PHYSIOLOGICAL MECHANISMS UNDERPINNING GROWTH AND AGING IN WILD

BIRDS

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ABSTRACT

Life-history trade-offs have been well-documented within the literature through correlational and experimental studies. However, the physiological mechanisms underlying these trade-offs are less understood. Currently, there is great interest in shared mechanisms, specifically endocrine mechanisms, that might underlie the variation in life-history traits. Insulin-like growth factor-1 (IGF-1) may be one shared mechanism that is particularly important. IGF-1 is a metabolic hormone that is part of a highly conserved insulin-signaling pathway known to influence multiple life-history traits including growth and longevity across taxa, however, little is known about these trade-offs outside of laboratory populations. This dissertation focuses on the role of IGF-1 as a hormonal mechanism underlying the life-history trade-off between growth and aging in wild birds. While the causes of aging are not fully understood, telomere dynamics (length and change in length) are a potentially important mechanism underlying lifespan.

To investigate the role of IGF-1 as a hormonal mechanism underlying the life-history trade-off between growth and aging in Franklin's gulls (*Leucophaeus pipixcan*) and house sparrows (*Passer domesticus*). In Franklin's gulls, dietary restriction reduced growth rate and IGF-1 levels but did not impact telomere dynamics. However, there was a significant negative correlation between IGF-1 levels and telomere length at the end of the post-natal growth period. In house sparrows, we found that nestling growth rates varied with respect to year, but IGF-1 levels did not. Telomere dynamics were not related to growth rates or IGF-1 levels, suggesting that during post-natal growth nestlings may be able to mitigate or even delay costs to later life stages. Finally, when exogenous IGF-1 was administered to house sparrow nestlings during the post-natal growth period, nestling growth was impacted but only in some years. Exogenous IGF-1 increased growth and final mass in 2016 and final mass in 2018. There was a trend suggesting

experimental birds had shorter telomeres in 2016. Similarly, in 2018, experimental birds had

significantly shorter telomeres than control birds. These effects were not observed in 2017,

suggesting that trade-offs between growth an aging might only be visible under certain

environmental conditions, which may vary with respect to year.

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CHAPTER 1: PHYSIOLOGY OF LIFE-HISTORY TRADE-OFFS: IS INSULIN-LIKE GROWTH FACTOR-1 AN IMPORTANT MEDIATOR?

Organisms are expected to allocate finite resources among competing life-history traits to maximize fitness [1,2]. In support of this idea, there is evidence both across and within species that investment in traits such as growth and reproduction often comes at a cost to longevity [1,2]. Across vertebrates, organisms tend to exist along a fast-slow life-history continuum, with species at one end displaying rapid development, high reproductive investment, and shorter lifespans and species at the other end exhibiting slower growth, lower reproductive output and greater longevity [3]. Within vertebrate species, there is also both correlational and experimental evidence that elevated investment in growth and/or reproduction often increases mortality and reduces lifespan [4–6]. Although these life-history trade-offs are well documented the underlying physiological mechanisms are less well understood [7].

Currently, there is great interest in how physiological mechanisms such as hormones integrate life-history traits and shape life-history strategies [8–10]. Hormones are signaling molecules that often have pleiotropic effects on morphological, physiological, and behavioral traits and underlie life-history trade-offs [11,12]. One hormonal mechanism that may be particularly important in this context is insulin-like growth factor-1 (IGF-1). IGF-1 is a polypeptide metabolic hormone that is part of a highly-conserved insulin-signaling pathway that is involved in regulating multiple life-history traits including growth, reproductive output, and lifespan in diverse organisms [10,13,14]. Recent cross-species comparisons in mammals and in birds suggest that in addition to mediating life-history trade-offs within species, IGF-1 integrates a suite of life-history traits along a fast-slow axis, which is expected to facilitate the evolution of traits along the axis (e.g., the evolution of a faster or slower life-history strategy), but constrain

the independent evolution of life-history traits orthogonal to the axis (e.g., the evolution of faster growth, greater reproductive output, and a *longer* lifespan)[15,16]. However, we still know little about the role of IGF-1 in mediating life-history trade-offs and shaping life-history strategies in most vertebrates, particularly outside of laboratory populations [10,17–19]. Here, we review what is known about IGF-1 in vertebrates, identify several research approaches that are likely to increase our understanding of the role that this conserved signaling pathway plays in shaping life-history trade-offs, and suggest how comparisons of IGF-1 across vertebrate groups could yield novel insight into the role of hormonal mechanisms in shaping the evolution of life-history strategies.

IGF-1: A highly conserved signaling pathway

IGF-1 is a metabolic hormone that is highly conserved across vertebrates and is one of three ligands in the insulin/insulin-like ligand signaling pathways (e.g. IGF-1, IGF-2, insulin) [10]. The secretion of IGF-1 is regulated primarily by the somatotropic axis or the growth hormone/IGF-1 axis ([20] Figure 1). In response to variation in life-stage and environmental factors (i.e., food abundance, temperature, and stressors) growth hormone releasing hormone (GHRH) is released from the hypothalamus, which then stimulates the secretion of growth hormone (GH) from the anterior pituitary. GH then enters the bloodstream and stimulates the production of IGF-1 by the liver [10,21–23]. Increasing levels of hepatic IGF-1 induce negative feedback, inhibiting the release of GHRH at the hypothalamus and GH at the pituitary [20]. IGF-1 was traditionally expected to only mediate the effects of GH, however it is now recognized that local production of IGF-1 also occurs independently of GH in non-hepatic skeletal and skin tissues, acting in both an autocrine and paracrine fashion [24,25].
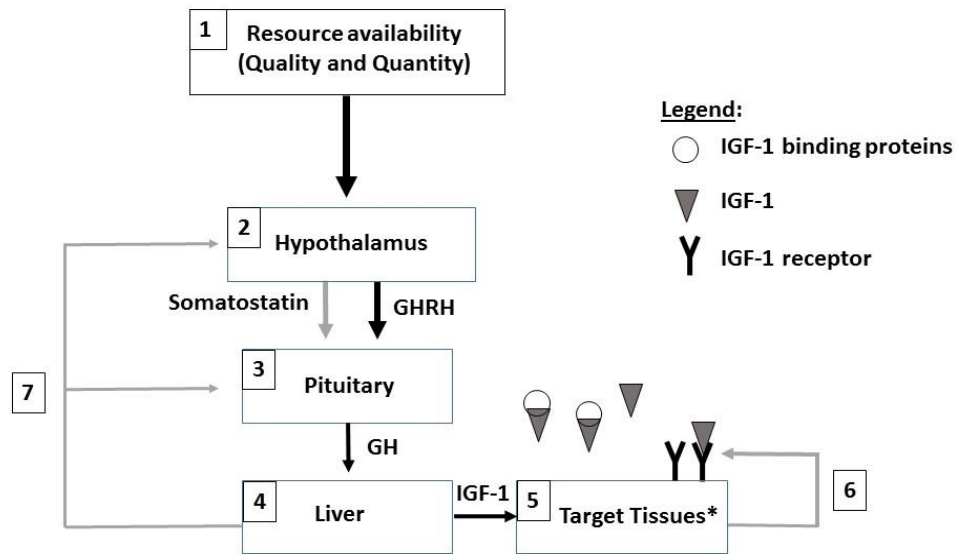
Figure 1. (1) Environmental factors (i.e. food abundance, temperature, stress etc.) and life-stage (2) induces the release of growth hormone releasing hormone (GHRH) by the hypothalamus, (3) stimulating the release of growth hormone by the anterior pituitary. (4) GH enters the bloodstream, stimulating production of insulin-like growth factor-1 (IGF-1) by the liver. (5) IGF-1 then enters the bloodstream exerting downstream effects on target tissues. While in circulation, bioactivity of IGF-1 is regulated by the actions of binding proteins, effects on IGF-1 can be inhibitory or excitatory (6) IGF-1 can be locally produced in certain tissues such as skeletal and skin tissue. (7) IGF-1 production is also subject to negative feedback, through increasing levels of hepatic IGF-1 which inhibit signaling by the hypothalamus and pituitary.

Activation of IGF-1 signaling is regulated through the actions of binding proteins, the IGF-1 receptor (IGF-1R), and the abundance of IGF-1 in tissue or circulation [26]. Most circulating IGF-1 (>90%) is bound to specific binding proteins [27], which have distinct roles in either enhancing or inhibiting IGF-1 actions by regulating the ability of IGF-1 to bind to receptors [27,28]. Binding proteins can transport IGF-1 to target tissues and extend the half-life of the molecule, protecting it from degradation in the bloodstream [20]. Once released from binding proteins, unbound IGF-1 can bind to insulin (INS), IGF-1, or IGF-2 receptors, although it binds to IGF-1 receptors (IGF-1R) with a much greater affinity [29].

Differences in the number and function of binding proteins have been identified across classes. For example, six IGF-1 binding proteins (IGF-1BP) have been identified in mammals [27], yet it is likely the avian equivalent of IGF-1BP6 is nonfunctional as a binding protein [18]. Furthermore, selection for differences in the IGF-1 sequence can alter interactions with binding proteins. For instance, the rate of IGF-1 sequence divergence is greater for reptiles (including birds) than mammals, with strong positive selection at sites that could be functionally important for binding affinities [18]. In turn, altered binding affinities could give rise to differences in IGF signaling between vertebrate taxa; for example, human IGF peptides (IGF-1 and IGF-2) have greater clearance rates than the avian IGF peptides, likely due to differences in interactions with binding proteins [30,31]. Together, these studies suggest that differences in binding protein interactions and receptor affinities may give rise to differences in IGF-1 signaling across vertebrates, which could have important functional consequences.

<div align="center">

IGF-1 and life-history traits

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IGF-1 and growth in vertebrates

Evidence both across and within species often demonstrates positive associations between IGF-1 levels and growth [10,14,17,32–34]. For example, research using knockout models suggests that IGF-1 signaling plays an essential role in both embryonic and postnatal growth in vertebrates [35–37]. Knockout mice with disrupted IGF-1 signaling experience significant reductions in circulating IGF-1 during embryonic stages, are significantly smaller at birth, and exhibit reduced postnatal growth [24,38–40]. Additionally, mice that are homozygous null mutants for both the IGF-1 gene and IGF-1 receptor (Igf-1 (-/-) and Igf1r (-/-) respectively, exhibit severe postnatal growth retardation and/or die at birth [38,41].

Positive associations between IGF-1 and growth are also often observed in artificial selection experiments [34,42–45]. For example, mice selected for elevated plasma IGF-1 exhibit increased growth and overall mass compared to individuals selected for low plasma IGF-1 [45]. Similarly, sheep and poultry selected for increased growth or development also exhibit elevated circulating IGF-1 levels compared to low growth selection lines ([36,45–47], but see [46]). Interestingly, in broilers (chickens selected for high meat production), the administration of exogenous IGF-1 during the post-natal growth period has mixed effects on growth ([47], but see [48]). Broilers are under intense artificial selection for maximal growth whereas other poultry breeds not selected for growth (i.e. White leghorns) and other wild birds show patterns more consistent with findings in mammals. For example, a single injection of exogenous IGF-1 in 7-day old white leghorn had altered muscle protein synthesis rates [49]. Likewise, free-living pied flycatcher (*Ficedula hypoleuca*) nestlings injected with IGF-1 exhibited faster growth, greater body mass, and longer tarsus length than controls [50].

There is also evidence that IGF-1 levels are influenced by environmental inputs (e.g., temperature, resource availability/abundance, stress) during development, which may allow individuals to plastically adjust growth rates. For example, in fish, environmental conditions during embryogenesis, such as hypoxia or arsenic exposure, can alter IGF-1 signaling, reducing growth [51,52]. Similar responses to environmental conditions during early development have also been observed in mammals. Nutritional restriction of maternal diet leads to reductions in circulating IGF-1 and IGF-1 signaling in offspring tissues and metabolism [53–55], potentially altering post-natal growth trajectories. Nutrition during the post-natal growth period has also been shown to alter IGF-1 signaling into adulthood [56].

IGF-1 and reproduction in vertebrates

Beyond growth, IGF-1 is essential for proper functioning of the reproductive systems and is often associated with the onset of reproductive events [17,32,57,58]. In vertebrates, IGF-1 stimulates the production of hormones involved in sexual maturation including gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) [59,60]. In both mammals and birds, IGF-1 promotes steroidogenesis, stimulating production of progesterone and estradiol by the ovary as well as inducing spermatogenesis and Leydig cell function in the testes [60–63]. In fish, IGF-1 signaling is also known to influence both reproductive hormone production and oocyte maturation [22]. The most direct evidence of the influence of IGF-1 of reproductive function comes from knockout mouse models in which targeted disruption of both copies of the IGF-1 gene or IGF-1R gene results in infertility [21,38–40,64]. Experimental interruption or repression of signals that stimulate IGF-1 production can also disrupt reproduction. For example, female mice that are homozygous for targeted disruption of the gene encoding the IGF receptor/IGF binding protein have reduced plasma IGF-1 and delayed puberty, parturition, and reduced litter sizes [41].

Correlative studies reveal positive associations between circulating IGF-1 and reproductive traits, such as age at first reproduction, reproductive stage, and reproductive output. [17,32,62,65,66]. For example, in brown house snakes (*Lamprophis fuliginosus*), IGF-1 peaks after first mating and declines just prior to egg laying [32]. In garter snakes (*Thamnophis elegans*) elevated IGF-1 concentrations were associated with enhanced reproductive output (total litter mass) [17]. Similarly, female mice selected for elevated plasma IGF-1 had greater mass, produced significantly larger litters, and had heavier mammary glands [58]. Within avian species, the specific links between IGF-1 levels and reproductive traits are still being

investigated. However, in chickens, IGF-1R is abundant within granulosa cells in the ovary and the concentration of this receptor increases with reproductive maturity [67]. Further, there is also evidence in chickens that certain IGF-1 genotypes may be predictive of egg productivity [68].

IGF-1 and longevity in vertebrates

Insulin-like peptides and insulin-like growth factors are also often associated with variation in lifespan in diverse taxa [13,69]. Among vertebrates, a reduction in IGF-1 signaling is generally associated with increased longevity [38,40,70,71]. Long-lived mouse models (calorie-restricted and Ames dwarf mice) share similar characteristics including reduced body size and lower plasma IGF-1 levels[72]. Further, a study of 31 inbred mouse strains found that IGF-1 was inversely correlated with median lifespan, with long-lived strains exhibiting lower levels of circulating IGF-1 [73]. Interestingly, mice with partial inactivation of IGF-1R in the brain exhibited reduced circulating IGF-1 and post-natal growth and longer median lifespan [40]. In humans, centenarians and their offspring have an overrepresentation of heterozygous mutations in the IGF-1R gene compared to individuals without a family history of unusual longevity [74].

There is also some evidence that variation in IGF-1 signaling is associated with cellular aging mechanisms such as oxidative stress and telomeres [75]. For instance, mice with lower levels of IGF-1 exhibit greater oxidative stress resistance than controls [38]. In addition, administration of exogenous IGF-1 induced telomere shortening in skin fibroblasts of humans with acromegaly, a condition that alters growth and reduces longevity [76]. Further, in humans, genome-wide association studies (GWAS) have revealed that genetic variation in the insulin/insulin-like growth factor and telomere maintenance pathways are associated with longevity [77]. Yet, whether this same relationship between IGF-1 and cellular aging exists in natural populations is unknown and is an important area of future research.

<center>**Future directions**</center>

Accumulating evidence suggests that IGF-1 is likely to be an important mechanism underlying life-history trade-offs and mediating variation in life-history strategies in vertebrates. A major limitation to our current understanding is that much of this information comes from a limited number of species often under controlled laboratory conditions. However, because IGF-1 is a signaling molecule that is responsive to environmental conditions and varies across the lifespan, a comprehensive knowledge of the role that IGF-1 plays in shaping life-history strategies will require studies examining the relationships between IGF-1 levels and life-history traits at different life stages and across environmental contexts in a wide range of species. Here we outline several approaches that we expect to be fruitful for increasing our understanding of the role of IGF-1 in mediating life-history strategies in vertebrates.

Natural variation

One approach that is likely to yield important insight into the relationships between IGF-1 and life history traits is measuring natural variation in these variables in free-living populations. Thus far, few studies have used this approach, but we highlight two recent ones here. Sparkman et al. [17] examined the relationship between IGF-1 levels in adult garter snakes (*Thamnophilis elegans*) from two different ecotypes that exhibit divergent life-history strategies. Snakes from the lakeshore ecotype generally show a faster pace of life, they grow more quickly, reproduce at a greater rate, and have reduced longevity compared to snakes from the meadow ecotype [78]. The relationship between IGF-1 and ecotype was somewhat complex and varied across years (see below for more detail), but lakeshore snakes with greater reproductive output consistently had greater IGF-1 levels than meadow snakes with lower reproductive output [32]. More recently, Lewin *et al.* [79] found that spotted hyenas (*Crocuta crocuta*) that had greater

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IGF-1 levels when they were juveniles were heavier and reached reproductive maturity at a younger age, but also had reduced longevity [79]. Taken together, these results are generally consistent with the idea that IGF-1 plays an important role in mediating differences in life-history strategies in free-living populations, but more studies are critically needed.

An important consideration for future studies will be at what life stage to collect hormone samples and some of this will be influenced by constraints of the organism, study design, or both. In the spotted hyenas (*Crocuta crocuta*), samples were collected during early life between 5.5-12.1 months of age, whereas in the garter snakes (*Thamnophilis elegans*), samples were collected in adulthood. In the hyenas, this age range was chosen because it is a time when the animals are old enough that they can be safely captured, but are still dependent on their mothers for food and thus are less likely to experience extreme resource fluctuations which can affect IGF-1 levels [80,81]. Interestingly, there is also evidence from other mammals that variation in IGF-1 levels during early life might be more strongly related to later life-history traits. For example, in mice IGF-1 at 6 months, but not at later life stages (12 and 20 months) was significantly negatively related to median lifespan [73]. Further, in pigs, IGF-1 levels during the juvenile period was a better predictor of body composition than levels measured in adulthood [82]. IGF-1 can also vary across life-history stages within adults. For example, in adult garter snakes, IGF-1 levels varied depending on reproductive status, and whether the animals were shedding, and accounting for these factors in the study design and analyses will be critical [17].

Another factor that is likely to be important is sex, particularly in species marked by strong sexual size dimorphism. For example, in species where males exhibit faster growth and greater muscle deposition than females, males are typically characterized by greater plasma IGF-1 levels [66,83,84]. There is also evidence that sexual dimorphism in lifespan is related to IGF-1

signaling. In humans, females tend to live longer than males and have reduced IIS (insulin/IGF-1 signaling) activity [85]. Further, in mice, females that are heterozygous for the IGF-1R live 33% longer than wild-type females, however, this lifespan extension was not observed in heterozygous males [38].

When the timing and level of IGF-1 signaling is strongly correlated between the sexes, it could potentially constrain the evolution of sex-specific optima of life-history traits influenced by IGF-1. Interestingly, a recent study in brown anoles (*Anolis sagrei*), a sexually dimorphic lizard in which males grow more rapidly and are larger than females, suggests that sex steroids may play an important role in modulating sex-specific differences in the expression of IGF-1 and IGF-2 genes during development [86]. During growth, males have greater expression of IGF-1 and IGF-2 genes and experimentally elevating testosterone in females during development increases the expression of genes in the IIS signaling pathway and results in more male typical growth patterns [86]. Interestingly, this pattern is not observed in mammals. For example, in ewes fed a phytoestrogen and given an estradiol -17β implant for 24 hours had increased circulating IGF-1 levels compared to controls [87]. There is also some limited evidence in birds that steroids play an important role in regulating sex-specific patterns of growth. For example, in turkeys, females are smaller than males and exhibit lower IGF-1 levels during the rapid, early growth period (1-7 weeks post- [44]) and in chickens, birds that are in the same family as turkeys, males that are treated with estrogen exhibit reduced IGF-1 levels [88]. Variation in glucocorticoids can also affect growth patterns by influencing IGF-1 signaling in fish, rodents and birds [99, 100, 101], which could also vary between the sexes.

Another important issue to consider when examining the relationship between natural variation in IGF-1 levels and life-history traits is that IGF-1 levels are dynamic and sensitive to a

range of environmental conditions including fluctuations in food availability, temperature, and stress exposure [56,90,91]. Thus, it will be essential to take this variation into account so that patterns between life-history traits and IGF-1 levels are not obscured. For example, in the garter snake study mentioned above, the relationship between IGF-1 levels and ecotype (i.e., fast lakeshore or slow meadow life-history) depended on interactions with date and body size, which likely reflected fluctuations in resource availability [17]. Moreover, given that hormones are expected to allow animals to plastically adjust their physiology and behavior to prevailing environmental circumstances, this flexibility in IGF-1 levels in response to environmental stimulus might also be an important source of variation that is intricately linked to fitness and life-history strategies rather than simply contributing confounding noise [92].

To measure IGF-1 flexibility, researchers could use a within-individual reaction norm and relate variation in the slope of IGF-1 levels across a relevant environmental gradient (i.e., low to high resource availability) or in response to an injection of its secretagogue, GH, to life-history traits [92]. Researchers could also examine changes in the speed of the IGF-1 response to an environmental variable or GH injection [92]. However, before using this approach researchers will first need to carefully characterize the time course over which IGF-1 changes in response to the environmental variables of interest or a GH injection in their chosen study species.

Although IGF-1 levels vary across life-history stages and in response to environmental variables, there is some evidence that IGF-1 levels are heritable [93] and have responded to selection for increased growth in several species [22,45,58]. Thus, there is good reason to expect that variation in IGF-1 levels can respond to selection, but this has yet to be studied in free-living populations and is an important area of future research. The heritability of IGF-1 levels could also be examined in long-term populations with pedigrees using animal models [94] and in some

cases, it may be possible to use historically collected blood samples. Exposure to previous freeze thaw cycles can negatively impact IGF-1 levels [95]. However, in the hyena study mentioned above, IGF-1 levels were measured in plasma samples that had been routinely collected as part of a long-term study and continuously frozen (some for over 20 years) and remained in excellent condition [79].

Experimental manipulation

Another approach that will compliment studies of natural variation and be essential for assessing the role of IGF-1 in mediating life-history trade-offs is experimental manipulations of IGF-1 levels. Both injections and mini-osmotic pumps have been routinely used to elevate IGF-1 levels in laboratory studies [62,96–101] and these techniques could also be readily adapted for work in the field. As mentioned earlier, Lodjak *et al.* [50] recently used daily injections of IGF-1 to experimentally elevate IGF-1 levels in growing, free-living pied flycatchers (*Ficedula hypoleuca*) and found that chicks with greater IGF-1 grew at a faster rate. Importantly, the long-term effects of experimentally manipulated IGF-1 levels on other life-history traits remains unknown and will be an important area of future study.

The pied flycatcher (*Ficedula hypoleuca*) study focused on experimental manipulations during post-natal growth, but it will also be important to examine the effects of experimental manipulations of IGF-1 levels on life-history traits at other life stages including pre-natal growth and in adulthood. For example, in both mammals and birds, there is evidence that mothers transfer IGF-1 levels to developing embryos [102,103]. In mammals, it is difficult to manipulate the physiology of the offspring during pre-natal development without also affecting the mother. However, in oviparous animals including fish, reptiles, and birds, numerous studies have successfully used direct hormone manipulations of egg constituents to investigate the functional

consequences of elevated hormone exposure during embryogenesis [104–107] . For example, this has been successfully demonstrated in chickens, where recombinant human IGF-1 was injected into eggs over the course of 2 weeks, resulting in alterations to chick growth [96]. In adults, it would also be possible to manipulate IGF-1 at a constant rate over a longer time period (up to at least 28 days) without the need for daily contact using mini-osmotic pumps [100]. Mini-osmotic pumps require minor surgery at implantation and removal but might work well for studies of free-living individuals.

Cross-species comparisons

Comparative studies can be used to assess whether the same patterns observed between IGF-1 and life-history traits within species also influence the evolution of life-history strategies across species. Currently, there is great interest in the role that hormones play in life-history evolution as they often have pleiotropic effects on suites of life-history traits [8–10,15]. At one extreme, selection that results in changes in plasma hormone levels could affect an entire suite of life-history traits sensitive to the hormone and facilitate the evolution of certain life-history trait combinations while constraining the evolution of others (i.e., phenotypic integration, evolutionary constraint, evolutionary conservation of hormone effects) [8–10,15]. At the other extreme, selection that results in changes in tissue sensitivity by changing hormone receptor densities and/or local hormone production would allow life-history traits to evolve more independently of one another (i.e., phenotypic independence, evolutionary potential, and no evolutionary conservation of hormone effects) [8–10,15].

When variation in plasma IGF-1 levels is related to a suite of life-history traits, it suggests that the independent evolution of those traits is potentially constrained. Swanson and Dantzer [15] recently tested this idea across mammals by examining the relationship between

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IGF-1 levels and several life-history traits related to development time, body size, reproduction and maximum lifespan. Generally, they found that species characterized by a faster pace of life had greater IGF levels than species with a slower life-history strategy. These interesting results suggest that changes in IGF-1 levels would facilitate the evolution of life-history traits along the axis (i.e., a faster or slower life-history strategy), but constrain the evolution of combinations of life-history traits orthogonal to the axis (e.g., selection to increase growth *and* longevity). These results are further corroborated by a more recent comparative study in birds that found that IGF-1 levels were negatively related to average lifespan and positively related to some measures of reproductive output, albeit in a more complex manner that interacted body mass [16]. However, there are also intriguing differences between these two studies that warrant further consideration.

One major difference between these two studies is the phylogenetic scope of the analyses, which influenced how much variation there was across species in many of the key life-history traits that were studied. The mammalian study included 41 species from 7 orders and 21 families, whereas the avian study included 63 species from 1 order and 22 families. The wider phylogenetic breadth of the mammalian study, made it possible for Swanson and Dantzer [15] to also examine the relationship between IGF-1 levels and the mode of development and they found that altricial species were characterized by greater IGF-1 levels than precocial species. Birds also exhibit diverse developmental modes that span the altricial-precocial spectrum, however because the avian study was restricted to passerines, an order that only includes altricial species, it was not possible to test whether this same pattern found in mammals is also present in birds and this should be investigated in future studies.

Another potentially important difference between these two studies is the degree of variation in body mass. Body mass is expected to influence many rate related processes (i.e.,

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metabolic rate, reproductive rate) and thus form an important constraint that plays a role in organizing species along the fast-slow life-history continuum [108,109]. In the mammalian study, body mass ranged from 0.023 kg in the house mouse (*Mus musculus*) to 3,180 kg in the Indian elephant (*Elephas maximus*), whereas in the avian study, body mass only ranged from 0.006 kg in the goldcrest (*Regulus regulus*) to 0.475 kg in the hooded crow (*Corvus cornix*). The relationship between IGF-1 levels and body mass was significant in both studies, but interestingly it was opposite in sign. IGF-1 was negatively related to body mass across mammalian species [15,110]. In contrast, IGF-1 was positively related to body mass across avian species, a pattern that is consistent with what is typically found within species [10]. It is unclear to what degree this seemingly divergent pattern between IGF-1 levels and body mass in mammals and birds is due to variation in study design versus potentially important biological differences.

In the mammalian study, IGF-1 appears to be more closely related to adult body mass, basal metabolic rate, and relative growth rate than the fast-slow life history axis itself [15]. This suggests that changes in plasma IGF-1 levels influence a species placement along the fast-slow life history continuum mainly through effects on these traits. Interestingly, a recent comparative study of mammalian life-histories found that in addition to body mass, several lifestyle factors define a second fast-slow life-history axis such that species with access to abundant and reliable food resources are characterized by a faster life-history, whereas species that have reduced death rates because they are volant, arboreal, or fossorial have a slower life-history [109]. However, we currently have no information about the relationship between IGF-1 levels and relative food abundance or lifestyle variables across species and this would be interesting addition for future comparative studies. There is also evidence, in birds that tropical species have lower mortality

than temperate species, which could also influence aspects of the pace of life [111] and it would be interesting to examine whether IGF-1 levels vary with latitude.

Another interesting extension of these two studies would be to examine the relationship between IGF-1 levels and life-history traits across these two vertebrate classes. For example, for a similar body size, some studies suggests that birds grow more quickly and age more *slowly* than mammals [112]. Birds also complete growth prior to reproduction, whereas mammals often have an extended development period that can overlap with and compete for resources during reproduction [113]. Because of differences in growth patterns, the independent evolution of certain combinations of life-history traits may be somewhat more malleable across mammals and birds than within classes. Alternatively, some comparative evidence suggests that both mammals and birds are organized along the same fast-slow life-history axis, with mammals exhibiting a faster overall life-history strategy than birds [3], which is consistent with the idea that IGF-1 could integrate the evolution of life-history strategies across mammals and birds as it does within classes.

Consistent with the idea that variation in IGF-1 signaling is involved in mediating differences in life-history strategies across mammals and birds, McGaugh *et al.* [18] found that although the core insulin/insulin-like signaling and target of rapamycin (IIS/TOR) network that includes IGF-1 was largely conserved across mammals and reptiles (a clade that includes birds), there were high levels of divergence and selection on extra-cellular hormones, receptors, and binding proteins within this network when compared to control genes [19]. Whether these changes in hormone signaling within this network map onto important transitions in life-history evolution within and across classes is currently unknown and will be an important area of future research. Expanding comparative analyses to also include reptiles (other than birds) would also

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make it possible to examine the relationship between IGF-1 and other important life-history transitions such as endothermy [19]. Given that hormone and receptor sequences vary among species, antibodies may bind inconsistently, and it will be essential for IGF-1 assays to be carefully validated for each species included in future comparative studies [19].

Conclusion

Growing evidence suggests that IGF-1 plays an important role in mediating life-history trade-offs and shaping life-history strategies both within and across vertebrates. Much of this research comes from a limited number of species often under controlled laboratory conditions, but recent studies in free-living populations generally support these observations. Given that IGF-1 is a signaling molecule that responds plastically to environmental conditions and changes across the lifespan, future research in a greater diversity of species at different life-stages and in diverse environments are critically needed, including studies that assess the relative importance of IGF-1 flexibility in contributing to these patterns.

Comparative studies in mammals and birds suggest that in addition to mediating trade-offs within species, variation in IGF-1 levels also contributes to the evolution of life-history strategies across species. Thus far, these studies suggest that changes in IGF-1 levels facilitate the evolution of species along a fast-slow life-history axis, but also likely constrain the independent evolution of some life-history trait combinations. Expanding these studies to examine the relationship between IGF-1 signaling (including receptor densities and binding proteins) and life-history traits across classes is likely to yield critical insight into the role of hormonal mechanisms in shaping life-history strategies across phylogenetic transitions.

In the following chapters, I will address questions regarding the relationship between IGF-1 and life-history traits in Franklin's gulls (*Leucophaeus pipixcan*) and free-living house

sparrows (*Passer domesticus*). Specifically, my dissertation focuses on the role of IGF-1 as a mediator between avian growth and aging. Here I provide preliminary information regarding the role of IGF-1 during growth and in cellular aging in a short and long-lived bird species.

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CHAPTER 2: EFFECTS OF GROWTH RATE ON INSULIN-LIKE GROWTH FACTOR-1 LEVELS AND TELOMERE DYNAMICS IN A LONG-LIVED SEABIRD

Abstract

Plastic modification of post-natal growth rate is essential to survival in developing organisms. Organisms may adjust their growth rate in response to a variety of environmental conditions. Yet, the physiological mechanisms that allow organisms to make these modifications and the costs that may underlie these modifications in growth rate are less well understood. IGF-1 is likely to be an important hormonal mechanism given its responsiveness to nutrition and role in post-natal growth. In the short-term, modification of growth may be beneficial, however, it may also incur long-term costs such as increased telomere attrition. Telomeres are repetitive, non-coding, protective caps on the end of eukaryotic chromosomes are thought to play an important role in cellular senescence. In this study, we experimentally altered growth rates by manipulating diet in Franklin's gull (*Leucophaeus pipixcan*) chicks. All chicks were fed *ad libitum* the first week post-hatch. At 7 days post-hatch food-restricted chicks were fed 60% of the control chick food intake for 10 days. After 10-days of restriction, all chicks were fed *ad libitum* for the duration of the study. Food-restricted Franklin's gull chicks reduced their growth rates significantly during restriction; however, structural growth did not differ between treatment groups, indicating our treatment did not impact body size, only individual growth rates. IGF-1 concentrations were significantly reduced in the restricted treatment group following the diet restriction. IGF-1 was positively related to body mass. There was no overall impact of treatment on telomere length, however, regardless of treatment group, IGF-1 concentrations were significantly negatively correlated with telomere length at day 40 post-hatch. Our findings

suggest that IGF-1 may mediate variation in growth in Franklin's gulls and influence telomere dynamics.

Introduction

Growing organisms often flexibly adjust their growth in accordance to prevailing environmental conditions. These modifications, for example, may be driven by predation pressure. For example, in birds, faster nestling growth rates minimize predation risk and within-nest resource competition [1,2]. However, equally important, is the ability of an individual to adjust growth based on variation in resources, for instance, by undergoing rapid compensatory growth following release from periods of inadequate nutrition [3]. Conditions experienced by growing organisms during early life can have significant impacts on an individual's development, growth, and physiology and long-term fitness [4–6]. However, the mechanisms which allow organisms to make these modifications and the underlying costs associated with growth modifications are less well understood.

In vertebrates, hormones influence suites of life-history traits which allow individuals to plastically adjust growth to prevailing conditions[7–9]. One hormonal mechanism that may be particularly important in this regard is insulin-like growth factor-1 (IGF-1). IGF-1 is a highly conserved, nutritionally responsive, metabolic hormone and has been posited as a mediator of plastic variation in growth [10]. IGF-1 is one of three ligands in the insulin/insulin-like signaling pathway (IIS) (e.g. IGF-1, IGF-2, and insulin), where secretion is primarily regulated by the somatotropic (growth hormone/IGF-1) axis in response to environmental conditions (i.e. stress, temperature, resource abundance etc.) [10–12] Several studies have demonstrated the importance of IGF-1 during the embryonic and post-natal growth periods in vertebrates. Experimental and correlational studies across vertebrates have found positive relationships between IGF-1 and

growth parameters [13–17]. For example, in captive populations of non-domesticated species IGF-1 levels are positively correlated with overall body size and overall growth rates [15,18]. Further, individuals selected for elevated IGF-1 levels exhibit increased growth rates [19]. In addition, knockout studies have shown that disrupted IGF-1 signaling during embryonic development results in smaller individuals at birth and reduced post-natal growth rates [20–22]. This effect is further amplified in mice with homozygous null mutations for the IGF-1 gene and IGF-1 receptor gene (Igf-1 (-/-) and Igf1r (-/-) respectively) in which individuals exhibit severe growth inhibition and/or die at birth [21]. In birds, injections of exogenous IGF-1 have positive effects on a range of growth parameters including muscle protein synthesis, muscle development, and overall growth rates [23–25].

While there may be clear advantages of rapid growth such as attaining adult body size to increase the chance of reproductive success, organisms are rarely observed growing at maximal rates. This may be because rapid growth can incur long-term costs[3]. For example, the increased metabolic activity associated with elevated growth rates may have cascading effects such as increased oxidative stress, which can damage macromolecules including DNA, thus accelerating biological aging [26]. Telomere length and loss rate (telomere dynamics) may represent a cost to modifications in growth as telomeres shorten in response to oxidative stress [26]. Telomeres are non-coding segments of DNA that form protective caps on the end of eukaryotic chromosomes, enhancing genome stability [27]. They shorten with each round of replication and, accordingly, telomere loss is greatest during periods of accelerated cell division [27,28]. In support of this, several longitudinal studies have demonstrated that telomere loss is greatest during early life when individuals are undergoing rapid growth and development [29–31]. Further, experimental elevation in growth rate has been demonstrated to increase telomere attrition [32]. In addition,

exogenous IGF-1 has been shown to shorten telomeres in skin fibroblasts[33], suggesting that IGF-1 may act as potential mechanism by which elevated growth incurs long-term costs.

In this study, we tested the hypothesis that IGF-1 is a hormonal mechanism by which individuals plastically modify their growth under food restriction and these modifications are costly. To experimentally modify growth, we manipulated food availability during post-natal development in Franklin's gulls (*Leucophaeus pipixcan*), a long-lived seabird [34]. Previously, this gull was found to have differences in embryonic growth phenotypes, in which late season chicks hatched earlier with shorter tarsi compared to early season chicks [35], thus we incorporated season in our experimental design. To manipulate food availability, we subjected early and late season chicks to a 40% food restriction over a 10-day restriction period. Thus, we predicted our diet manipulation would significantly reduce growth rate in food-restricted individuals. Further, those food-restricted individuals would have shorter telomeres and greater IGF-1 plasma concentrations during the alimentation period. Lastly, in this system, given that late season chicks have an overall 'faster' embryonic growth phenotype, we predicted late season chicks to have shorter telomeres and increased concentrations of IGF-1 compared to early season chicks.

Materials and methods

Study species

In May 2015, we collected freshly laid Franklin's gull eggs from breeding populations in Rush Lake, North Dakota. [35]. Early season eggs (n=25) were collected on May 7th, 2015, and late season eggs (n=25) were collected on May 25th, 2015. We only collected the first egg from each clutch. To ensure an egg was the first egg of a clutch, we only collected eggs from nests that contained a single egg. Incubation by both sexes occur after the laying of the second egg

[34]. The egg length (±0.1mm) and breadth (±0.1mm) were measured in the laboratory within 8 hours of collection. Eggs were then randomly assigned to an incubator maintained at 37.5° C and 65% relative humidity with automatic egg turners (Brinsea Mini EX high performance egg incubator).

Figure 2. Early and late season Franklin's gull (*Leucophaeus pipixcan*) chicks, approximately 7- and 17-days post-hatch. **Photo credit:** Aurelia Kucera

Incubators were then placed in one of two environmental chambers maintained at 24°C that differed only by photoperiod. Environmental chambers were equipped with full spectrum light bulbs set to timers. In one chamber, early season eggs were incubated under a short-day photoperiod (14:10 light: dark 24-h cycle) and in the other chamber late season eggs were incubated under a long day photoperiod (18:6 light: dark). We selected these photoperiods following established methods [35]. After 21 days of incubation, eggs were removed from automatic turning and checked daily for signs of hatching. After hatching, chicks were immediately removed from the incubator, weighed, assigned to either a control or restricted treatment group, and placed in a brooder for approximately 12 hours before moving to their respective groups. Early season sample sizes totaled n = 10 control and n = 10 restricted birds. Late season sample sizes totaled 9 control and 9 restricted birds.

Husbandry

Gull chicks were raised in plastic bins (~ 3sq.ft) in groups of 3 to 4 individuals. Plastic bins were fitted with rubber mats to prevent slipping and aid in sanitation. Heated floor mats and a one heat bulb (120W) were attached above each bin. The temperature of each bin was monitored daily during the first week and supplemental heat was provided for approximately 2 weeks until chicks could thermoregulate at room temperature.

Throughout the duration of the study, birds were fed a high protein diet consisting of kitten food (Royal Canin, Mother & BabyCat©) moistened with water. During th first week post hatch, all chicks were fed *ad libitum*, by hand, until they were able to feed themselves. To estimate food intake, chicks were weighed before and after each feeding and the difference in mass was recorded. Chicks were fed daily every 2 hours from 7:00 am to 7:00 pm totaling 6 feedings per day. All chicks had constant access to fresh water.

Diet manipulation

At the start of the second week post-hatching, chicks were exposed to either an experimental food restricted or a control treatment. In the food restricted group, chicks received 60% of the *ad libitum* food intake (a 40% restriction) for 10 days. Chicks were weighed before and after each feeding to the nearest 0.1g to determine food intake. To determine how much food (g) restricted chicks received at each feeding we used 60% of the food intake from the oldest control group. A new amount was calculated for each feeding using the same control group throughout the duration of the restriction. After the 10-day restriction, the restricted treatment group resumed *ad libitum* feeding until the end of the experiment. Control chicks were *ad libitum* throughout this experimental period. We did not observe any severe health problems due to diet restriction.

Sample collection

      Growth measurements were collected from all chicks at 7 a.m. prior to feeding on days 7,

17, 27, and 40 post-hatching. At all sampling points, we measured mass to the nearest 0.1g with

a digital scale, culmen length and wing chord were recorded to the nearest 0.1cm. Fasted plasma

samples were collected from chicks on days 7, 17, 27, and 40 post-hatching. All samples were

collected prior to the first feeding. Blood was immediately stored on ice upon collection. All

samples were then centrifuged at 10,000 x g for 10 minutes, plasma removed, and stored at -

80°C within 30 minutes of collection until further analysis.

Hormone measurements

      We analyzed total IGF-1 concentrations using an enzyme-linked immunosorbent assay

(ELISA) multispecies IGF-1 kit from Immunological and Biochemical Test Systems GmbH

(distributed by Eagle BioSciences). This kit has been validated for chickens and has a relative

binding of 70%. We validated the multispecies IGF-1 kit for Franklin's gulls using serial plasma

dilutions and a modification of the kit protocol. Samples were assayed in duplicate. The inter-

assay and intra-assay variation were 2.2% and 2.1% respectively. The sensitivity of the assay

was 1ng/mL.

Telomere measurements

      We extracted DNA from red blood cells using Macherey Nagel Nucleospin Blood kits

following the manufacturers protocol (Genomic DNA from Blood). DNA concentration and

purity were assessed using the Nanodrop 8000 (ThermoScientific). Samples with 280/260 and

260/230 ratios above 1.8 were used for telomere analysis. Relative telomere length was

measured at day 7,17, 27, and 40 post-hatch for each treatment group using quantitative

polymerase chain reaction (qPCR), and validated for Franklin's gulls on an Mx3000P

(Stratagene) [36,37]. We calculated the relative length (T/S) of the samples as the ratio of the telomere repeat copy number (T) to that of a single copy control gene (S), relative to the reference sample.

We used the following gull-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward and reverse primers as the single copy control gene (Integrated DNA Technologies): 5'-CGGAGCACCGCTTACAATTT-3' and 5'-GCATCTCCCACTTGATGTTG-3' respectively. The following primers were used to measure telomeres: TEL1b: 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' and Tel2b: 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'. The qPCR program for the GAPDH gene was as follows: An initial denaturing step at 95°C for 10 minutes was followed by 40 cycles 30s at 95°C, then 30s at 60°C. For telomeres, an initial denaturing step at 95°C for 10 minutes was followed by 27 cycles for 15s at 95°C, 30s at 58°C, and 72°C for 30s.

Reactions for GAPDH and telomeres were run on separate plates. The number of PCR cycles (C_t) required for the products to accumulate enough fluorescence to cross a threshold was determined. Thus, individuals with relatively long telomeres are characterized by low C_t values and individuals with relatively short telomeres are characterized by high C_t values. The reaction conditions for each were as follows: 20ng of DNA in a final volume of 25µl containing .25µl forward and reverse primer, 12.5µl SYBER green Master Mix (Quanta Bio), 6µl water, and 6µl DNA sample. Each sample was run in triplicate and the average values were used to determine the T/S ratio. To determine the T/S ratio, we used the following formula: $2^{\Delta\Delta CT}$, where $\Delta\Delta CT = (C_t^{Telo} - C_t^{GAPDH})$ reference $- (C_t^{Telo} - C_t^{GAPDH})$ [38]. Water was used as a non-template control sample on each plate. The average intra-assay variation for the ΔCt values for the GAPDH

assays were .21% and 1.05% for the telomere assays. The average inter-assay variation for the

ΔCt values was 6.85%.
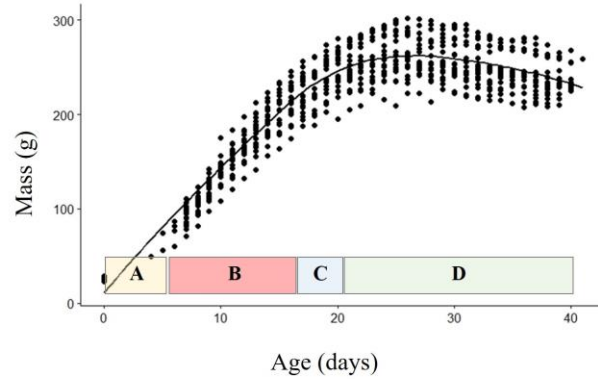
Figure 3. Chick growth and feeding periods for Franklin's gulls (*Leucophaeus pipixcan*). (A) Pre-restriction period: 0-7 days post-hatch. (B) Restriction period: 7-17 days post-hatch. (C) Alimentation period: 17-20 days post-hatch. (D) Post-restriction period: 20-40 days post-hatch.

Statistical analysis

To assess the impact of the treatment on chick growth we calculated growth rates within

distinct feeding periods. Feeding periods were defined as: restriction, alimentation, and post-

restriction following. The restriction period lasted from the start of the restriction treatment (i.e.

day 7 post-hatch) to 10 days later (i.e. day 17 post-hatch). The alimentation period was defined

as the end of the restriction period to 3 days later [39]. Finally, the post-restriction period lasted

from the end of the alimentation period until the end of the experiment (i.e. day 40 post-hatch)

(figure 3). Within these feeding periods we calculated median instantaneous daily growth rate

and median absolute daily growth rate for each individual. Instantaneous daily growth rate was

calculated as follows: $ln\left(\frac{Mass_i+1}{Mass_i}\right)$, where i represents the age of the individual at a specific day.

Daily absolute growth rate was calculated as $Mass_{i+1} - Mass_i$. We then, used a linear mixed

effect model (LMM) to assess the relationship between absolute and instantaneous median

growth rates and treatment, where median growth rate was treated as the dependent variable. Feeding period, season, and sex were included as fixed effects and nest ID was included as a random effect with chick ID included as a repeated variable. The *lsmeans* function in the R package *lsmeans* was used to run Tukey's HSD post-hoc pairwise comparisons[40].

To evaluate the impact of treatment on overall body size, we measured several structural growth characteristics including culmen length, wing chord, and head to bill length taken specific sampling periods (day 7, 17, and 27 post-hatching). To reduce the dimensionality of the structural growth variables, structural growth was defined as the first principal component from a principal component analysis (PCA) of the structural measures (culmen length, head to bill length, and wing chord length) of chicks taken at specific sampling periods: Pre-restriction (day 7), Restriction (day 17), and Post-Restriction (day 27). PC1 explained 96.4% of the variance (table 1.). We then used an LMM to evaluate the relationship between treatment and structural growth (PC1), where PC1 was the dependent variable. Treatment, mass, age, sampling period, season and sex were included as fixed effects with nest ID and chick ID included as random effects.

We examined the potential effects of treatment on IGF-1 levels at four separate sampling period (i.e. day 7, 17, 27, and 40). To assess the impact of treatment on IGF-1 levels, IGF-1 levels were treated as the dependent variable. Treatment, mass, sex, sampling period, and season were all included as fixed effects and chick ID and nest ID were included as random effects. We also examined the effect of treatment on telomere dynamics (length and change in telomere length). Telomere length was treated as the dependent variable with treatment as a fixed effect. In addition to treatment we also included initial telomere length, age, sampling period (i.e. day 7, 17, 27, 40), sex, and season as fixed effects. Chick ID was included as a random effect. We also

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examined the impact of maximum instantaneous growth rate on overall change in telomere length. Change in telomere length was calculated as: (telomere length at sampling point 4 (day 40) – telomere length at sampling point 1 (day 7)). In this model change in telomere length was the dependent variable and treatment, sex, and season were included as fixed effects with nest ID as a random effect. We used Pearson's correlations tests to determine whether IGF-1 levels and telomere length were correlated across sampling periods.

Data were analyzed using R v.3.2.3 (R Development Core Team 2015) statistical software. The *lmer* function in the R package *lme4* was used to run LMM models [41]. To meet LMM model assumptions for normality, all hormone and telomere data were log transformed. The *lsmeans* function in the R package *lsmeans* was used to run post-hoc pairwise comparisons[40]. The PCA was performed using SPSS 24.   In every LMM model, individual chicks (early season: n=19; 10 control, 10 restricted, late season: n=20; 9 control, 10 restricted) values for each treatment group (n=2) were used as independent data points.

Table 1. Loadings for PCA on structural growth variables and the percentage of structural growth variation explained by the PC axis

Structural Growth Variables	*PC1*
% of variance exp.	96.4
Culmen length (mm)	.991
Head to Bill Length (mm)	.962
Wing chord length (mm)	.973

Table 2. Linear mixed effect models with body mass as the response variable

Sources of variation	SE	df	F	p-value
Pre-Restriction: 7 days post-hatch				
Treatment	2.77	10.17	.05	.82
Season	3.10	10.17	.51	.48
Sex	2.16	32.03	19.24	.90
Age	2.81	32.94	.013	<.001*
Nest (random effect)				

Results

Growth manipulation

Prior to the start of the restriction period (day 7 post-hatch), body mass did not differ significantly between treatment groups ($F_{1,10.17}=0.428$, $p=0.82$, table 2.) nor in chicks produced from eggs collected during the early or late season ($F_{1,15.12}=0.51$, $p=0.48$, table 2.). During the restriction period, there was a significant effect of treatment on chick median instantaneous ($F_{1,9.02}=40.5$, $p<0.001$, figure 4) and absolute growth rates ($F_{1,9.04}=41.6$, $p<0.001$, figure 4). During the 10-day restriction period (days 7 to 17 post-hatch), diet restricted chicks had significantly reduced growth compared to controls (*Tukey's HSD*, $p<0.001$, figure 4).

Whereas, during the alimentation period (i.e. the defined 3-day period after restriction), chicks in the diet restricted treatment group increased their median instantaneous (*Tukey's HSD*, $p<0.001$) and absolute growth rates (*Tukey's HSD*, $p<0.001$) relative to controls. However, median instantaneous and absolute growth rates during the alimentation period never surpassed those of the control group during the restriction period (*Tukey's HSD*, p=.74) and (*Tukey's HSD*, p=.69). Median instantaneous and absolute growth rates during the post-restriction period did not significantly differ between groups (*Tukey's HSD*, *p=.59*) and (*Tukey's HSD*, *p<.001*, figure 4.). In addition, structural growth measures (PC1) did not differ significantly between treatment

$F_{1,10.73}=0.0017$, p=0.96) or seasons ($F_{1,10.01}=2.20$ p=0.16) at any of the sampling periods (i.e. re-restriction, Restriction, Post-Restriction). Regardless of treatment group, there was a trend for males to be overall structurally larger than females ($F_{1,29.16}=3.25$, p=0.08).

IGF-1

Prior to manipulation, IGF-1 levels did not differ between treatment groups *Tukey's HSD post-hoc t_{134} = .586, p=.99*, CI$_{95}$ = 3.74-7.53, figure 5). IGF-1 levels were significantly lower in restricted chicks after the restriction period (*Tukey's HSD*, p=.03, figure 4). Further, IGF-1 levels and body mass were significantly positively related, where larger individuals had greater concentrations of IGF-1 ($F_{1,134}=6.90$, p=0.009) and females, that are smaller than males, also had overall lower levels of IGF-1 compared to males ($F_{1,134}=8.57$, p=0.004). Season did not have a significant effect on IGF-1 concentrations ($F_{1,134}=0.039$, p=0.84).

Telomere dynamics

There was no effect of treatment on overall telomere length ($F_{1,30.73}=.809$, p=0.37). In addition, there was no effect of treatment on overall change in telomere length ($F_{1,8.11}=.809$, p=0.53). However, late season chicks had longer telomeres compared to early season chicks ($F_{1,33.21}=4.73$, p=0.03, figure. 6a). Telomere length was not related to maximum instantaneous growth rate ($F_{1,31.66}=.793$, p=0.37). However, there was a trend indicating that maximum instantaneous growth rate was associated with greater positive change in telomere length ($F_{1,27.34}=3.76$, p=0.06). Lastly, we also observed a significant negative correlation between IGF-1 concentrations and telomere length chicks regardless of treatment at day 40 post-hatch (r=-0.38, p=0.02, figure. 6b).
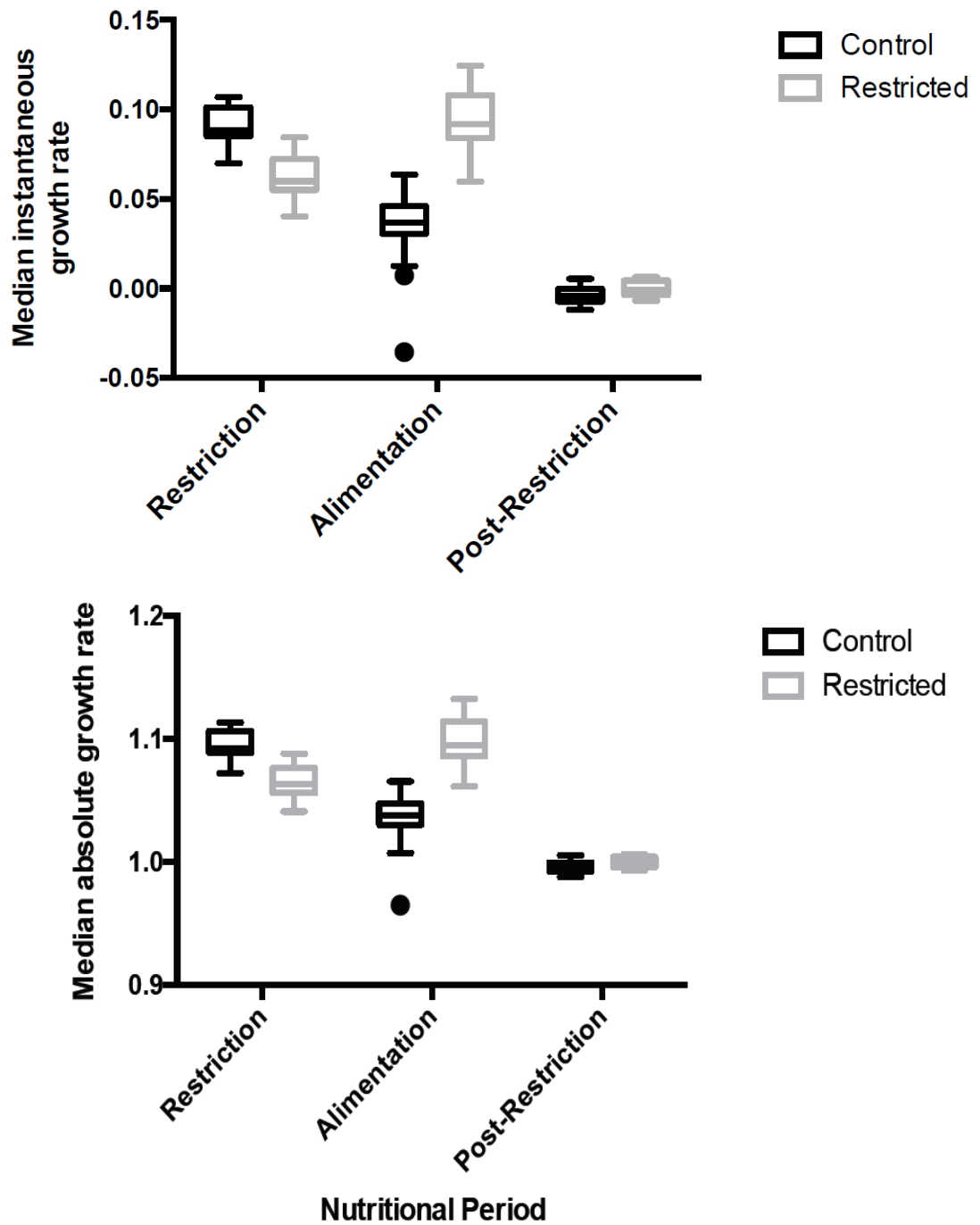
Figure 4. Median instantaneous and absolute growth rates during the selected feeding periods in Franklin's gull chicks (mean±SEM).
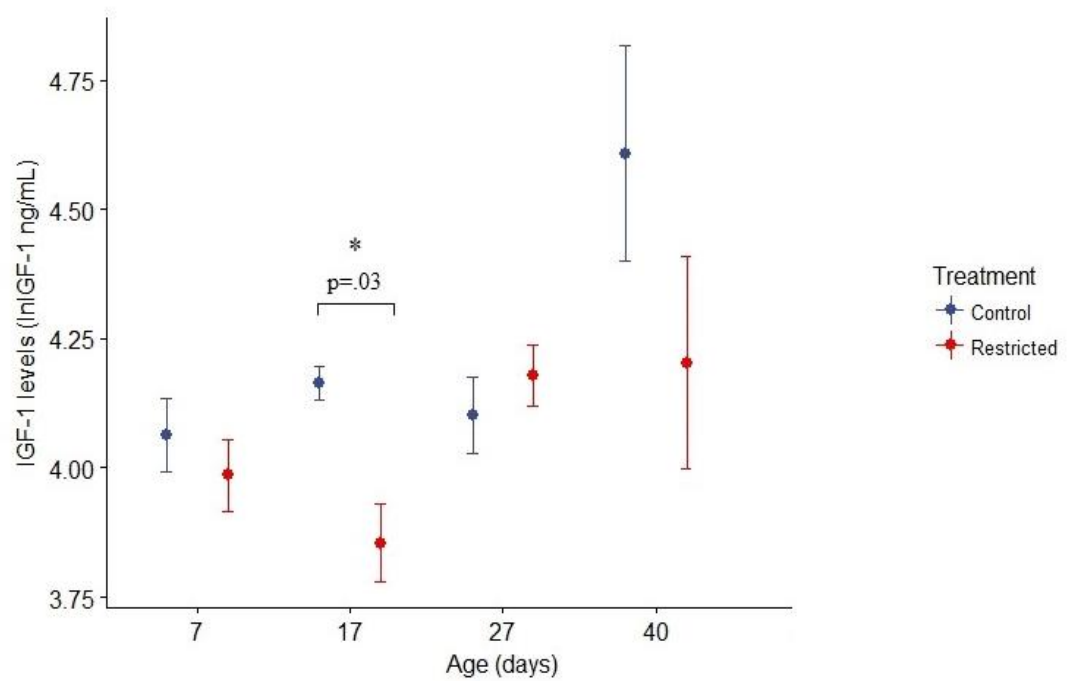
Figure 5. Relationship between IGF-1 levels and age in gull chicks. IGF-1 was measured at four different sampling periods: Pre-restriction (day 7), Restriction (day 17), Post-restriction (day 27) and a final measurement (day 40). IGF-1 levels between treatments did not differ at day 7 (P=.99). Restricted birds had lower IGF-1 levels at day 17 compared to controls (P=.03). IGF-1 levels did not differ between treatments at day 27 (P=1.00) and day 40 (P=.10). P-values are based on Tukey's HSD post-hoc test and significant differences, *P<.05.
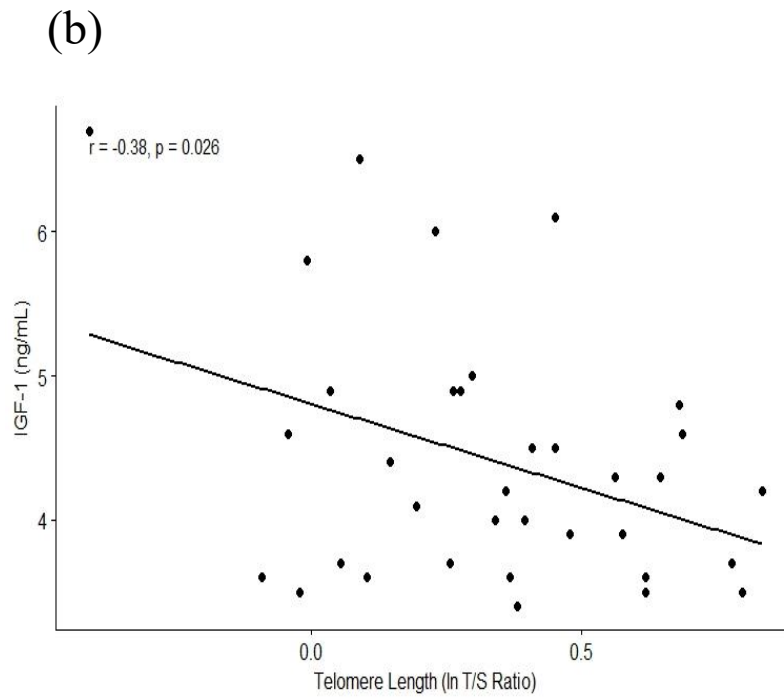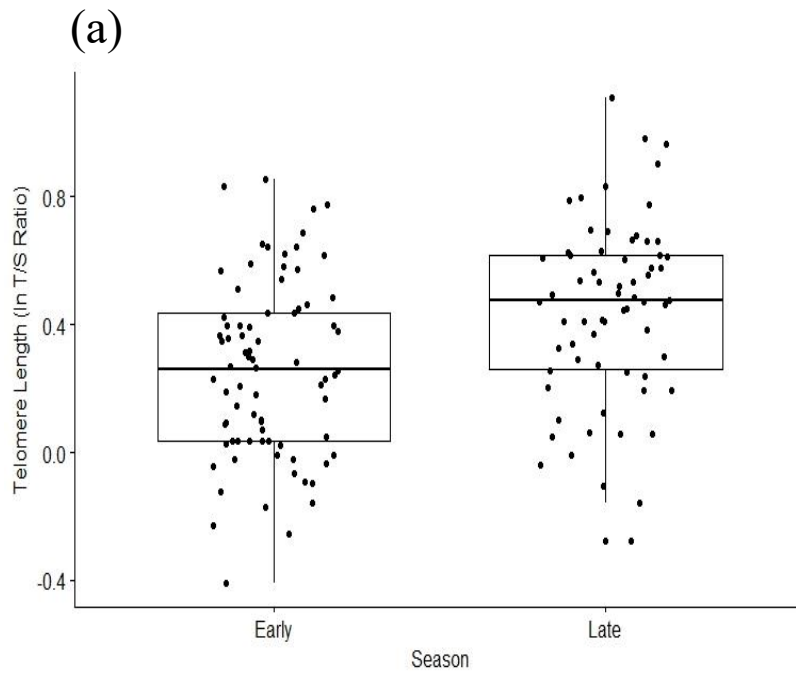
Figure 6. (a) Relationship between telomere length (ln T/S ratio) and season. (mean±SEM). (b)Correlation between telomere length (ln T/S ratio) and IGF-1 concentrations at day 40 post-hatch in early and late season Franklin's gulls.

Discussion

The diet manipulations significantly reduced growth rates during the restriction feeding period (figure. 2). However, median growth rates of restricted individuals during the alimentation feeding period never surpassed that of the control chicks during the restriction feeding period, suggesting that individuals in the restricted treatment group did not undergo compensatory growth[39]. As predicted, during the alimentation period, chicks in the restricted treatment group had increased median growth rates during the alimentation period, however median growth rates did not differ between treatments during the post-restriction period (figure 4.). Further, structural growth measures (PC1) did not differ significantly between treatments (table 1.), indicating that while individuals in the restricted treatment lost a substantial amount of mass during the restricted period, they were able to maintain normal structural growth. Chicks may have been able to maintain structural growth in line with controls during the restriction period by assimilating organ and muscle tissue [42,43].

At 7 days post-hatch, prior to restriction, there was no difference in IGF-1 concentrations between treatment groups (figure 5). However, after the 10-day restriction period (day 17 post-hatch), restricted individuals had significantly lower concentrations of IGF-1 compared to controls (figure 5). Such reductions in IGF-1 concentrations following food-restriction are well-documented within the literature across vertebrates [44–48]. We also observed a positive relationship between body mass and IGF-1 concentrations, where heavier individuals had greater concentrations of IGF-1 compared to smaller individuals. Previous studies have reported similar results across vertebrates [49,50]. We did not observe any differences in IGF-1 concentrations in relation to season, differences in mass or structural growth were observed in relation to season through the duration of the study (table 1,2).

There was no effect of treatment on telomere length or change in telomere length. This could suggest that the period over which our study took place was not sufficiently long enough for telomere attrition to occur. However, many studies have detected telomere loss within 30 days or less [51–53] including a study in black-backed gull chicks [54]. Further, in birds, the lifespan of red blood cells is estimated at 30-45 days, depending on the species [55], suggesting that if attrition occurred, we should have been able to measure it. Telomerase, an enzyme responsible for adding DNA to the end of telomeres, acts to reset telomere length at fertilization [56,57]. However, while telomerase activity is expected to be down-regulated in post-mitotic somatic tissues following embryonic development [58], there is evidence in long-lived birds indicating that telomerase activity can be maintained throughout life [59], and may be one reason why telomere loss did not differ between treatment. Further, when selecting individuals for this experiment, we targeted the first laid egg in each clutch. In European storm petrels (*Hydrobates pelagicus*), a pelagic seabird, nestlings reared under more favorable conditions experienced little change in telomere length, compared to those reared in unfavorable conditions [60]. Therefore, it may be that the duration and severity of our treatment, while it resulted in a significant reduction in mass and growth rate (figure 4), was not sufficiently long or taxing enough to effect telomere length. It also may be that since gulls are a long-lived seabird, and they may be able to modify their growth within a specific range that does not induce long-term costs such as telomere attrition.

Interestingly, late season chicks had significantly longer telomeres compared to early season chicks, regardless of treatment (figure 6a). Previous work in Franklin's gulls have suggested that there may be differences in maternal investment in egg constituents, impacting offspring development [35]. Thus, it is plausible that differences in maternal investment in egg

constituents (i.e. hormones, carotenoids, antibodies) contributed to differences in telomere lengths by season. Differences in yolk constituents during embryonic development have been shown to impact telomere dynamics. For example, domestic chickens (*Gallus domesticus*) exposed to increased concentrations of glucocorticoids during embryonic development had shorter telomeres compared to control birds [61]. However, more data regarding specific yolk constituents and how they vary across the season in Franklin's gulls are needed to understand their impacts on telomere dynamics.

In addition to egg constituents, parental age might also impact late season telomere length. In several species, older individuals lay earlier in the breeding season compared to younger individuals [62–66]. Further, there is evidence to suggest that offspring produced by older parents have shorter telomeres and increased telomere attrition compared to younger parents [67,68]. In our study, late season eggs were collected approximately 3 weeks after early season eggs. It is plausible in this scenario that late season eggs were produced by younger parents, which later impacted overall offspring telomere length.

While treatment itself did not impact telomere length, IGF-1 concentrations were significantly negatively correlated with telomere length at day 40-post hatch (figure 6b). That is, individuals with greater concentrations of IGF-1 had shorter telomeres at day 40 post-hatch, suggesting that IGF-1 may influence cellular aging in through its effects on telomere dynamics. This is in line with findings from Matsumoto *et al.* 2015 which demonstrated that exogenous insulin-like growth factor-1 induced telomere shortening in skin fibroblasts in humans with acromegaly, a condition that alters growth and reduces longevity, suggesting IGF-1 may play an important mechanistic role in influencing telomere dynamics specifically during periods of rapid growth. Though our manipulation was insufficient to induce compensatory growth, IGF-1 levels

were significantly lower during the restriction feeding period. The mechanism by which IGF-1 induces telomere shortening is unclear, however, there is some evidence to suggest that activation of the IGF-1 pathway may enhance reactive oxygen species (ROS) production, ultimately damaging telomeres[33,69].

Overall, our results suggest that IGF-1 may act as a hormonal mechanism by which individuals can plastically modify their growth when restricted. In addition, IGF-1 may also play an important mechanistic role in influencing telomere dynamics. Thus, IGF-1 may be an important shared-mechanism that underlying variation in life-history traits.

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CHAPTER 3: NATURAL VARIATION OF IGF-1 AND TELOMERE DYNAMICS ACROSS MULTIPLE YEARS IN HOUSE SPARROW NESTLINGS

Abstract

Post-natal growth is a life-history trait that often has important fitness consequences. Individuals may increase growth to maximize survival, but faster growth may incur long-term costs. Endocrine pathways often enable organisms to integrate and respond to environmental cues, yet the physiological mechanisms that underlie variation in growth and associated costs are less well understood. Insulin-like growth factor-1 may be one potential hormonal mechanism underlying variation in growth and contribute to the long-term costs of growth through its effects on cellular aging. Telomeres are protective, non-coding caps found on the end of eukaryotic chromosomes that are thought to play an important role in cellular senescence. In this study, we examined the relationships between IGF-1, growth rate, and telomere dynamics across three years in a free-living population of house sparrows (*Passer domesticus*). We measured nestling IGF-1 levels, growth, and telomeres during the post-natal growth period (day 2 – day 10 post-hatch). IGF-1 was sampled at day 6 and day 10 post-hatch. Telomere measurements were sampled at day 2 and day 10 post-hatch. Nestling growth rates differed among years, however, IGF-1 levels did not. Mass was weakly positively related to mean IGF-1 levels and male nestlings tended to have greater IGF-1 levels than female nestlings. Lastly, telomere dynamics (change in telomere length, and telomere length at day 10) were not related to either growth rate or IGF-1 levels, suggesting that individuals may be able to mitigate, or delay costs associated with fast growth.

Introduction

Post-natal growth is an important life-history trait strongly linked to survival and reproductive success across vertebrates[1]. Increasing growth rates may be one way individuals can minimize time spent in vulnerable stages of development to maximize survival and reproductive success[1,2], however, fast growth can be constrained by physiological or environmental costs[3]. For example, differences in resource quality or quantity can cause substantial variation in offspring growth rates [4–7], however, rapid growth may incur long-term costs on individual reproduction, immunity, and senescence [8–13]. In recent years there has been much interest in identifying and understanding how physiological mechanisms mediate variation in life-history traits [13–17]. Endocrine pathways transduce information from environmental cues [13,18,19] yet, the physiological mechanisms underlying variation in growth and their associated costs are less well understood, particularly outside of laboratory populations.

Recent evidence has suggested that insulin-like growth factor-1 (IGF-1) may be one potential mechanism underlying variation in growth. IGF-1 is a highly conserved metabolic hormone and highly plastic to environmental cues, specifically, to changes in nutritional status and content [19–22]. IGF-1 is one of three ligands in the insulin/insulin-like signaling pathway (i.e. IGF-1, IGF-2, insulin) [19,21,23]. In vertebrates, the secretion of IGF-1 is regulated via the somatotropic axis and secretion of IGF-1 occurs in response to variation in environmental cues (e.g. nutrition, temperature, stress etc.) [19,24,25]. Secretion of IGF-1 is primarily regulated by the somatotropic (growth hormone/IGF-1) axis, where the release of growth hormone (GH) from the anterior pituitary stimulates secretion of IGF-1 by the liver ([19] and see references therein). In addition to systemic secretion of IGF-1 by the liver, local production of IGF-1 can occur independently of GH in many other tissues, acting in an autocrine and paracrine fashion [26–28].

The growth promoting effects of IGF-1 during embryonic and post-natal growth are well documented within the literature. IGF-1 is expressed in a range of tissues throughout development [29–31], but is typically greater during the postnatal than the embryonic period [32–34] and levels of IGF-1 are often positively associated with growth rates [35,36]. Experimental research using knockout mice has demonstrated that IGF-1 signaling is vital for embryonic and postnatal growth [37–40]. Further, in birds, injections of IGF-1 have been shown to promote muscle development and increase feed efficiency during post-natal growth[41–43].

Among vertebrates, a reduction in IGF-1 signaling generally corresponds to increased longevity and reduced senescence[44–48]. One way, IGF-1 might impact an individual's longevity is through its effects on cellular aging mechanisms, such as telomeres. Telomeres are highly conserved, non-coding, regions of DNA at the end of eukaryotic chromosomes [49,50]. Telomeres shorten with age, and this shortening tends to be greatest during periods of rapid growth [51]. Growth requires cell proliferation and protein synthesis. Free radicals, such as reactive oxygen species (ROS) are important cell-signaling molecules that occur as a by-product of aerobic metabolism [52,53]. However, in excess, they can result in increased oxidative stress, damaging macromolecules such as proteins, lipids and DNA including telomeres [49]. In support of this, a recent study found that exogenous IGF-1 accelerated telomere shortening in skin fibroblasts, potentially by enhancing ROS production [54,55].

Although there is a substantial literature examining the relationships between IGF-1, growth and longevity in domesticated and laboratory animals, we currently have little information about the role of IGF-1 in mediating life-history trade-offs in free-ranging animals (but see[35,36,56–58]). In this study, we examined the relationships between IGF-1 during post-natal growth, growth rate, and telomeres in nestling house sparrows (*Passer domesticus*). We

predicted that IGF-1 would be positively correlated with nestling growth rate and that chicks that grew more quickly would have shorter telomeres.

Materials and methods

Study species

This study was carried out during the breeding seasons of 2016-2018 on a well-established population of free-living house sparrows (*Passer domesticus*) in Fargo, ND (figure 7.). The study area consists of 130+ nest boxes maintained on North Dakota State University agricultural areas. Nest boxes are mounted on several agricultural storage buildings and barns and maintained year-round. Each breeding season (April through July), boxes are regularly monitored to determine precise laying, hatching, and fledging dates.

Figure 7. House sparrow nestling (*Passer domesticus*). House sparrows breed in North Dakota from early April to August.

Sample collection

To follow individual nestlings throughout the post-natal growth period, nestlings were marked with a unique color using Sharpie© markers at hatching. The following growth measurements were collected on days 2, 6, 8, and 10 ±1 post-hatching: body mass, wing chord, and rectrix length. In 2018, tarsus length was also measured.

To measure telomere length in red blood cells, small blood samples were collected from each nestling at 2- and 10-days post-hatching between 9- 11 am. At 2-days post-hatching, a much smaller volume of blood (approx. ~5µl), was taken from the brachial vein using heparinized capillary tubes, transferred into 20µl of phosphate buffered saline (PBS), and then frozen -80°C for later analyses. At 10-days post-hatching, blood was collected from the brachial vein using heparinized capillary tubes, centrifuged, separated, and frozen at -80°C until analyses.

Telomere analysis

DNA was extracted from blood cells using Macherey Nagel Nucleospin Blood kits following the manufacturer's instructions (Genomic DNA from Blood, Macherey Nagel). Following extraction, DNA purity and concentration were assessed using the Nanodrop 8000 (Thermo Scientific) and all samples had 280/260 and 260/230 ratios above 1.8±0.1. To measure relative telomere length we used qPCR (quantitative polymerase chain reaction) following established protocols [59,60] and modified for use in house sparrows. The telomere forward and reverse primers were as follows: Tel1b (5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'), Tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3') and GAPDH was used as the single copy control gene GAPDH-F (5'CTCCAGTAGATGCTGGGATAATG-3'), GAPDH-R (5'-CATCACAGCCACACAGAAGA-3'). The telomere and GAPDH reactions were carried

out on separate plates and run in duplicate with a water sample run as a negative template control. A separate sample was run on every plate in triplicate to assess average intra plate variation.

For each assay the efficiency of each reaction was measured using a standard curve of a serial dilution of a reference sample. The reference sample was pooled DNA from several nestling house sparrows collected in 2016. Average values of each sample were then used to determine the T/S ratio according to the following formula: $2^{-\Delta\Delta Ct,}$ where $\Delta\Delta Ct = (C_t^{Telo}-C_t^{GAPDH})$ reference $- (C_t^{Telo}- C_t^{GAPDH})$ (Agilent Technologies, 2012). All samples were run against a standard curve of 40, 20, 10, 5, and 2.5 ng produced by serially diluting a reference sample. All plate efficiencies were within an accepted range of $100 \pm 15\%$. Across all three years, average plate efficiency for telomere and GAPDH plates were 96.0% and 98.1% respectively. For the 2016 assays, the average inter-plate variation for the T/S ratio was 14.4% and the average intra-plate variation of the Ct values was 1% for the GAPDH assays and 3% for the telomere assays. For the 2017 telomere assays, the average intra-plate variation of the Ct values was 0.3% for the GAPDH assays and 2.0% for the telomere assays, and the inter-plate variation for the T/S ratio was 14.1%. For the 2018 telomere assays, the average intra-plate variation of the Ct values was 1.3% for the GAPDH assays and 2.1% for the telomere assays and the inter-plate variation for the T/S ratio was 13.0%.

Hormone analysis

Free IGF-1 levels were measured using an enzyme-linked immunosorbent assay (ELISA). While almost 90% of IGF-1 is bound to one of six binding proteins which regulate bioavailability of the hormone [61,62], some studies consider free- IGF-1 (i.e. the percentage of hormone that is not bound to binding protein) of greater biological relevance [63]. Free plasma

IGF-1 levels were measured in duplicate using a competitive ELISA developed at University of Debrecen. The assay procedure was as follows: 96-wells NUNC microplates were coated overnight at 4°C with 100µl of an antibody raised against IGF-1 in rabbits. The capture antibody was incubated for 2 hours at room temperature (24°C) with 20 µl known concentrations (in serial dilutions starting at 500 ng/ml) of synthetic chicken IGF-1 or 20µl of sample and 100 µl biotinylated IGF-1 as a trace. After incubation, the microplate was washed three times with 250 µl of PBS buffer (8g NaCl, 0.2g KCl, 1.44g Na2HPO4 and 0.24g KH2PO4 in 1000 ml ddH2O, pH 7.4) containing 0.025% Tween 20. After washing, 100 µl of streptavidin-horseradish peroxidase conjugate was added to all wells and incubated at room temperature at 30 minutes, followed by another washing cycle (3 times). Then, 100 µl of tetra-methyl-benzidine was added to the wells and incubated at room temperature for 30 minutes. The enzymatic reaction was stopped by adding 100 µl of 1M H2SO4, and optical density was measured at 450 nm (reference at 620 nm). The calibration curve was fitted using a 4-parametric log-logistic curve, and concentrations of unknown samples were read off from this curve. For these assays, the mean inter-assay coefficients of variation were 13.6% and the mean intra-assay variation of coefficients were 4.3%. Free IGF-1 plasma levels were measured using a competitive binding ELISA developed at the University of Debrecen, Hungary and validated for house sparrows through serial plasma dilutions (figure 8).
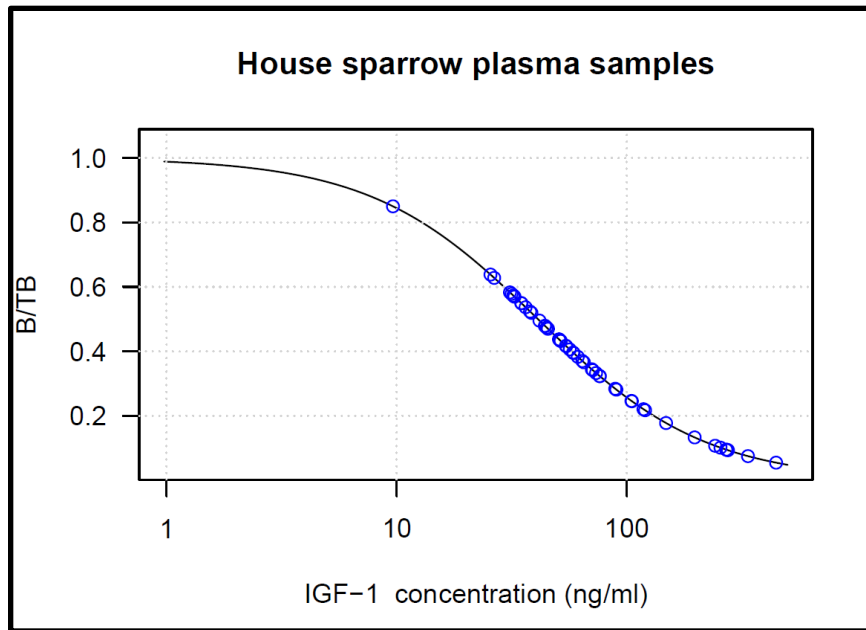
Figure 8. Validation of house sparrow plasma samples via serial dilution.

Molecular sexing

All individuals were sexed using molecular techniques following established methods [64]. The following forward and reverse primers were used to amplify the highly conserved chromobox-helicase-DNA-binding genes (CHD-W and CHD-Z) in birds: P8 (5'--CTC-CCAAGGATGAGRAAYTG-3') and P2 (5'-TCTGCATC- GCTAAATCCTTT-3'). The PCR amplification was carried out in a total volume of 20μl. The final reaction conditions were as follows: 10μl of Dreamtaq Green PCR 2x Mastermix (Thermo Scientific), 1.0μl P2 and P8 primers, 6.0μl ddH$_2$0, 2μl template. Between 4 and 40ng of DNA was used as template. PCR was performed in an Eppendorf Mastercycler Gradient thermal cycler. Products were then run out on 2% agarose gel and individuals were identified as either male or female.

Statistical analysis

All data were analyzed using R. v.3.2.3 (R Development Core Team 2015) statistical software. To test for relationships between IGF-1 and growth rate, and telomere dynamics we used restricted maximum-likelihood to fit linear mixed effect models using the R package
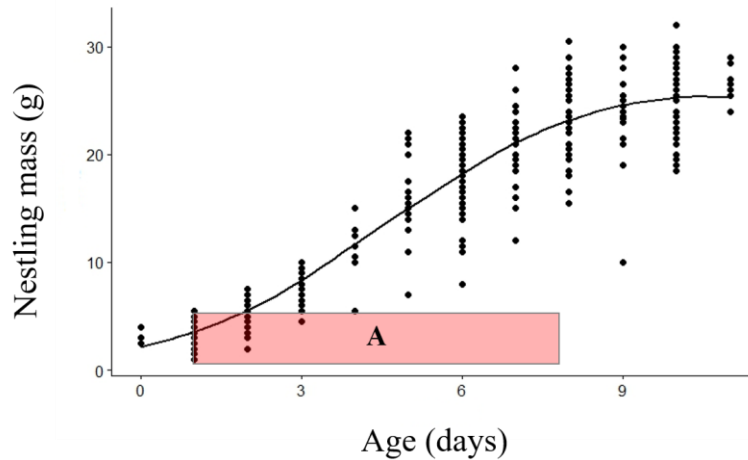
Figure 9. (A) Growth curve for house sparrow nestlings, A represents the period of linear growth; approximately from day 2-day 8 post-hatch.

package lme4 [65]. The sample size for these analyses were 13 broods (n=57 individuals) for

2016, 7 broods (n=29 individuals) for 2017, and 10 broods (n=43 individuals) for 2018. To meet

the linear mixed model assumptions for normality, the hormone and telomere values were log-

transformed prior to running the analyses. To assess overall change in telomere length, telomere

values were corrected for 'regression to the mean' following established methods to account for

the impact of baseline telomere length on its attrition rate within an individual [66]. We also

included a mean IGF-1 level in our analyses for each nestling by averaging an individual's IGF-1

level at day 6 and day 10 post-hatch. The R package *lsmeans* [67] was used to conduct multiple

post-hoc comparisons among groups using Tukey's HSD.

Nestling growth rate was calculated as the slope of the linear phase of the growth curve

during post-natal development for each nestling. Slopes for each nestling were calculated as the

change in mass with age from day 2 to day 8 post-hatch (figure 9). Then, to determine whether

nestling growth rate varied across years, we ran a model that included nestling growth rate as the

dependent variable and year, sex, brood size, and Julian date as fixed effects. Lastly, individual

nest ID and nest location were included as random effects to account for variation in nest boxes and spatial variation between nesting sites.

For the analysis investigating the relationship between nestling growth rate and IGF-1 we ran models that included nestling growth rate as the dependent variable (table 1). In these models, IGF-1 levels (either day 6 or day 10 post-hatch, or mean IGF-1), sex, brood size, and Julian date were treated as fixed effects (table 1).To investigate the effect of year on IGF-1 levels we also included a year by treatment interaction. All non-significant interaction terms were dropped from the final model. To investigate the relationships between IGF-1 and age across development we ran a model that treated nestling IGF-1 levels (either combined day 6 and 10 or mean IGF-1) as the dependent variable. In this model, nestling age at sampling, mass at sampling, sex, brood size, and Julian date were treated as fixed effects and nestling ID was included as a repeated variable. In all the above models, we also included nest ID, nest location, and year as random effects to account for temporal and spatial variation in nesting sites and nest boxes across years. Lastly, we used Pearson's correlation to determine whether IGF-1 levels at day 6 post-hatch were correlated with IGF-1 levels at day 10 post-hatch within an individual.

For analyses investigating the relationship between nestling growth rate and telomere dynamics we ran a model that included either telomere length or change in telomere length as the dependent variable and sex, brood size, and Julian date as fixed effects. For the analysis of the relationship between IGF-1 levels and telomere dynamics, we ran a model that included final telomere length (telomere length at day 10 post-hatching) was treated as the dependent variable. In these models, IGF-1 levels (either day 6 post-hatch, day 10 post-hatch, or mean IGF-1), initial telomere length, sex, brood size, and Julian date were treated as fixed effects (table 2). In each of

these models, we also included nest ID, nest location, and year as random effects to account for temporal and spatial variation in nesting sites and nest boxes across years.

Results

Nestling growth rates varied significantly among years ($F_{2,23.87}$=5.15, p=0.01). Specifically, nestling growth rates in 2018 were significantly higher compared to nestling growth rates in 2016 (Tukey's HSD, p=0.01, figure 10), however, they did not differ between years 2017 and 2018 (Tukey's HSD, p=0.38, figure 10). Growth rates did not differ with respect to sex ($F_{1,72.34}$=.486, p=0.48) or brood size ($F_{1,15.97}$=.147, p=0.70). Nestling mass at day 2 post-hatch was significantly different among years, in which nestlings in 2016 were significantly lighter than nestlings in both 2017 and 2018 ($F_{2,35.42}$=7.98, p<.001). Final mass (i.e. mass at day 10 post-hatch) also differed by year with respect to sex ($F_{2,26.47}$=5.43, p=0.01). Specifically, male nestlings in 2017 were significantly heavier at their final mass than male nestlings in 2016 (*Tukey's HSD* p<.001, figure 11). Further, male nestlings in 2017 were also significantly heavier than females in 2016 (*Tukey's HSD* p<.001, figure 11). However, the final mass of female nestlings did not differ between females in 2016 or 2017 (*Tukey's HSD* p=.25, figure 11). Nestling mass did not differ between the sexes in 2018 (Tukey's HSD p=.80) or between years 2016 and 2018 (*Tukey's HSD*, p=.96).

IGF-1 levels at day 6 post-hatch were not related to nestling growth rate ($F_{1,56.22}$=0.02, p=0.88, table 3), or day 10 post-hatch ($F_{1,56.22}$=0.02, p=0.88, table 3). Further, nestling growth rate was not related to mean IGF-1 levels ($F_{1,65.14}$=0.17, p=0.67, table 3). Similarly, final mass was not related to IGF-1 levels at day 6 post-hatch ($F_{1,58.44}$=2.13, p=0.15, table 3) nor at day 10 post-hatch ($F_{1,64.54}$=0.255, p=0.62, table 3). IGF-1 levels did not vary significantly with nestling age ($F_{1,85.07}$=1.54, p=0.21, table 3), however, there was a significant effect of sex on overall IGF-

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1 levels, where males had significantly greater IGF-1 levels compared to females ($F_{1,115.69}$=4.19, p=0.04, table 1, figure 12). Similarly, there was a significant effect of sex on average IGF-1 levels, where males had significantly greater mean IGF-1 levels compared to females ($F_{1,72.29}$=6.73, p=0.01). In addition, there was a trend suggesting mean IGF-1 levels were positively, related to nestling mass at day 10 post-hatch, however, this was not significant ($F_{1,52.51}$=3.23, p=0.07). IGF-1 levels at day 10 post-hatch were not correlated with IGF-1 levels at day 6 post-hatch (r=.032, p=0.79).

Final telomere length (i.e. telomere length at day 10 post-hatch) was not related to nestling growth rate ($F_{1,48.44}$=0.04, p=0.83). Similarly, we found no relationship between change in telomere length and nestling growth rate ($F_{1,47.86}$=0.03, p=0.86, table 4). Telomere length was not related to IGF-1 levels at day 10 post-hatch ($F_{1,54.28}$=0.671, p=0.41, table 4) or IGF-1 levels at day 6 post-hatch ($F_{1,36.54}$=0.17, p=0.68, table 4). Likewise, change in telomere length was not related to IGF-1 levels at day 10 post-hatch ($F_{1,52.55}$=.641, p=0.42, table 4) or IGF-1 levels at day 6 post-hatch ($F_{1,40.67}$=.116, p=0.68). However, there was a trend suggesting that the average IGF-1 levels for an individual were negatively related to telomere length ($F_{1,50.54}$=3.11, p=0.08, table 2, figure 13), but not change in telomere length ($F_{1,52.95}$=1.51, p=0.22, table 4).
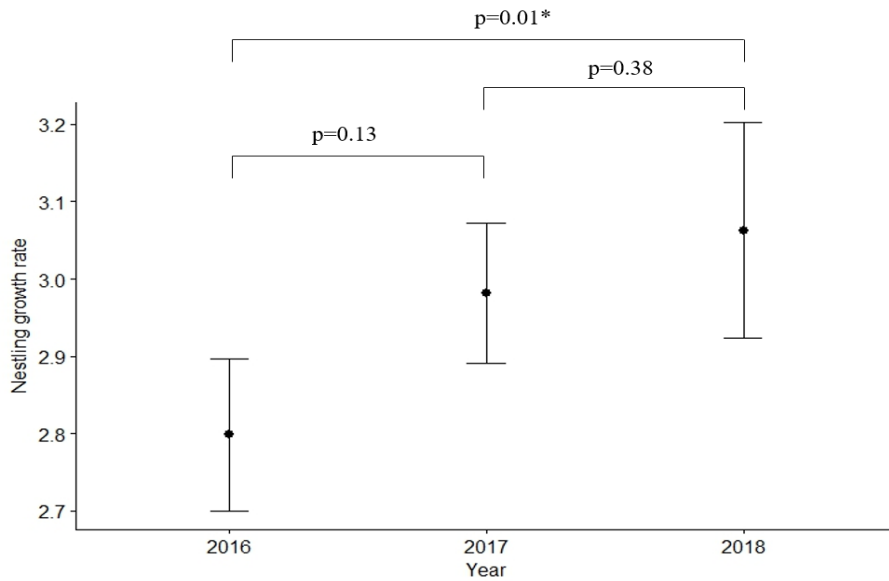
Figure 10. Nestling growth rates across years in house sparrows (mean±SEM). Nestling growth rates were calculated as individual slopes of the change in mass with age during the linear phase of the post-natal growth period. P-values are based on Tukey's HSD post-hoc test and significant differences, *P<.05.
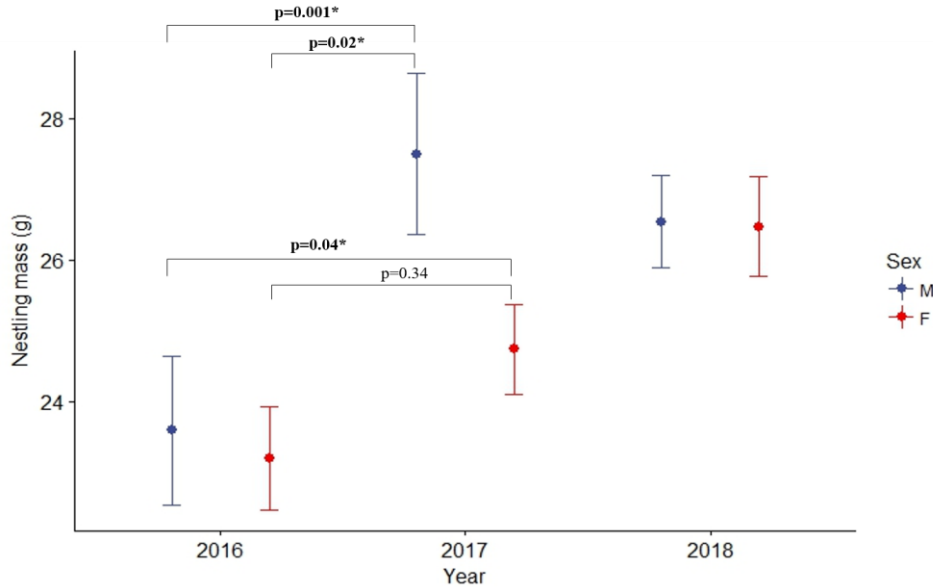
Figure 11. Nestling mass at 10 days post-hatch levels at day 10 post-hatch by sex across all years in house sparrow nestlings (mean±SEM). P-values are based on Tukey's HSD post-hoc test and significant differences, *P<.05.
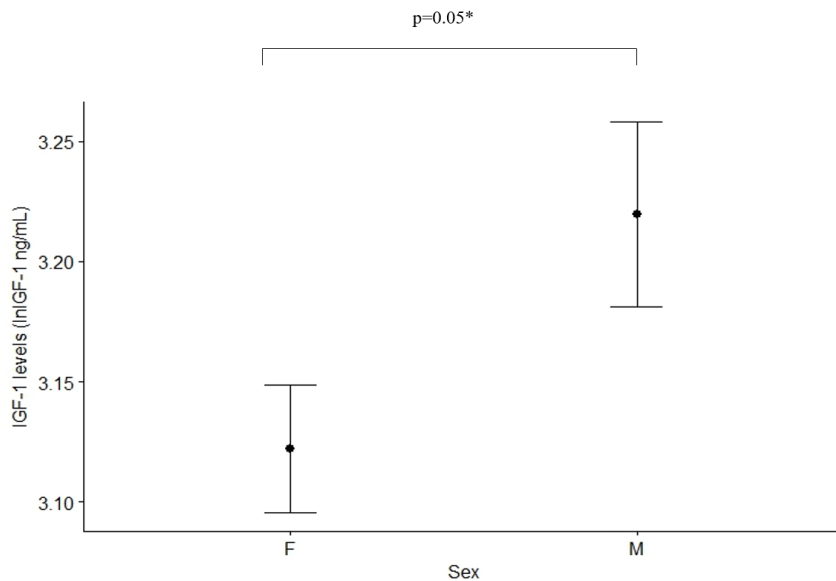
Figure 12. The relationship IGF-1 levels and sex across all years in house sparrow nestlings (mean±SEM).

$F_{1,50.54}=3.11, p=0.08$
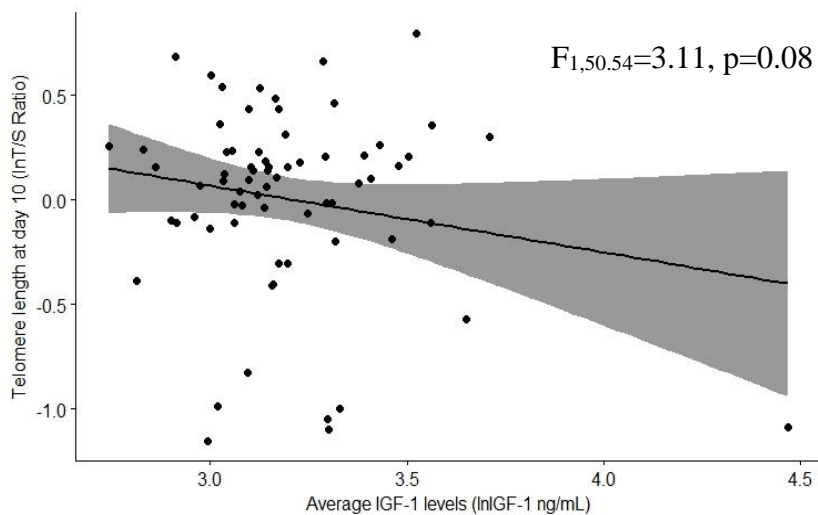
Figure 13. Nestling telomere length at day 10 post-hatch and average IGF-1 levels of an individual (mean±SEM). P-values are based on Tukey's HSD post-hoc test and significant differences, *P<.05.

Table 3. Relationship between growth rate, final mass, and nestling IGF-1 levels at each time point (day 6 and day 10 post-hatch). Nest ID, location, and year was included as a random factor in each model. Non-significant two-way interactions were removed from the model. Parameter estimates ± s.e. (standard errors) are reported and significant variables are bolded, while p-values below p=0.10 are italicized. For day 6 (n=80 individuals) and day 10 (n=82).

Dependent	Independent variable/factor	β± s. e.	F	p
Growth rate	IGF-1 (day 10)	0.167±0.214	0.612	0.43
	Sex	-0.0256±0.105	0.059	0.80
	Brood Size	-0.025±0.004	0.031	0.86
	Julian day	-0.010±0.056	5.11	**0.03**
Growth rate	IGF-1 (day 6)	-0.019±0.131	0.022	0.88
	Sex	0.001±0.094	3.98	*0.06*
	Brood size	-0.049±0.083	0.35	0.56
	Julian day	-0.009±0.004	0.002	0.98
Final mass	IGF-1 (day 6)	1.11±0.761	2.12	0.15
	Age	1.53±0.398	14.79	**<.001**
	Sex	-0.65±0.513	1.61	0.22
	Brood size	-0.118±0.524	0.051	0.82
	Julian day	0.075±0.028		**0.01**
Final mass	IGF-1 (day 10)	-0.556±1.10	0.25	0.61
	Age	1.505±0.397	14.37	**<0.001**
	Sex	-0.564±0.510	1.22	0.29
	Brood Size	-0.210±0.527	0.159	0.69
	Julian day	0.064±0.027	5.75	**0.03**

Table 4. Relationship between telomere dynamics and nestling IGF-1 levels at each time point (day 6 and day 10 post-hatch). Nest ID, location, and year was included as a random factor in each model. Non-significant two-way interactions were removed from the model. Parameter estimates ± s.e. (standard errors) are reported and significant variables are bolded, while p-values below p=0.10 are italicized.

Dependent	Independent variable/factor	β± s. e.	F	p
Telomere length (day 10)	IGF-1 (day 10)	-0.125±0.152	0.672	0.41
	Initial telomere length (day 2)	0.451±0.088	26.00	**<.001**
	Sex	0.039±0.077	0.258	0.61
	Brood size	0.029±0.047	0.369	0.55
	Julian day	-0.003±0.002	1.53	0.23
Telomere length (day 10)	IGF-1 (day 6)	-0.047±0.114	0.168	0.68
	Initial telomere length (day 2)	0.365±0.093	15.44	**<.001**
	Sex	0.086±0.085	1.03	0.31
	Brood size	0.042±0.059	0.516	0.49
	Julian day	-0.002±0.003	0.261	0.62
Change in telomere length	IGF-1 (day 10)	-0.137±0.168	0.666	0.41
	Sex	0.079±0.086	0.858	0.36
	Brood size	0.012±0.055	0.054	0.81
	Julian day	-0.002±0.003	0.786	0.39
Change in telomere length	IGF-1 (day 6)	-0.059±0.127	0.218	0.64
	Sex	0.118±0.092	1.63	0.21
	Brood size	0.012±0.058	0.042	0.84
	Julian day	-0.002±0.003	0.390	0.54
Telomere length (day 10)	Average IGF-1 levels (6&10)	-0.284±0.161	3.11	*0.08*
	Initial telomere length (day 2)	0.372±0.087	26.00	**<.001**
	Sex	0.056±0.084	0.258	0.61
	Brood size	0.026±0.054	0.369	0.55
	Julian day	-0.001±0.003	1.53	0.23

Discussion

The results of this study indicated that nestling growth rates were not related IGF-1, either at day 6 post-hatch, day 10 post-hatch, or mean nestling IGF-1 levels. Similar results were observed with respect to final mass (i.e. mass at day 10 post-hatch), where final mass was not related to mean IGF-1 levels, or IGF-1 levels at day 6 or day 10 post-hatch. A previous study in great tits (*Parus major*) found that relationships between IGF-1 levels and growth rates can vary with nutritional conditions [68], in part because growth hormone (GH) stimulates the secretion of IGF-1 by the liver, however, the ability of that tissue to respond to GH is also regulated by the nutritional status of an individual[69]. It's important to note, while we controlled for sampling time in our study, we were unable control for whether nestlings were fasted at the time of sampling. As a result, the nutritional status of the nestling at the time of sampling (i.e. whether they had eaten) is unknown, which could have impacted IGF-1 levels in our study.

In addition to nutritional status of individuals, IGF-1 levels within a nestling could be altered by actions of IGF-1 binding proteins (IGFBP). IGFBPs play an important role in regulating the biological actions of IGF-1 by influencing its ability to bind to receptors [70]. When bound, IGFBPs extend the half-life of IGF-1 in circulation and provide transport to target tissues [21]. In our study, we measured free IGF-1, or the portion of IGF-1 that remains unbound in circulation, and biologically active. Most of IGF-1 is remains bound in circulation (<90%) [71], and changes in binding proteins in response to environmental factors could impact the amount of free-IGF-1 in circulation. Circulating concentrations of IGFBP3 (the most common IGFBP) and IGFBP2 enhance the IGF-1 actions for bone and muscle growth. In mammals, fast growth rate and nutrient intake is often associated with increased IGFBP3 and IGFBP2 in circulation [71–74]. It is also important to note that not all IGFBP actions are associated with

growth. For example, IGFBP 4,5, and 6 often inhibit IGF-1 actions, attenuating the hormone in circulation[71].   Thus, for future studies it may be important to consider not only IGF-1 levels in circulation but also the proportions of specific binding proteins as they may provide greater insight into the relationship between growth and the IGF-1 system.

Regardless of IGF-1 levels, our study also demonstrated that nestling growth rates differed significantly across years (figure 3). Final nestling mass (i.e. mass at day 10 post-hatch) also differed among years and with respect to sex. Specifically, in 2017, male nestlings were significantly heavier than both male and female nestlings in 2016. However, the final mass of females in 2016 and 2017 did not significantly differ. There were also no significant differences in nestling mass (males and females) in 2017 and 2018. Further, nestlings in 2016 were significantly lighter compared to nestlings in 2017 and 2018 regardless of sex at our initial sampling point (day 2 post-hatch). These differences in initial (day 2 post-hatch) and final mass (day 10 post-hatch) across years and between sexes may be the result of variation in nutritional resources and/or parental condition. For example, in house sparrows, nutritional conditions within a nest could be mediated by parental feeding rates. For example, in house sparrows, males with a larger badge size (i.e. an indicator of condition) had proportionally greater feeding rates and greater offspring survival compared to small badge males [66]. In our study, our calculations for growth rate reflect the linear phase of growth (i.e. day 2- day 8 post-hatch), thus it is possible that differences in parental feeding rates prior to and after this time period could have influenced initial and final mass of nestlings. In addition, there is some evidence to suggest that maternal condition and age can impact nestling condition at hatch, where females in poorer condition or those that are older produce poorer condition nestlings [67–69].

When we examined how IGF-1 changed with respect to nestling age we found no significant difference between IGF-1 levels at day 6 post-hatch or day 10 post-hatch. Total IGF-1 (i.e. bound and unbound) has been shown to decrease with age, and is generally highest during periods of rapid growth [38,68,75]. In humans, free IGF-1 also seems to decline with age [76], however, in our study, we only sampled IGF-1 at two age groups and were unable to characterize IGF-1 levels prior to day 6 post-hatch. Males had greater IGF-1 levels compared to females (figure 6A). This observation is regularly reported within the literature, particularly in those species that exhibit strong sexual dimorphism [77–79]. Mass was weakly positively related to mean IGF-1 levels; however, this was not significant. Though, this trend is in line with comparisons made within a species, in which larger individuals have greater levels of IGF-1 compared to smaller individuals [20,58,80,81]. Interestingly, IGF-1 levels at day 6 were not correlated with IGF-1 levels at day 10 post-hatch and relationships between IGF-1 and growth rate and year were only seen in when using mean IGF-1 levels. This suggests that sampling period will be important for discerning relationships between IGF-1 and life-history variables.

Lastly, we did not observe a relationship between telomere dynamics (length at day 10 post-hatch and change in telomere length) and nestling growth rate, however, there was a trend suggesting nestlings with greater mean IGF-1 levels had shorter telomeres (figure 6). Rapid growth during the post-natal growth period is often associated with increased telomere loss, [9,82,83], however, this relationship is not always observed [84]. While there may be consequences to fast post-natal growth, it is possible that this trade-off is not visible under conditions where nestlings are able to mitigate costs associated with growth or if costs are paid over a longer time scales, or at a later life stage [85]. For example, zebra finches (*Taeniopygia guttata*) with the fastest growth rates were more susceptible to oxidative damage nearly two

months after the post-natal growth period compared to slower growing individuals[11]. Similarly, Atlantic salmon delayed reproduction after undergoing a bout of increased growth as juveniles [86]. Thus, it is possible that nestlings in our study were able to mitigate any negative cost of growth during the post-natal growth period and/or potentially delay the cost to an alternate life stage.

In conclusion, our results demonstrated that nestling growth rate was not related to free IGF-1 levels or telomere dynamics in house sparrows. However, nestling growth rates and mass differed with respect to year, suggesting that there could have been some variation in resource availability/quality. These findings suggest that if there are costs to growth, house sparrow nestlings may be able to mitigate them during the post-natal growth period or delay them to later life stages. IGF-1 levels at day 6 and day 10 were not correlated and a weak relationship between IGF-1 and mass was only discernable when using mean IGF-1 values, suggesting that values collected across development may be inconsistent, thus it will be important for future studies to consider when and how to sample their study organism. In addition, future studies should attempt to characterize how different parts of the GH/IGF-1 axis varies across post-natal development as this may be variable across species. Lastly, there was a trend suggesting that individuals with greater mean IGF-1 levels also had shorter telomeres, however, this was not significant (figure 13), This finding may suggest a role for IGF-1 as a shared mechanism underlying life-history trade-offs, however, manipulation experiments will be imperative for discerning causal relationships between IGF-1, growth, and aging.

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CHAPTER 4: MANIPULATION OF INSULIN-LIKE GROWTH FACTOR-1 ON NESTLING GROWTH AND TELOMERE DYNAMICS ACROSS YEARS IN HOUSE SPARROW NESTLINGS

Abstract

Correlational and experimental studies have demonstrated both across and within taxa that investment in growth comes at a cost to longevity. Despite this, the physiological mechanisms linking growth and aging are not well understood. One hormonal mechanism that may act as an important mediator underlying the relationship between growth and aging is insulin-like growth factor-1 (IGF-1). IGF-1 is essential for normal growth and development and has been linked with longevity across species. One-way IGF-1 may influence aging and longevity in vertebrates is through its effects on telomeres. Telomeres are non-coding repetitive segments of DNA that enhance genome stability and have been shown to be predictive of lifespan in natural populations. To test whether IGF-1 accelerates growth and cellular aging, we manipulated circulating IGF-1 in house sparrow (*Passer domesticus*) nestlings during the post-natal growth period over a period of three years. In 2016 and 2017, all of the chicks within nests were assigned to either an experimental or control treatment. Experimental nests were injected with a physiologically relevant dose of recombinant human IGF-1 (rhIGF-1) in a gelatin carrier matrix from day 3- day 10 post-hatch. Control nestlings were injected with only the gelatin carrier matrix. In 2018, we controlled for nest by assigning individual nestlings within the nest to either experimental or control treatments. To measure IGF-1 levels and telomere length, blood samples were collected at day 3, 6, and 10 post-hatching. We collected growth measurements every 3 days until day 10 post-hatch in all years. In 2018, we extended sample collection until day 12 post-hatch. Nestling IGF-1 levels were successfully raised following an injection of

rhIGF-1. Experimental chicks had longer culmens than controls in all years. However, feather development and growth rate were only significantly greater than controls in 2016. Nestling growth rates did not differ between treatments in 2017 or 2018. However, in 2016 and 2018, experimental birds were significantly heavier than controls at their final mass. Further, in 2016, IGF-1 injected nestlings had significantly shorter telomeres at day 10 post-hatching than controls, but there was no significant relationship between treatment and nestling telomere length in 2017 and 2018 at day 10 post-hatch. However, at day 12 post-hatch experimental birds had significantly shorter telomeres than controls in 2018. Overall, the results of this study suggest that IGF-1 may impact nestling growth and telomere dynamics, however, the degree to which IGF-1 affects these traits varies across years.

Introduction

In accordance with life-history theory, organisms are predicted to allocate resources among competing life-history traits (i.e. growth, longevity etc.) to maximize fitness [1,2]. In vertebrates post-natal growth rate is an important life-history trait often predictive of survival and fitness[2]. Faster growth may be favored is to allow individuals to attain a larger body size as quickly as possible and escape vulnerable early life stages [3–5]. Yet, individuals generally do not grow at their maximal rates even when conditions are favorable [3], possibly because rapid growth come at a cost to longevity [6]. In support of this, studies have demonstrated that investment in growth is associated with increased mortality or reduced lifespan [7–9]. Further, experimentally elevated growth has been shown to reduce median lifespan in some species [10]. However, the physiological mechanisms linking growth and reduced longevity are less well understood.

Hormonal mechanisms have pleiotropic (i.e. multiple) effects on morphological, physiological, and behavior traits and often mediate life-history trade-offs [11–14]. One mechanism that may be particularly important in this regard is insulin-like growth factor-1 (IGF-1), a highly conserved and nutritionally sensitive metabolic hormone. Numerous laboratory and field studies, have implicated IGF-1 as an essential regulator of growth [15–21] and it has been associated with variation in lifespan across taxa [22–25]. Recently, Swanson and Dantzer [26] found that elevated IGF-1 was associated with elevated growth and reduced longevity across 41 species of mammals. Further, in a recent study in passerines, IGF-1 was found to be negatively related to lifespan[27]. Thus, this supports IGF-1 as a putative mediator of the trade-off between growth and aging.

One mechanism by which IGF-1 might influence cellular aging and longevity is through its effects on oxidative stress and telomere dynamics. Telomeres are non-coding segments of DNA that form protective caps on the end of eukaryotic chromosomes, enhancing genome stability [28,29]. Telomeres shorten as cells divide and once telomeres reach a critically short length the cell begins to senesce [29]. Telomere length and loss (dynamics) have been shown to be predictive of mortality in natural populations of mammals [30,31] and birds[32]. Further, rapid growth early in life can increase telomere loss [33,34]. Elevated IGF-1 is expected to increase growth, but in doing so may lead to greater reactive oxygen species (ROS) production as a byproduct of aerobic metabolism [35]. When ROS production exceeds the ability of the anti-oxidant defense system to combat it, it can damage macromolecules such as lipids, proteins, and DNA including telomeres [36]. In support of this, there is evidence that exogenous IGF-1 accelerates telomere loss in skin fibroblasts, but whether this is observed *in vivo* is unknown [37]. In this study, we tested the hypothesis that IGF-1 accelerates post-natal growth and

increases cellular aging (i.e. telomere dynamics) by experimentally elevating IGF-1 in house sparrows (*Passer domesticus*) chicks during post-natal development. We predicted that chicks with elevated IGF-1 would grow faster, reach a larger mass, and have shorter telomeres than controls.

Materials and methods

Study species

The study was carried out on a well-established population of free-living house sparrow in Fargo, ND during the breeding season (May-July) in 2016,2017, and 2018. The study area consists of agricultural areas maintained by North Dakota State University. Nest boxes are mounted on barns and agricultural storage buildings and are maintained throughout the year. Over the study period nest boxes were checked regularly to determine precise laying and hatching dates. At hatching, all nestlings were aged, and each marked with a unique color using a Sharpie © marker.

Manipulation protocol (Years 2016 and 2017)

All nests were carefully monitored and at hatching, each nest was randomly assigned to one of two treatments: experimental (2016: n=7 nests, n=31 individuals; 2017: n=10 nests, n=45 individuals) or control (2016: n=6 nests, 28 individuals; 2017: n=8 nests, n =40 individuals). Experimental and control groups received daily intramuscular injections (into the right breast muscle near the keel of the sternum) using 27G disposable syringes beginning 3 ±1-days post-hatch and continuing until day 10±1 post-hatch for an average of 7 days of treatment. Experimental chicks were injected with recombinant human IGF-1 (National Hormone and Peptide Program, Los Angeles, California) diluted in a sterile gelatin solution (Sigma Aldrich) following methods outlined in [21]. The hormonal medium was diluted to 100µg/mL based on a

previous study in great tits [38] and pied flycatchers [21] and is likely within the biological range for house sparrows given they are of similar size and life history. We administered 50µl of rhIGF-1 in the gelatin carrier matrix, which resulted in a 5µg daily dose [21] and successfully elevated IGF-1 levels within a physiologically relevant range. The range for IGF-1 levels in non-manipulated chicks (natural variation) was between 13.41 and 45.56 ng/mL, with the mean value at 24.10 ng/mL. The mean value for experimental birds following IGF-1 injection was 31.0 ng/mL with the maximum value at 43.6 ng/mL. Control chicks received injections of only the sterile gelatin solution.

Manipulation protocol (Year 2018)

In 2018, at hatching, each nestling within a nest was randomly assigned to one of two treatments: experimental (n=25 individuals) or control (n=17 individuals) from 11 nests. House sparrows can hatch asynchronously [39], but we only assigned nestlings within a nest to a treatment when they were within ±1 day of hatching of one another to control for variation in age.. Experimental and control chicks were then treated as described above.

Sample collection

To measure IGF-1 and telomeres, blood samples were collected from the brachial vein of experimental and control nestlings on days 2, 6, and 10 post-hatching. In 2016, blood taken for hormone analyses at day 10 was collected 30 minutes after injection to confirm whether our treatment successfully elevated IGF-1 levels. This time course was chosen based on established methods in broiler chickens [40]. Blood samples taken at day 6 post-hatching were collected prior to injection. Blood was collected by venipuncture using 26.5-gauge needles and heparinized capillary tubes, centrifuged, and then separated. Red blood cells and plasma were then stored at -80°C until further analysis. Body mass, wing chord, and rectrix length were

measured in experimental and control groups on days 2, 6, 8, and 10 post-hatching. Body mass was measured to the nearest 0.5g using a Pesola. Culmen, wing chord, and rectrix length were measured to the nearest 1.0mm.

Hormone and telomere analysis

We extracted DNA from red blood cells using Macherey Nagel Nucleospin Blood kits following the manufacturer's protocol (Genomic DNA from Blood, Macherey Nagel). DNA purity and concentration were assessed using the Nanodrop 8000 (Thermo Scientific). Samples with 280/260 and 260/230 ratios above 1.8±0.1 were used for telomere analysis. Relative telomere length was measured at day 2 and day 10 post-hatch for each treatment group using quantitative PCR following the established protocols [32,41]. The following forward and reverse primers were used to amplify the telomere: Tel1b (5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'), Tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3') and GADPH was used as the single copy control gene GADPH-F (5'CTCCAGTAGATGCTGGGATAATG-3'), GAPDH-R (5'-CATCACAGCCACACAGAAGA-3'). The telomere and GADPH qPCR reactions were carried out on separate plates and run in triplicate with a water sample run as a negative template control. Average values of each sample were then used to determine the T/S ratio according to the following formula: $2^{\Delta\Delta Ct}$, where $\Delta\Delta Ct = (C_t^{Telo}\text{-}C_t^{GAPDH})$ reference $- (C_t^{Telo}\text{-} C_t^{GAPDH})$ [42]. All samples were run against a standard curve of 40, 20, 10, 5, and 2.5 ng produced by serially diluting a reference sample. For the 2016 assays, the average inter-plate variation for the T/S ratio was 14.4% and the average intra-plate variation of the Ct values was 1% for the GAPDH assays and 3% for the telomere assays. For the 2017 telomere assays, the average intra-plate variation of the Ct values was 0.3% for the GAPDH assays and 2.0% for the telomere assays,

and the inter-plate variation for the T/S ratio was 14.1%. For the 2018 telomere assays, the

average intra-plate variation of the Ct values was 1.3% for the GAPDH assays and 2.2% for the

telomere assays and the inter-plate variation for the T/S ratio was 13.9%.

We measured free IGF-1 levels using an enzyme-linked immunosorbent assay (ELISA).

Free IGF-1 refers to the percentage of hormones that is not bound to binding proteins. Typically,

most of IGF-1 is bound to specific binding proteins which can have distinct roles in either

enhancing or inhibiting IGF-1 actions as well as extending the half-life of the molecule in

circulation [43,44]. However, some studies consider free IGF-1 of greater physiological

relevance compared to total IGF-1 (i.e. bound and free IGF-1)[45–47]. Plasma IGF-1 levels were

measured in duplicate using a competitive ELISA developed at University of Debrecen. 96-well

NUNC microplates were coated overnight at 4°C with 100µl of an antibody raised against IGF-1

in rabbits. The capture antibody was incubated for 2 hours at room temperature (24°C) with 20

µl known concentrations (in serial dilutions starting at 500 ng/ml) of synthetic chicken IGF-1 or

20µl of sample and 100 µl biotinylated IGF-1 as a trace. After incubation, the microplate was

washed three times with 250 µl of PBS buffer (8g NaCl, 0.2g KCl, 1.44g Na2HPO4 and 0.24g

KH2PO4 in 1000 ml ddH2O, pH 7.4) containing 0.025% Tween 20. After washing, 100 µl of

streptavidin-horseradish peroxidase conjugate was added to all wells and incubated at room

temperature for 30 minutes, followed by another washing cycle (3 times). Then, 100 µl of tetra-

methyl-benzidine was added to the wells and incubated at room temperature for 30 minutes. The

enzymatic reaction was stopped by adding 100 µl of 1M H2SO4, and optical density was

measured at 450 nm (reference at 620 nm). The calibration curve was fitted using a 4-parametric

log-logistic curve, and concentrations of unknown samples were read off from this curve. We

used a chicken plasma in quadruplicate to determine intra- and inter-assay coefficient of variation (4.8% and 9.7% respectively).

Molecular sexing

Individuals from all years were sexed using established methods[48]. We amplified the highly conserved chromobox-helicase-DNA-binding genes: CHD-W and CHD-Z using the following forward and reverse primers: P8 (5'--CTCCCAAGGATGAGRAAYTG-3') and P2 (5'-TCTGCATCGCTAAATCCTTT-3'). PCR was performed in an Eppendorf Mastercycler Gradient thermal cycler. Reaction conditions for PCR amplification were as follows: 10µl of Dreamtaq Green PCR 2x Mastermix (Thermo Scientific), 1.0µl P2 and P8 primers, 6.0µl ddH$_2$0, 2µl template for a total volume of 20µl. PCR products were run out on 2% agarose gel and then males and females were determined via band size following established methods[48].

Statistical analysis

All data were analyzed using R v.3.2.3 (R Development Core Team 2015) statistical software. The R package lsmeans was used to conduct multiple post-hoc comparisons using Tukey's HSD[49]. We used restricted maximum likelihood to fit linear mixed effect models using the R package lme4[50]. For all years, we evaluated nestling growth rate as the slope of the linear phase of the growth curve during post-natal development for each nestling. Slopes for each nestling were calculated as the change in mass with age from day 2 to day 8 post-hatching. We then used linear mixed effects models to assess the relationship between growth rate, treatment, and telomere dynamics. To meet the linear mixed model assumptions for normality, the hormone and telomere values were log-transformed prior to running the analyses.

For analyses evaluating the effect of treatment on nestling growth rate we treated nestling growth rate as the dependent variable and treatment, sex, Julian date, year, and brood size were

treated as fixed effects. To investigate the effect of year on treatment we also included a year by treatment interaction term. All non-significant interaction terms were dropped from the final model. Lastly, we included nest ID and site location as random effects to account for variation in nest boxes and nesting sites.

For analyses evaluating the effect of treatment on nestling growth measures (i.e. culmen length, wing chord, and rectrix length) we treated either wing chord, culmen length, or rectrix length as the dependent variable and treatment, mass, age, sex, year, Julian date, and brood size as fixed effects with nestling ID as a repeated variable. To investigate how treatment impacted nestling growth measures over time and across years we also included two interaction terms: treatment*age, treatment*year, and treatment*age* year. All nonsignificant interaction terms were dropped from the final model. Lastly, we included nest ID and site location as random effects.

For analyses evaluating the effect of treatment on telomere dynamics (length and change in telomere length). To evaluate the effect of treatment on final telomere length we ran models with final telomere length as the dependent variable. Treatment, sex, brood size, and year were included as fixed effects. We included a treatment by year interaction term to evaluate the effect of year on treatment. All non-significant interaction terms were dropped from the final model. In the model with final telomere length as the dependent variable we also included initial telomere length as a fixed effect to account for random variation in starting length. To evaluate the effect of treatment on change in telomere length we ran a model with telomere length as the dependent variable and treatment, time (the number of days between sampling events), sex, Julian day, brood size, and year as fixed effects. We included a treatment*year*time interaction to evaluate the effect of year and time on treatment. All non-significant interaction terms were dropped from

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the final model. Lastly, we included nest ID, assay, and nestling site location as random effects with nestling ID included as a repeated variable.

Results

IGF-1 treatment

To verify our treatment was effective, in 2016, all nestlings received an IGF-1 or control injection at 10 days post-hatch and then a blood sample was taken approximately 30 minutes following the injection. Nestlings injected with IGF-1 had significantly greater levels of IGF-1 compared to control nestlings (*Tukey's HSD*, p<0.001, figure 14).

Nestling growth

At the start of the experiment, initial mass did not differ between treatments ($F_{1, 115.08}$=0.007, p=0.93) or across years ($F_{1, 76.22}$=0.389, p=0.67). At the start of the experiment, initial mass did not differ between treatments ($F_{1, 115.08}$=0.007, p=0.93) or across years ($F_{1, 76.22}$=0.389, p=0.67). During the experiment, we observed a significant interaction between year and treatment on nestling growth rate, where experimental birds had significantly greater growth rates compared to control birds, only in 2016 (*Tukey's HSD*, p=0.02, figure 15).

During the experiment, we observed a significant interaction between year and treatment on nestling growth rate, where experimental birds had significantly greater growth rates compared to control birds, only in 2016 (*Tukey's HSD*, p=0.02, figure 15). Similarly, we observed a significant year by treatment interaction on mass at day 10 post-hatching, where experimental birds were significantly heavier than control nestlings, but only in 2016 (*Tukey's HSD*, p=0.05, figure 16). Mass at day 10 post-hatching did not differ significantly with respect to treatment in 2017 (*Tukey's HSD*, p=0.99, figure 16) or 2018 (*Tukey's HSD*, p=1.00, figure 16). However, in 2018, we extended growth measurements until day 12 post-hatching and experimental birds were

significantly heavier at day 12 compared to control birds ($F_{1, 11}$=12.05, p=0.005, figure 17). In all years, the culmens of experimental chicks grew significantly faster as there was a significant interaction between treatment and age, ($F_{1, 511.70}$=4.05, p=0.04). In addition, there was a significant effect of treatment on rectrix length, but only in 2016 (*Tukey's HSD*, p=0.03), where experimental birds had longer tails compared to control birds. Although there was no overall effect of treatment on nestling wing chord, there was a weak interaction between treatment and age, which suggests that experimental birds tended to have longer wing chords over time than control birds ($F_{1, 582.11}$=3.03, p=0.08).
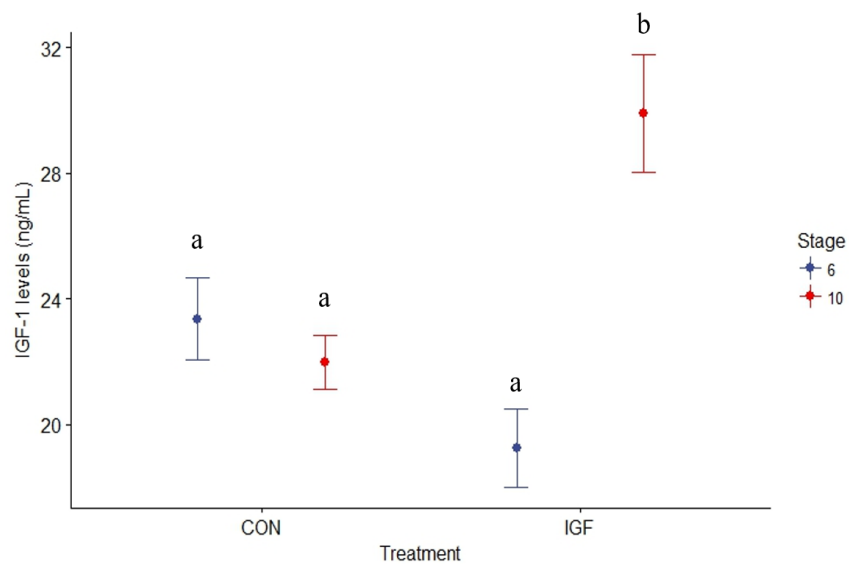

Figure 14. The relationship between free IGF-1 levels and treatment with respect to age in house sparrow nestlings in 2016 (mean±SEM). Dissimilar letters denote statistical differences between groups (Tukey's post-hoc test).
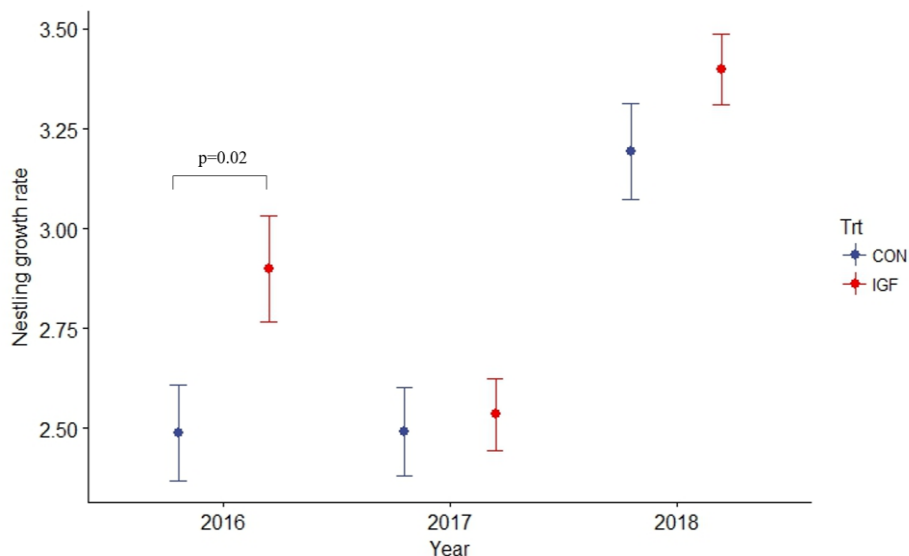
Figure 15. The relationship between growth rate (mean±SEM) and treatment by year in house sparrow nestlings during post-natal development. Growth rate was calculated as the slope of the change in mass with age from day 2 to day 8 post-hatch for each nestling. P-values are based on Tukey's HSD post-hoc test and significant differences, *P<.05

Telomere dynamics

Initial telomere length did not differ between treatments ($F_{1,61.21}=1.75$, p=0.19). However, there was a trend suggesting that initial telomere length differed by year ($F_{2,38.76}=3.01$, p=0.06), specifically, initial telomere length in nestlings was shorter in 2016 compared to 2017 (*Tukey's HSD*, p=0.06), though this was not significant. We observed a significant interaction between treatment and year, suggesting that effects of treatment on telomere length varied with respect to year ($F_{1,96.47}=3.58$, p=0.03, figure 18). However, multiple comparisons using Tukey's HSD revealed no significant differences with respect to treatment and year (figure 18). In 2018, at day 12 post-hatch, telomere length was significantly shorter in experimental birds compared to control birds ($F_{1, 1.91}=84.35$, p=0.01, figure 19). Treatment did not impact change in telomere length ($F_{1,64.81}=1.65$, p=0.20). However, telomere length varied significantly with respect to year ($F_{2,53.40}=8.21$, p=<.001), where nestlings in 2016 might experience a greater negative change in

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telomere length compared to nestlings in 2017 (*Tukey's HSD*, p=0.02), though this was not significant.

There was no significant difference in IGF-1 levels (prior to injection) at day 6 (*Tukey's HSD*, p=0.25, figure 14). In 2017 and 2018 all blood samples were collected prior to injection of IGF-1 or the control. In 2017, we observed no differences in IGF-1 levels with respect to treatment ($F_{1, 13.59}$=1.03 p=0.32) or age (i.e. day 6 or day 10 post-hatch) ($F_{1, 106.68}$=0.765 p=0.38). Similarly, in 2018, IGF-1 levels did not differ between treatment groups ($F_{1, 106.68}$=0.765 p=0.38) or age ($F_{1, 106.68}$=0.765 p=0.38).
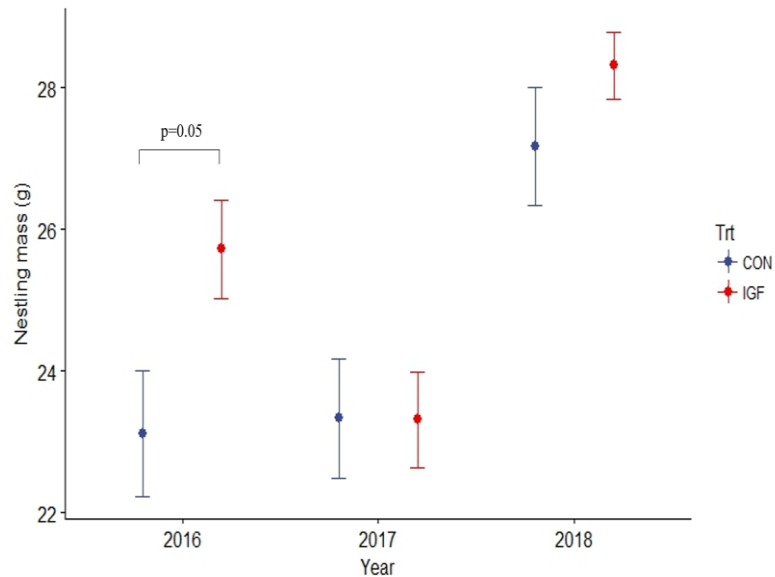
Figure 16. The relationship between mass at day 10 post-hatch (mean±SEM) and treatment by year in house sparrow nestlings during post-natal development. P-values are based on Tukey's HSD post-hoc test and significant differences, *P<.05
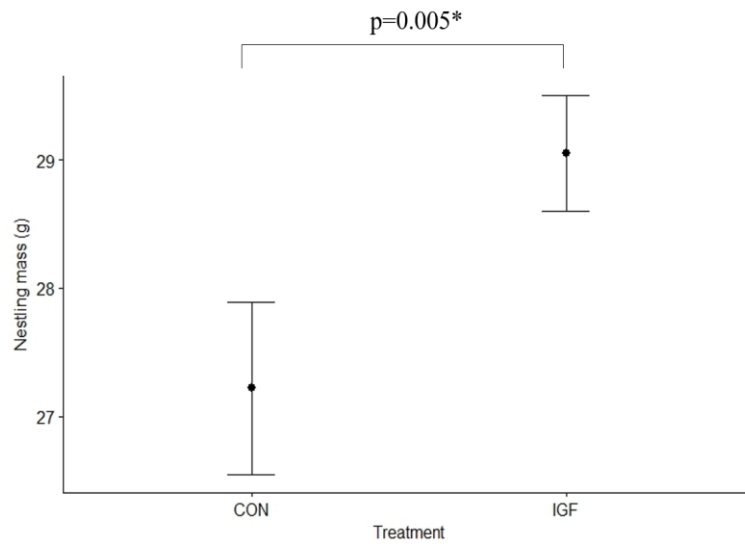
Figure 17. The relationship between mass at day 12 post-hatch (mean±SEM) and treatment in 2018 in house sparrow nestlings during post-natal development. P-values are based on Tukey's HSD post-hoc test and significant differences, *P<.05
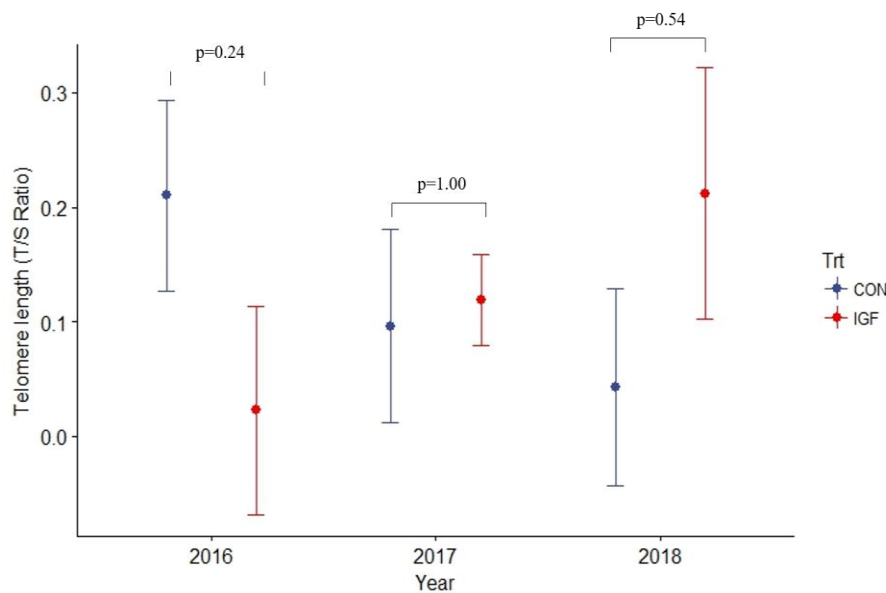
Figure 18. The relationship between telomere length at day 10 post-hatch (lnT/S ratio) and treatment by year in house sparrow nestlings in 2016 (mean±SEM). P-values are based on Tukey's HSD post-hoc test and significant differences, *P<.05
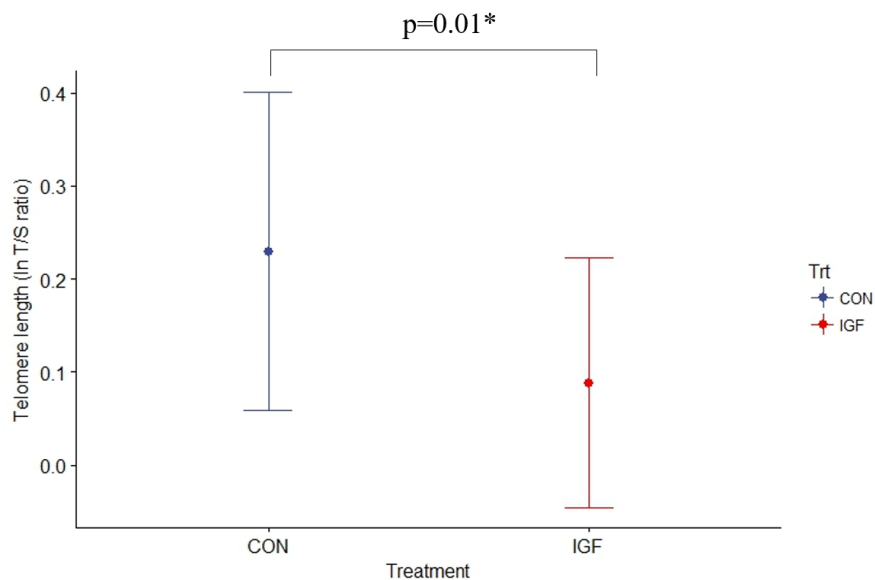
Figure 19. The relationship between telomere length at day 12 post-hatch (lnT/S ratio) and treatment by in house sparrow nestlings in 2018 (mean±SEM). *P<.05

Discussion

The results of this study suggest that manipulation of IGF-1 hormone through exogenous injections of rhIGF-1 affects nestling growth in house sparrows, but, the magnitude of these effects varies by year. Across years, experimental nestlings had significantly longer culmens over the post-natal growth period compared to control nestlings. Similarly there was trend for experimental nestlings to also have longer wing chords over the post-natal growth period, regardless of year. We also observed significant effects of treatment on final mass and nestling growth rate, with respect to year. In 2016, experimental nestlings had significantly greater growth rates and were heavier at day 10 post-hatching compared to controls and in 2018 experimental birds were significantly heavier than controls at day 12 post-hatch (figure 15,16,17). These findings are consistent with previous studies in birds administered exogenous IGF-1 and also with observed positive effects on growth [21,51]. However, these effects were not observed in 2017 (figure 15,16). The administration of exogenous IGF-1 often has mixed

results on growth in poultry strains that have been selected for fast growth [52], suggesting that if indvidiuals are already growing at maximal rates, administration of additional IGF-1 may have little effect. Lastly it's also important to note in our study that we were unable to identify whether increased mass was due to increased fat deposition or an increase in muscle mass, though previous studies in poultry have found relationships between IGF-1 injections and increased muscle development [51,53,54].

One factor that may contribute to variation in our experimental results among years is variation in resource availability. Thus, it may be that the effects of exogenous IGF-1 on nestling growth were more pronounced in 2016 as a result of a reduction in resource abundance or quality. Biological actions of IGF-1 are in part regulated by growth hormone (GH)[15], and when individuals are energy restricted, GH levels increase while IGF-1 levels decrease[55], thus if individuals were energy restricted in 2016 or during the latter part of the growth period in 2018, exogenous IGF-1 may have had a larger effect on nestling growth rates. Further, it also may be that nestlings in 2016 were under greater stress (potentially a result of variation in resources) compared to other years, and as a result we may only see trade-offs under these conditions. This scenario has been demonstrated in a long-lived strain of mutant flies, that under calorie restricted conditions flies were unable to maintain high fecundity, suggesting that tradeoffs may only be visible under stressful conditions [56].   Similarly, when the experimental period was extended to day 12 post-hatch in 2018, we observed a significant difference in nestling mass between experimental birds and control birds, where experimental birds were significantly heavier than control birds (figure 17). One reason we might have observed this effect in later nestling stages could be due to the fact that nestlings finally exceeded a threshold in which they were able to grow quickly and mediate damage to telomeres.

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In 2016, there was evidence suggesting that experimental nestlings also had shorter telomeres at day 10 post-hatch compared to their control counterparts, specifically when 2016 data is isolated from 2017 amd 2018 ($F_{1,96.47}$=3.58, p=0.03, figure 18). These findings suggest that IGF-1 might acclerate telomere shortening through it's effects on nestling growth rate. Previous studies have reported similar findings *in vitro*, where exogenous IGF-1 also resulted in shortened telomeres in skin fibroblasts [37]. However, there was no impact of treatment on change in telomere length within or across years. Interestingly, there was a trend suggesting that nestlings in 2017 experienced a greater negative change (i.e. loss) in telomere length, however, this was not significant.

We also took blood samples in 2017 and 2018 at day 6 and day 10 post-hatch prior to the injection of IGF-1. IGF-1 levels did not differ between treatment groups, age, or year. This is in contrast to a previous study in an altricial bird, the Pied flycatcher (*Ficedula hypoleuca*), demonstrated that IGF-1 levels were significantly greater in samples taken during the middle of the nestling growth period compared with samples taken at the end of the growth period [21]. One reason we might not have observed a difference between IGF-1 levels and age could be that we measured free levels of IGF-1, which represents the unbound fraction of the hormone in circulation. While free and total IGF-1 levels are positively related [47], there is evidence in humans that the ratio of free to total IGF-1 can vary with age and with respect to nutritional status [57,58], and thus changes in the proportion of bound hormone are not reflected in our data. It's also important to note that most data regarding the relationship between free IGF-1 and age comes from human studies in which free IGF-1 is measured over the course of many decades. It is currently unknown in free-living populations how free and total IGF-1 vary across

post-natal development, and whether free or total IGF-1 is more relevant within the context of life-history theory is largely unexplored.

Our study suggests that IGF-1 may act as mechanism underlying variation in growth and body size in house sparrow nestlings, however, the degree to which exogenous IGF-1 may impact nestling growth can vary with respect to year, possibly owing to differences in resource abundance. Further, while treatment with exogenous IGF-1 significantly increased mass at day 10 post-hatch and growth rate in 2016, and final mass (mass at day 12 post-hatch) in 2018. In addition, there was evidence suggesting that wing chord and culmen were longer in IGF-1 treated groups over the post-natal growth period compared to controls. In addition, there was some evidence to suggest that nestlings in 2016 also had shorter telomeres at day 10 post-hatch and day 12 post-hatch in 2018, though no differences in telomere length were observed in 2017. These results are suggestive of IGF-1 as a hormonal mechanism underlying the trade-off between growth and aging. However, future studies in free-living populations should consider additional ways to quantify nutritional status of individuals prior to and during any experimental manipulations.

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CHAPTER 5: CONCLUSION AND FUTURE DIRECTIONS

Major findings

In accordance with life-history theory, investment in growth is predicted to come at a cost to longevity [1,2]. In support of this, there are several correlational and experimental studies documenting a reduction in lifespan with increased investment in growth [3–5, see references therin]. Currently, there is great interest in elucidating the physiological mechanisms, such as hormones, that underlie these life-history tradeoffs [6–11]. One such hormone, insulin-like growth factor-1 (IGF-1), is likely to be particularly important due to its effects on multiple life-history traits including growth, reproduction, and lifespan across vertebrates [12–15]. My research investigated the role of insulin-like growth factor-1 as a mediator of the trade-off between growth and aging in wild birds. Specifically, I focused on four main questions: 1) Is IGF-1 correlated with growth and other life-history traits including lifespan across species? 2) If growth is experimentally manipulated via diet restriction does it influence IGF-1 and telomere dynamics? 3) Is natural variation in IGF-1 during post-natal development related to growth and telomere dynamics? and 4) Does experimentally elevated IGF-1 accelerate growth and telomere shortening during early life? To answer these questions, I conducted a series of manipulation and correlational studies using Franklin's gulls and house sparrows as a model system (a long-lived and short-lived bird species respectively).

In chapter 2, I manipulated growth rates of Franklin's gulls in the laboratory via diet. For our diet manipulation, chicks in the restricted treatment group were fed 60% *ad libitum* for a period of 10 days. Following the 10-day restriction, restricted birds were fed *ad libitum* for the duration of the study (until 40 days post-hatch). We found that our manipulation modified chick growth rate, however, we were unable to induce compensatory growth. Prior to restriction, IGF-1

levels did not differ between groups, however, IGF-1 levels were significantly reduced in the restricted birds following the diet restriction. IGF-1 levels were also positively related to mass and males tended to have greater levels of IGF-1 compared to females. These results are consistent with patterns reported in the literature across vertebrates [16–20]. Our study found no effect of treatment on telomere length or change in telomere length, suggesting that our diet manipulation was not sufficiently long enough or severe enough for telomere attrition to occur. However, an alternative explanation may be that gulls, as a long-lived seabird, may be able to modify their growth to changing conditions, but are unwilling to exceed a threshold that may induce long-term costs such as telomere attrition. Lastly, while treatment did not impact telomere dynamics, we found a significant correlation between IGF-1 levels and telomere length at day 40 post-hatch. Together, our results from chapter 2 suggest that IGF-1 may play an important mechanistic role underlying growth and telomere dynamics in a long-lived seabird.

Chapter 3 further investigates the relationship between IGF-1. growth rate, and telomere dynamics over the course of three years in a free-living population of house sparrows. We found that although nestling growth rates significantly differed among years, IGF-1 levels did not. Further, IGF-1 levels (either at day 6, 10 post-hatch, or mean IGF-1 levels) were not significantly related to nestling growth rate. In addition, telomere length and loss rate were not significantly related to nestling growth rates or IGF-1 levels at either day 6 post-hatch or day 10 post-hatch. However, there was a trend suggesting that nestlings with greater mean IGF-1 levels also had shorter telomeres, though this was not significant. However, future studies could determine whether this finding is biologically important. These results suggest that nestlings may have been able to mitigate, or delay costs (i.e. telomere length/loss) associated with growth to later life-stages. Uncontrolled variables could reduce the magnitude of a trade-off in natural populations,

and correlations alone provide only limited information [21]. For example, we were unable to control whether an individual was fasted or fed during our experiment. In previous studies in chickens, calorie restriction can reduce IGF-1 levels substantially, but it may take several days to restore these levels back to the baseline sample[22].

Thus, in Chapter 4, we investigated whether IGF-1 accelerates growth and cellular aging in part though effects on telomere dynamics. In this experiment we experimentally elevated IGF-1 levels by injecting nestlings with a either a daily dose of recombinant human IGF-1 (rhIGF-1) (experimental chicks) or the adjuvant only (control chicks) and collected growth measurements every 3 days until day 10 post-hatch. The results of this study were somewhat ambiguous. While our treatment was successful in altering growth rates and final mass of individuals in 2016, where experimental birds grew faster and were heavier, however, this effect was not observed in 2017. In 2018, we extended the experimental period to day 12, and we observed similar effects on final mass as in 2016, where experimental birds were heavier relative to controls. In addition, we observed differential effects of the treatment on telomere dynamics (both length and change in telomere length) looking across the three years. In 2016, there was a trend suggesting experimental birds in 2016 had shorter telomeres compared to control birds. In 2018, experimental birds had shorter final telomere length compared to control birds. One reason we may not have seen consistent effects could be due to variation in environmental conditions across years. In line with this idea, there is evidence in the literature to suggest that in some cases, trade-offs might only be visible under restricted conditions[23].

Future directions

This dissertation provides baseline knowledge regarding the relationships between growth, IGF-1, and telomere dynamics in two species of birds, one precocial and the other

altricial during post-natal development. Future studies might consider further investigation into key components of the IGF-1 system such as receptors, binding proteins, and how these components change with age. Firstly, it's important to note, that in chapters 3 and 4, we measured only free-IGF-1. Currently, most studies on free-living species have only measured total IGF-1, which is comprised of both the bound and unbound levels of the hormone[24–28]. Most of IGF-1 is bound in circulation (<90%) to one of six IGF-1 binding proteins (IGFBP), which have distinct roles in either enhancing or inhibiting IGF-1 actions [29]. For example, IGFBP3 is often associated with increased growth rates and nutrient intake an [18,30,31], whereas IGFBP4,5, 6 often inhibit IGF-1 actions by attenuating the hormone in circulation[29]. When bound, IGF-1 is inactive, though understanding the proportions of binding proteins in circulation and they respond to environmental conditions may give a better understanding of the role of IGF-1 and it's impacts on life-history traits in free-living populations.

Once released from a binding protein, biological actions of IGF-1 are regulated through the IGF-1 receptor (IGF1R) and to a lesser extent, the IGF-2, and insulin receptor (IR)[32]. In general, hormone receptor densities and expression are known to change with tissue type, age, seasonally, and in response to stress[33–38]. In chickens, IGF1R densities varied substantially between embryonic breast muscle and thigh muscle, with breast muscle having significantly greater IGF-1R concentrations[37]. However, little is known how IGF1R densities and binding changes during post-natal growth, with very few in non-model systems. Understanding how receptor densities and IGF-1 binding varies with age, season, and stress will be imperative to understanding the role of IGF-1 as a hormonal mechanism underlying life-history trade-offs.

Further, it is also important to understand how IGF-1 changes with respect to age for a specific study organism. Currently, most information available resides in laboratory or

domesticated species, and while the literature generally shows a decrease in free and total IGF-1 levels with age [39–41] but see[42], the time course for when IGF-1 levels begin to decline may vary between species. For example, in turkeys, a precocial bird species, total IGF-1 levels steadily increased until 40 days post-hatch and then began a slow decline until 200 days post-hatch[43]. Similar patterns are observed in chickens, where total IGF-1 levels have yet to decrease significantly by 11 weeks post-hatch [44]. In a study in altricial pied flycatchers, researchers found that total IGF-1 levels were greater at day 8 post-hatch (the middle of the growth period) compared to day 15 post-hatch (fledging) [26], however, it is unknown how IGF-1 levels changed prior to and in between those sampling periods. In contrast, in our study (Chapter 3), in altricial house sparrows, we found no difference between free IGF-1 at day 6 and day 10 post-hatch. We chose day 6 post-hatch to sample IGF-1 levels in part because it resided in the middle of the linear phase of growth, however, having a full understanding of how IGF-1 levels (either free or total) change over the post-natal growth period will provide a better idea of when to sample. Further, importantly, many of the studies in poultry can control for the nutritional status of an individual. It is likely important to know for free-living species whether fasted or non-fasted IGF-1 levels are related to life-history traits, however, implementation of this in the field will require careful planning.

In chapter 2 and 3 our studies did not find a relationship between telomere dynamics and growth rate, though previous studies have demonstrated telomere loss with respect to growth [45–47]. Future studies might consider investigating additional factors that might influence telomere length. For example, telomerase is an enzyme that actively restores DNA to the end of telomeres, and acts to reset telomere length at fertilization [48,49]. Typically, telomerase is down-regulated by the end of embryonic development[50], while telomerase activity is expected

to be down-regulated in post-mitotic somatic tissues following embryonic development [51]. There is some evidence in birds that suggests long-lived birds may not down-regulate telomerase in post-mitotic tissues [52]. However, little is known about when telomerase is down regulated during post-natal development and whether variation in telomerase during early life can influence telomere length. Interestingly, while telomere length may not have been strongly related to IGF-1 levels in these chapters there is evidence to suggest that genetic variation in the IGF-1/Insulin pathway and telomere maintenance pathways (which includes telomerase) is associated with human longevity[53], suggesting IGF-1 may still play an important role underlying variation in life-history traits such as aging.

This research is a preliminary step to investigating the role of IGF-1 as a mediator of life-history trade-offs in both a short and long-lived species. Given that this hormone and its components (i.e. binding proteins and receptor densities) respond plastically to environmental conditions more studies assessing the role of IGF-1 in a greater diversity of species across different life stages in a variety of environments are critically needed.

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