**Abstract**

1. The insulin-like signaling (IIS) network plays an important role in mediating variation in growth and other life-history traits in vertebrates. Although both insulin-like growth factor 1 (IGF1) and insulin-like growth factor 2 (IGF2) are key hormones in the IIS network, research on IGF2 has been largely neglected. This is likely because early biomedical research was conducted on rodent models, which do not express IGF2 after pre-natal development. Unfortunately, this historical bias has translated to research in functional ecology and the typical expression patterns of IGF2, particularly in postnatal life, across amniote species is virtually unknown.

2. Here we challenge this assumption and ask to what degree IGF2 is expressed during postnatal development and adulthood in amniotes (mammals, reptiles, and birds) by quantifying the relative liver gene expression of *IGF1* and *IGF2* using two approaches: (1) analysis of juvenile and adult liver RNAseq data from NCBI for 82 amniote species, and (2) qPCR on liver cDNA at embryonic, juvenile, and adult stages for two lizard, two bird, and two mice species.

3. The results of this cross-species comparison clearly demonstrates that *IGF2* is expressed in embryonic, juvenile, and adult life stages–often at a higher relative expression than IGF1–in nearly all amniotes tested, contradicting accepted patterns from laboratory rodent models. Further, we find evidence of adult sex-biased *IGF2* expression in some species, potentiating its role in sex-specific differences in growth, reproduction, and senescence.

4. We also found that outbred mouse strains across two families lack postnatal *IGF2* expression, consistent with the lab-selected inbred strains of mice and rats, indicating this pattern is due to phylogenetic placement not due to inbreeding or artificial selection for biomedical research.

5. Thus, our results clearly demonstrate that IGF2 expression is typical for amniotes throughout life and suggests that a comprehensive understanding of the mechanisms that mediate variation in growth and other life-history traits in vertebrates will require studies that measure IGF2 as well as IGF1.

**Keywords [In ABC order, no more than 8 and exclude words that are in the title]:** bird, mouse, gene expression, IGF1, insulin-like growth factor, liver, non-model system, reptile

**Data Availability**:

All data are available on NCBI or our GitHub. For the purpose of double-blinded peer-review, since the reviewers cannot have access to our GitHub, we upload as supplemental files an excel sheet with each data files as a tab (with the first as a readme) along with the code for analysis.

**Introduction**

“*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”*

*~ Classic Tales of Mulla Nasreddin, Farzad* (1989)

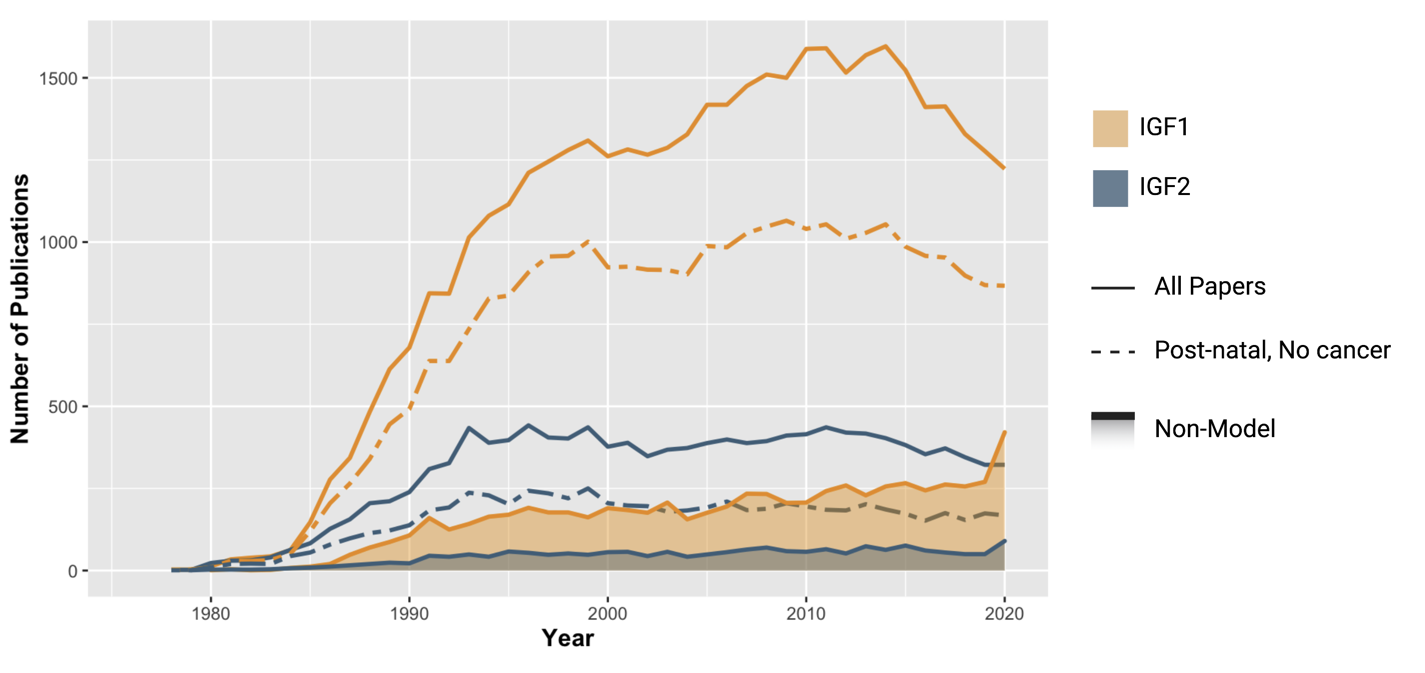
The fable above describes a phenomenon defined as the “street-lamp” effect (Freedman, 2010), where there is a tendency to search for answers where it is easy to look, which may not always be the correct place to search. Once a scientific discovery illuminates a hypothetical street-lamp, it often defines the focus of the research community, and inadvertently discourages researchers from searching outside the pool of light. This effect initiates 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; Kenyon, 2010; Papaconstantinou, 2009; Swanson & Dantzer, 2014). This network is activated by the paralogous hormones insulin, insulin-like growth factor 1 (IGF1) and insulin-like growth factor 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 these 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; Hiney et al., 1996; Stewart & Rotwein, 1996; Yakar & Adamo, 2012). Decreased signaling through the IIS network is associated with stress resistance and increased longevity (Ashpole et al., 2017; Greer et al., 2011; Holzenberger et al., 2003; Leroi, 2001; Tazearslan et al., 2011). IGFs were first studied in the laboratory rodent models, *Mus musculus* and *Rattus norvegicus*, (Rinderknecht & Humbel, 1976; Salmon & Daughaday, 1957), and it has since been established that *IGF1* is 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; 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 higher than 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, which 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 **(Figure 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 function and evolution of the mammalian placenta (Carter, 2012; 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 (**Figure 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 physiological ecology and functional genomics. While the levels of publication are significantly lower in species outside of rodents and humans, the proportion of studies on IGF1 remains consistently higher than IGF2 **(Figure 1; shaded areas)**. Recently, it has been documented that *IGF2* is expressed postnatally in reptile species (Beatty & Schwartz, 2020; Cox et al., 2017; McGaugh et al., 2015; Reding et al., 2016). In light of these results, here we ask, “In amniote vertebrates, is the postnatal expression of *IGF2* the exception, or the norm?”



**Figure 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 postnatal 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 reptiles, birds, and mammals, 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**). 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. Runs that had low numbers of cleaned 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 DataFile, Tab: MetaData\_Counts\_Cleaned; **Table 1**).

For visual comparison we present the level of *IGF1* and *IGF2* expression 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.

**Table 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 DataFile “MetaData\_Counts\_Cleaned” 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 | rhesus macaque: Transcript: IGF1-201 ENSMMUT00000065439.2  rhesus macaque: 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 | 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 |  |

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 [*Taeniopygia guttata*] and house sparrow [*Passer domesticus*]), two lizards (brown anole [*Anolis sagrei*] and Eastern fence lizard [*Sceloporus undulatus*]), and two rodents (house mouse [*Mus musculus*] and deer mouse [*Peromyscus maniculatus*]) 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 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

**Table 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 | 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%  R2= 0.996  E= 110.3%  R2= 0.998 |
| Juvenile - 4 Unk |
| Adult - (2M:2F) |
| house sparrow (*Passer domesticus*) | Juvenile - (4M:4F) | 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%  R2= 0.990  E= 116.7%  R2= 0.985 |
| Adult - (1M:3F) |
| Squamates | Eastern fence lizard  (*Sceloporus undulatus*) | Juvenile - 4 Unk | 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%  R2= 0.982  E= 103.0%  R2= 0.987 |
| Adult - 4M |
| brown anole (*Anolis sagrei*) | Embryonic - 4 Unk | 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%  R2= 0.993  E= 99.4%  R2= 0.997 |
| Juvenile - (2M:2F) |
| Adult - (2M:2F) |
| Mammal | house mouse  (*Mus Musculus*) | Embryonic$ - 4 Unk | 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%  R2=0.996  E= 101.8%  R2= 0.997 |
| Adult\* - (2M:2F) |
| deer mouse (*Peromyscus maniculatus*) | Juvenile# - 4 Unk |
| Adult# - (2M:2F) |

**$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.**

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 Files.

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, Figure 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*) (**Figure 2**). Strikingly consistent across all the three inbred and five outbred mouse strains, there was no postnatal expression of *IGF2* detected (**Figure 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, **Figure 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.

Diagram

Description automatically generated

**Figure 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 Files 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.

Chart

Description automatically generated

**Figure 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.

*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.

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) (**Figure 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) (**Figure 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) (**Figure 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) (**Figure 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. To do so, we used samples from inbred house mouse C57BL/6Jstrain 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 (**Figure 3b; Table 3**).

Diagram

Description automatically generated with low confidence

**Figure 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 see the relative expression levels more clearly 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 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 the IGF1R and insulin receptor to activate the IIS network (Denley et al., 2005). As such, both of these hormones may be critical for mediating variation in life history traits such as postnatal growth, reproduction and senescence, yet the focus has been on IGF1. The lack of IGF2 expression in rodents after birth has led to a research bias towards IGF1 in amniotic postnatal life and has limited the interpretation and understanding of the functional impacts of the IIS network. Here, we clearly demonstrate that *IGF2* is expressed during postnatal development in reptiles, birds and mammals. We also provide evidence that in some species IGF1 and IGF2 age-related expression patterns are sex specific. Taken together, these results suggest that examining variation in IGF2 alongside variation in IGF1 will be crucial for understanding the physiological mechanisms that mediate variation in growth and other life-history traits in vertebrates.

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 species, are an exception in the context of the amniote phylogeny. When examining the expression of the *IGF* hormones across the lifespan via quantitative gene expression analysis, we again confirmed that *IGF2* was expressed in 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* gene expression and IGF2 proteinin 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 IGF1and IGF2protein levels were similar three weeks post-hatching with IGF2remaining detectable at high levels in circulation through 20 weeks post-hatching (McMurtry, 1998). Similarly, in brown anoles *IGF2* was expressed at the embryonic, juvenile and adult stages (Beatty & Schwartz, 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 transcriptomic survey and qPCR results show distinct patterns in the *IGF1*:*IGF2* expression ratios among 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 examined (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 *IGF1* displayed extreme variation in expression levels (but not statistically different in mean expression to *IGF2*), while male *IGF2* expression was highly variable in the brown anole. Sex-specific expression of these hormones potentiate them having unique roles in regulating sex-specific differences in growth, reproductive status, and senescence. 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 rodent 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 nine rodent samples with publicly available RNAseq data, nor the postnatal samples collected from inbred laboratory house miceor outbred deer mice examined through quantitative gene expression displayed detectable *IGF2* expression. While the lack of mouse and rat postnatal *IGF2* expression is similar to what has been shown previously (Smith, CM et al., 2019; Yue et al., 2014), our results demonstrate persistence of this rodent pattern regardless of inbreeding status or evolutionary clade across rodent families (Muridae and Cricetidae). These 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 postnatal *IGF2* expression.

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) (**Figure 1**). Yet the limitations of biomedical rodent models have focused our attention on IGF1 while our level of understanding of IGF2 remains clouded. In consideration of the complexity of such a prolific physiological network, both evolutionarily and systematically, the concentrated focus on 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 across species and life stages. 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. 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 researchers to look beyond the current pool of light. We hope the results presented here, showing near ubiquitous postnatal expression of IGF2 across the amniote clade, encourages the functional ecology community to start talking about, and studying, IGF2.

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