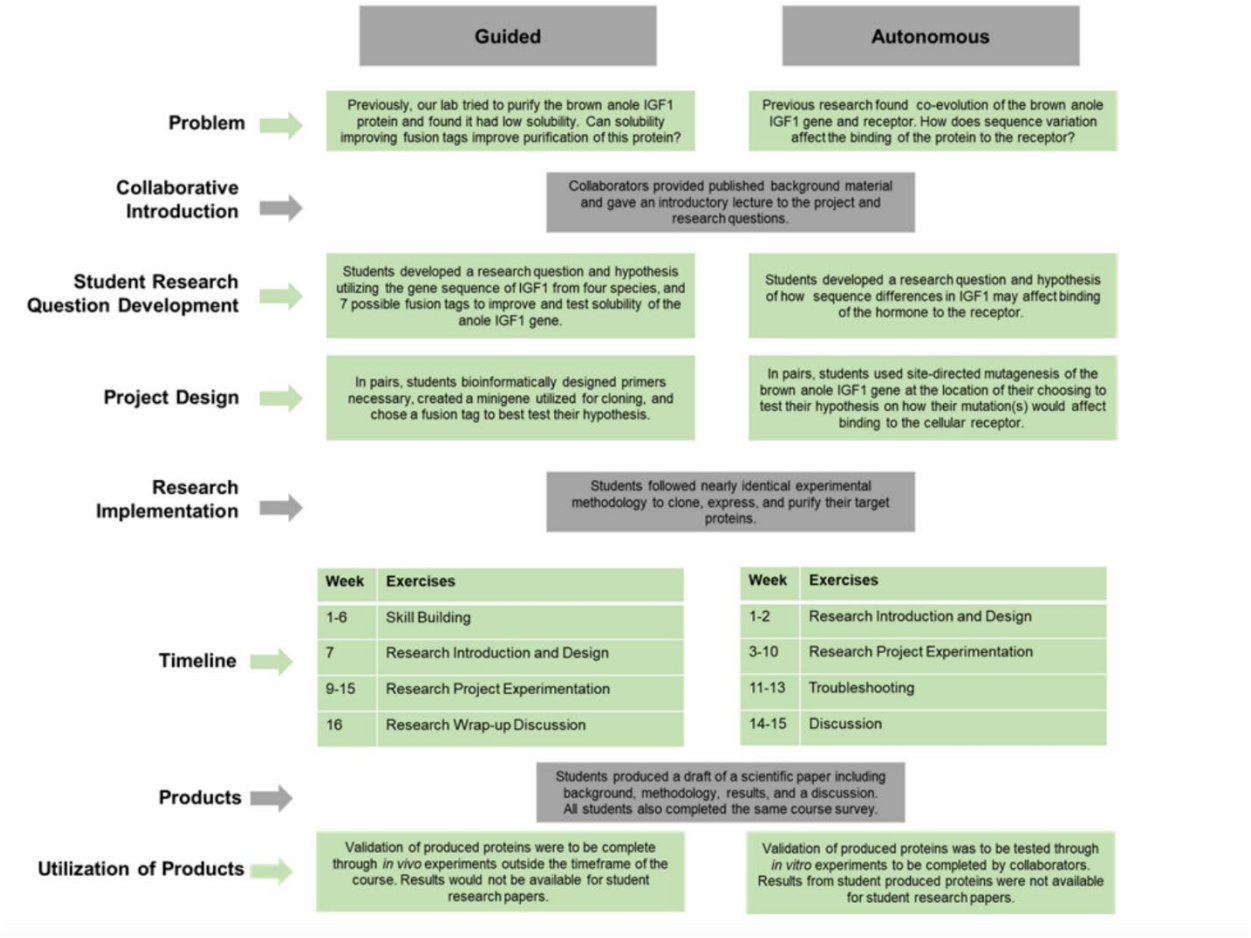

Figure S4.1: Detailed Research Design. The presented problem, student goals, project implementation and timelines are displayed in both the Guided and Autonomous Formats. Green boxes highlight differences between the two laboratory learning experiences, while gray boxes show similarities.

CONCLUSION

In many ways, functional genetics and genomics can work as a linchpin in the understanding of adjacent fields within biology. The understanding of how genotype leads to functional characteristics can address major questions within fields such as life-history evolution, ecology, biomedical sciences, and even be used to address needs within biology education (**Fig. 5.1**). Because presence of the IIS network is nearly ubiquitous across species, and the signaling cascade effects a plethora of physiological characteristics, gaining a clearer understanding of its function across species has the potential to largely impact many subdisciplines within the life sciences.

Despite the pervasive nature of IIS signaling across animals and its widespread downstream activations affecting physiological processes, very little is known about one of the networks main hormones, IGF2, and the functional effects of the IIS network in non-model species. IGF1 and IGF2 compete for binding to molecular IIS components, such as binding proteins and cellular receptors (Daza et al., 2011; Denley et al., 2005; Firth & Baxter, 2002), indicating that the presence of both hormones concurrently in a system may have different effects functionally than the presence of a single hormone. Yet, because IGF2 post-natal expression is not present in lab-rodents, the primary vertebrate model for studying IIS signaling, this has created a “street-lamp” effect on the sole function of IGF1. A primary goal of this dissertation was to understand the frequency of post-natal IGF2 expression across amniotes to assess the possible consequences of such a spotlighting effect.

In doing so, it was found that 92.5% of amniote species, with appropriate publicly available RNAseq data, expressed IGF2 post-natally (in juveniles and adults), with 70% of species expressing IGF2 at a higher level than IGF1. It was also verified that IGF2 was expressed across all studied life-stages in two lizard and two bird species. Additionally, it was confirmed through RNAseq data mining and quantitative gene expression that ***both*** lab and wild mice turn off gene expression of IGF2 following embryonic development. In combination, this indicates that the across amniotes post-natal expression of IGF2 is the norm, rather than the exception, and indicates our need for the development of vertebrate models that are more representative of the amniotic clade for study of IGF2 in adulthood.

As the brown anole lizard (*Anolis sagrei*) is a commonly used evolutionary and ecological model, characterizing the gene expression of the IIS network across space and time builds the foundation for its use as an additional model species for the study of IIS. Within the brown anole, it was found that IGF2 was expressed across all life stages and at a higher level than IGF1, which is more comparable to those patterns seen in humans in a way that mice and rats are not. Because IGF1 and IGF2 compete for binding proteins (Daza et al., 2011; Denley et al., 2005; Firth & Baxter, 2002) and binding proteins play an important role in normal and diseased physiology (Baxter, 2014; Gleason et al., 2010; Koutsaki et al., 2011; McQueeney & Dealy, 2001; Salih et al., 2004; Sureshbabu et al., 2012; Thai et al., 2015), understanding the expression profile of the binding proteins was also essential in understanding the implications of co-expression of IGF1 and IGF2. The ubiquitous expression of the binding proteins, with the exception of the variability detected in IGFBP5, is an interesting and important step in understanding the function of the IIS network as a whole.

Discovering the normality of IGF2 expression across amniotes brings into question the health implications of our current understandings of IIS signaling implications. With the street-light effect of IGF1 in rodent models that currently challenges the biomedical field, we know very little about the role IGF2 plays in human health even though the IIS network is commonly implicated in human cancer biology (Baxter, 2014; Ghoussaini et al., 2014; Perks & Holly, 2003; Slater et al., 2019; Sureshbabu et al., 2012; Thai et al., 2015; Yu et al., 2017), growth and development (Chao & D'Amore, 2008; Fisher et al., 2005; Pontén et al., 2008; White et al., 2018), and many other diseased physiologies such as cardiovascular disease (Berry et al., 2015; Higashi et al., 2019), obesity (Hedbacker et al., 2010; Nam et al., 1997), and neurodegenerative disorders (Álvarez et al., 2007; Gasparini & Xu, 2003) (**Fig. 5.1**; Chapters 1 and 2; Purple and Pink). Additionally, the functional relationships within the IIS network are likely to be affected by the presence of a second extracellular hormone. In order to examine the cellular and systemic effects of the IIS network in a model displaying postnatal IGF2 expression, we first must understand the genetic breakdown of IIS expression within that system. The development of the brown anole lizard as an additional model for IIS expression, explored in the first two chapters of this dissertation, opens an entire field of research in understanding the IIS network in a biomedical, ecological, and evolutionary context, bridging the gap between molecular biology and functional ecology.

With a better understanding of IIS network gene expression in reptiles, the potential functional impacts of IIS network regulation became of interest. As the IIS network plays a vital role in cellular division, growth and development, reproduction, and tissue regeneration, and brown anoles possess the ability to completely regenerate tail tissue, the potential role of the IIS network

in a reproduction-regeneration tradeoff was explored by experimentally testing if a tradeoff occurs within the species. Reproductive output, survival, offspring size, egg size, sex ratio, and the rate of tail regeneration were collected longitudinally for 8 weeks. While the predicted tradeoff between reproduction and regeneration was not detected, an interesting pattern arose. Females that had invested in regeneration produced larger eggs and hatchlings in comparison to non-regenerative animals, without negatively affecting clutch size or survival. The increased offspring size beginning at peak regenerative investment and continuing following the regenerative period suggests that regeneration may cause increased energetic efficiency or utilize shared physiological pathways with reproductive investment. While tradeoff experiments utilizing reptilian models are often done on clutch-laying income breeders, these findings indicate that there may be an important interaction between regeneration and reproduction over time that would be missed when studying a single clutch. Despite the fact that we didn't see the expected tradeoff based on life-history theory, the IIS network is still a possible shared mediator through the "shared physiological pathway" hypothesis and warrants further investigation through longitudinal studies utilizing income breeders with continuous vitellogenesis.

As life-history theory lies at the heart of biology (Stearns, 1992), broadening understanding *between* fields, it is often used to understand phenomenons such as animal behavior, natural selection, and genetic variation, it holds a lot of explanatory power within the field (Stearns, 1992). However, much like all biological concepts, our understandings are often context dependent, and the complexities of life-history relationships can take time to expose. As presented here (**Fig. 5.1**; Chapter 3; Green), the use of the brown anole as a model has the potential to change our perception of what was previously considered to be a fairly straight forward concept based on life-history

theory, the cost of tissue regeneration on reproduction. However, by using a model with continuous vitellogenesis, it became clear that the cost of regeneration on reproduction may actually be context and specifically, species dependent. Beyond our direct understanding of this relationship in the brown anole, our grasp of life-history has direct effects of decisions that are made in adjacent fields, such as ecology. In this case, an event that is considered to be stressful and known to negatively impact balance, locomotion, climbing, swimming, and survival (Arnold, 1984; Bateman & Fleming, 2009; Naya et al., 2007) in reptiles actually positively impacted reproductive investment of an invasive species. As plans are developed to address the growing concerns of ecological stability, including species decline and species invasion, our understanding of behavior and life-history strategy influence policy development. Therefore, our understanding needs to be well developed from the genetic level to the phenotypic level- taking into account interactions with the environment, variation by species, and study design (longitudinal studies vs. cross-sectional studies).

The use of the brown anole as a research model extends beyond the research laboratory and into the biology classroom as well. As biology education advances, there is a continuing call for exposure of undergraduate students to a more realistic depiction of research practices. At large research institutions, there are rarely enough faculty to accommodate student research needs on a 1-1 basis. However, implementations of CUREs allow students to participate in novel research in their traditional curriculum in a way that also supports faculty in advancement of their research. As a species with evolutionary, ecological, and genetic importance, IIS network research in the brown anole is ideal for integration in the classroom due to its broad relevance, ease of maintenance, and invasive species status. In order to use the IIS network and meet the goal of

species-specific protein production, the Recombinant DNA Technologies course on campus participated in a CURE in which they were tasked with expressing species-specific IIS network genes for use in functional experimentation. In the process, the novelty of IIS research in reptiles was used as a teaching tool to evaluate the effectiveness of CURE formats in upper-level biology courses. While it was found that student reported approval and appreciation for the CURE regardless of format, students did report that the use of skill-building led to more positive impacts of the curriculum. IIS network research in reptiles is a newly developing field and it creates many opportunities for integration into the classroom, although the novelty may mean that students need slightly more guidance than previously reported. By integrating this field of research into the classroom, students benefit from exposure to novel research, and instructors are able to use their time as educators to double as research advancements.

This addresses the needs of two closely related fields that are rarely studied concurrently. Biology education is influenced on an instructor-by-instructor basis, and often includes the perspectives and biases of the instructor's disciplinary specialty. It has been previously reported that students relate to materials at a higher level and are more likely to retain information when their instructors are actively engaged and enthusiastic about the materials they are teaching (Martin & Bolliger, 2018; Zhang, 2014). Additionally, students respond well to the knowledge that their work has significant impact on the field, rather than the traditional "cook-book" outcomes within the classroom (Auchincloss et al., 2014). Integrating novel research within the classroom has the potential to address the needs to multiple fields simultaneously. In this dissertation, a Course-Based Undergraduate Research Experience (CURE) was presented utilizing novel questions related to the IIS network (**Fig. 5.1**; Chapter 4; Yellow). As a result, students produced species-

specific proteins that allow for direct testing of functional effects of IGF1 and IGF2 in reptilian models, including *in vitro* and *in vivo* students, expanding our knowledge of the functional effects of the IIS network. Lastly, the developed proteins directly addressed the need for molecular tools available to study the IIS network outside of rodent models. As one of the greatest benefits to continued study of the IIS network in lab-rodents are the numerous resources available, such as knockout strains, antibodies, and quantification tools, there is a critical need for the development of tools that will function in alternative vertebrate species.

Together, the chapters in this dissertation display the importance of organismal model selection in the study of the IIS network, and lay the groundwork for use of the brown anole as a reptilian model. The need for alternative model species integrating the post-natal study of IGF2 with our "street-lighted" IGF1 research has been conveyed through a comprehensive study across amniotic clades, and the foundation for the use of the brown anole as an IIS model has been described through a comprehensive gene expression study. Beyond this, the usefulness of a reptilian model has been described in the context of biology education, the advancement of biomedical, evolutionary, and functional ecology research. Investment into expanding the brown anole to a commonly used IIS model organism has the potential to positively impact many fields of biology, and advance our understanding of the intricate IIS network interactions in ways that the existing models currently cannot.

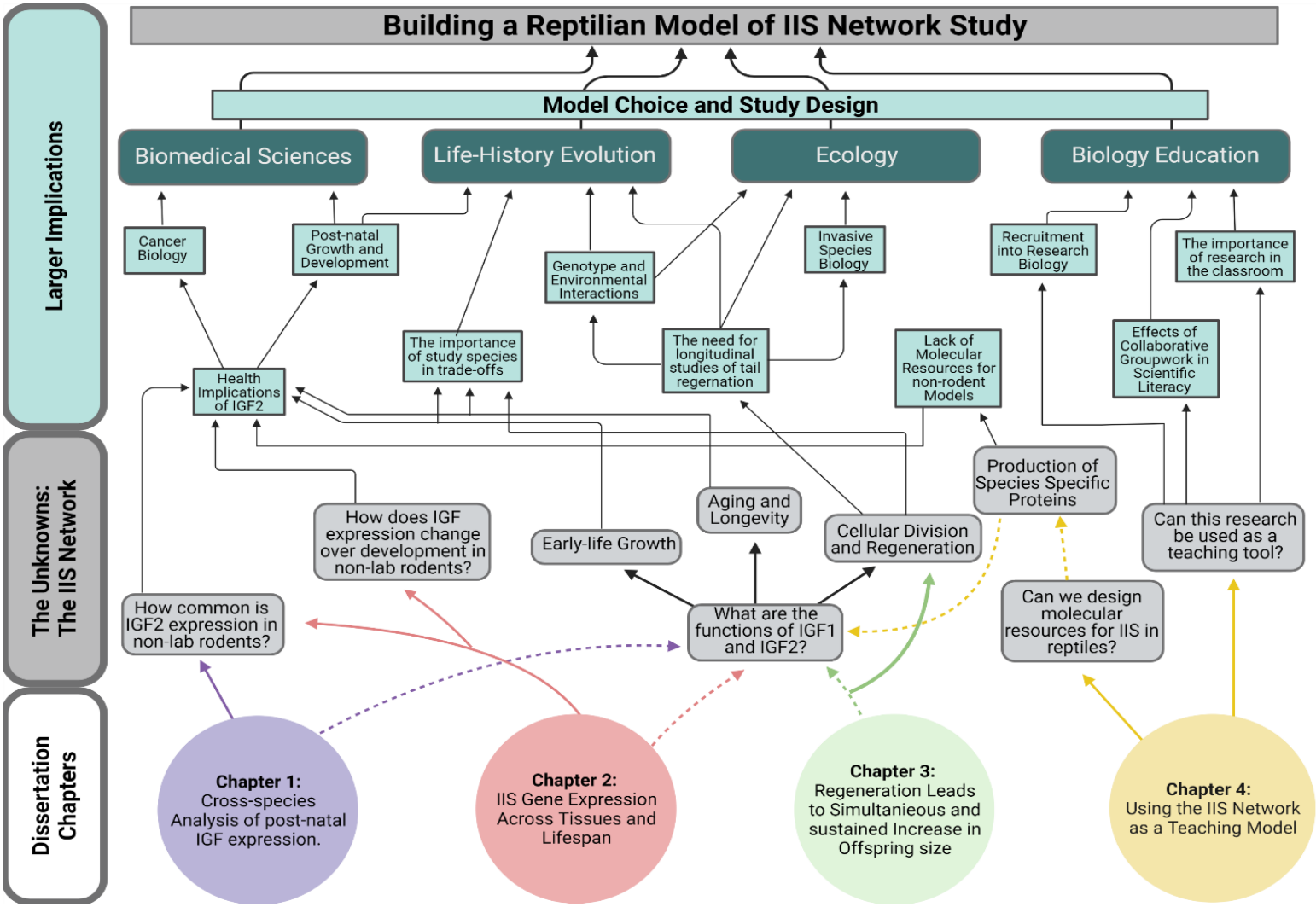

Figure 5.1: **Overview of Dissertation and Interconnectivity with Life Sciences**. Dissertation chapters are shown at the bottom, and are color coded by chapter. Arrows are then used to connect the chapters to questions within the field of IIS study. Solid arrows represent questions that are directly tested within the chapter, and dashed arrow represent questions which are directly related, but not directly tested by the chapter. The questions within the IIS network are then connected to larger questions within four main fields of biology: Biomedical Sciences, Life-history Evolution, Ecology, and Biology Education. Addressing these questions then leads to the importance of model choice and study design in IIS network study.

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