Az inzulin-szerű növekedési faktor-1 szerepe a barkóscinege életmenet döntéseiben

The role of insulin-like growth factor-1 in life-history decisions in the Bearded reedling

Thesis for the Degree of Doctor of Philosophy (PhD)

by **Zsófia Tóth**

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Debrecen, 2023

I hereby declare that I prepared this thesis within the Doctoral Council of Natural Sciences and Information Technology, Juhász Nagy Pál Doctoral School, University of Debrecen in order to obtain a PhD Degree in Natural Sciences at the University of Debrecen.

Debrecen, 2023. . . . . .

……

Zsófia Tóth

I hereby confirm that candidate Zsófia Tóth conducted her studies with my supervision within the Biodiversity Doctoral Program of the Juhász- Nagy Pál Doctoral School between 2015 and 2018. The independent studies and research work of the candidate significantly contributed to the results published in the thesis.

I also declare that the results published in the thesis are not reported in any other theses.

Debrecen, 2023. . . . . . ……...

Dr. Ádám Zoltán Lendvai

The role of insulin-like growth factor-1 in life-history decisions in the Bearded reedling

Dissertation submitted in partial fulfilment of the requirements for the doctoral (PhD) degree in Biology.

Written by **Zsófia Tóth** certified Biologist
in the framework of the Juhász-Nagy Pál Doctoral School of the University of Debrecen (Evolution and Biodiversity programme)

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Table of Contents

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"Changing your mind in the light of new ideas or better evidence constitutes scientific progress"
Tim Birkhead

Part 1.

In this part, I briefly overview the background for the field of my thesis, followed by a brief outline of the objectives, methods, and results of the publications that belong to my thesis. In the end, I summarise the implications of these publications.

1.1. General Introduction

1.1.1. Life-history theory

Life-history theory is one of the branches of evolutionary biology, which aims to explain the causes and consequences of variation in life cycles (Flatt and Heyland, 2011; Stearns, 1993). Life-history theory examines the variation in genotypes and phenotypes that determine the organisms' survival and reproductive success (Roff, 2002). Life-history traits are characteristics that affect the life course and, ultimately, the fitness of an organism. The most often investigated life-history traits include size at birth, age, and size at maturity, number, size, and sex ratio of offspring, reproductive investment, and lifespan.

These traits fundamentally influence life-table parameters such as juvenile and adult survival or the number of offspring. During evolution, these traits became highly variable among- and within species in response to the given circumstances to maximise their fitness (relative reproductive success) (Stearns, 2000). However, due to the limitation of resources, it is not possible to maximise all life-history traits. These limitations lead to resource allocation dilemmas between current and future reproduction and survival, resulting in energetic trade-offs between life-history traits that may cause negative phenotypic correlations (Stearns, 1989; Zera and Harshman, 2001). However, the variation in the acquisition and allocation of resources among individuals (e.g. some individual has more resources than others) can lead to a positive relationship between life-history traits, when a negative correlation is expected (van Noordwijk and de Jong, 1986).

One of the most robust patterns is that fecundity is inversely related to lifespan. This antagonistic relationship between survival and reproduction is a central tenet in life-history theory and is explained by the "costs of reproduction" (Williams, 1966). Cost can be defined as an advantageous change in a fitness trait that negatively impacts another (Stearns, 1989). The costs of reproduction can be either ecological (due to reproductive activity, when the parents are more exposed to predators or infections) or intrinsic (trade-off between the different life-history traits for a share of limited energy reserves) (Harshman and Zera, 2007). Because resources are often

limited in nature, the idea that life-history trade-offs are the result of resource constraints became a fundamental concept of life-history theory (Roff and Fairbairn, 2007). This concept is described as the “Y-model”, where the resources enter at the base of “Y” and will limit the possible allocations into different traits (de Jong and van Noordwijk, 1992; Reznick et al., 2000; Zera and Harshman, 2001). Even though the variation in life-history strategy could be endless, we can see that the life-history strategies are placed along a “fast-slow” continuum (Stearns, 1983). Along this continuum, we can find organisms that invest more energy to survival over reproduction (i.e. slow life history) and vice versa (i.e. fast life history) (Bauwens and Diaz-Uriarte, 1997; Blackburn, 1991; Promislow and Harvey, 1990; Saether, 1988). These differences in life-history strategies among individuals, species, or populations are also influenced by physiology or behaviour. The concept that integrates how the variation in life history, behaviour, and physiology is interconnected with one another is named “pace-of-life” syndrome (POLS) (Fig1.; Dammhahn et al., 2018; Mathot and Frankenhuis, 2018; Réale et al., 2010; Ricklefs and Wikelski, 2002). This thesis focuses on unraveling the intricate role of physiology – specifically, the impact of hormones – in actively shaping life-history strategies within the 'pace-of-life' syndrome framework.

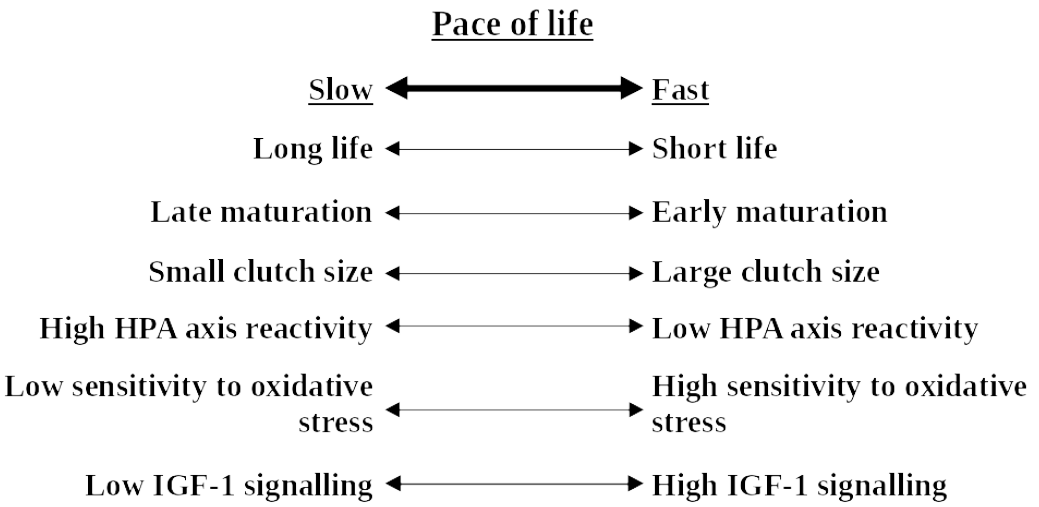

Figure 1: Schematic graph of the variation in different traits along the fast-slow continuum. Double arrows between the traits mean continuous variation in traits among individuals in a population. HPA-axis = Hypothalamic-pituitary-adrenal axis. IGF-1 = Insulin-like growth factor 1.

1.1.2. Hormonal mediation of life histories

Hormones are chemical messengers travelling through the bloodstream and influence almost all aspects of phenotype, such as metabolism, immune functions, morphology, development, or life-history. Hormones integrate information from both external (i.e. ecological or demographic processes) and internal (i.e. nutritional condition, age-specific survival) environments and act as a mediator to shape the best response to the given circumstances (Zera and Harshman, 2001). These factors together determine the differences in transitions between life-history stages and the investment to competing life-history traits that influence fitness. optimising life-history strategies involves the energy transitions between competing life-history traits (i.e. reproduction and self-maintenance), which involves multivariate selection (Ricklefs and Wikelski, 2002; Roff and Fairbairn, 2007). Traditionally, it has been thought that the cause of these trade-offs is the limited internal energy reserves (van Noordwijk and de Jong, 1986). However, later studies showed that the surgical removal of gonads increased the longevity of *Caenorhabditis elegans*, which suggests that the trade-offs might not be the cause of limited resources but rather the molecular signal originating from the gonads (Hsin and Kenyon, 1999). Therefore, another view has emerged, which states that the "pleiotropic" effects of regulators cause the trade-off between life-history traits (Leroi, 2001). Because hormones can simultaneously regulate many traits (hormonal pleiotropy), they are key mediators of life-history trade-offs between reproduction and lifespan (Martin et al., 2011; Wingfield et al., 1998). Since many traits of the phenotype are under endocrine control, hormones might be major determinants of pleiotropy, life-history correlations, and trade-offs. Although many signalling pathways have a role in shaping phenotype, in this thesis, I will focus on the role of insulin-like growth factor-1 (IGF-1) in the determination of life-history strategies in a common passerine, the Bearded reedling (*Panurus biarmicus*).

The insulin/insulin-like signalling pathway (IIS pathway), is an evolutionarily highly conserved molecular mechanism, and the ligands that belong to this pathway can be found in invertebrates and vertebrates as well (Brogiolo et al., 2001; Butler and LeRoith, 2001). Invertebrates have many insulin-like ligands, while vertebrates have only three: insulin, insulin-like growth factor-1 (IGF-1), and insulin-like growth factor-2 (IGF-2)(Brogiolo et al., 2001; Butler and LeRoith, 2001; Jones and Clemmons, 1995; Leevers, 2001). Insulin's main role is to regulate glucose homeostasis, IGF-2 regulates the development of the embryo and fetus, while the primary function of IGF-1 is to coordinate growth and development in already hatched/born individuals. In vertebrates, the IIS pathway is integrated into the Hypothalamic-Pituitary-Somatotropic (HPS) axis. Briefly, the pituitary secretes growth hormone (GH) which stimulates the secretion of insulin-like growth factor-1 (IGF-1), primarily from the liver, but IGF-1 secretion can occur in other tissues/organs such as gonads. IGF-1 travels through the bloodstream to regulate growth in the target tissues and mediate anabolic and catabolic processes (Roith, Scavo, and Butler, 2001). IGF-1 is one of the key factors regulating the metabolism and development of vertebrates concerning their nutritional status

(Dantzer and Swanson, 2012; Lodjak et al., 2016; Taguchi and White, 2008). IGF-1 production and secretion are suggested to decrease due to low nutrient/energy availability, which leads to an upregulation of cell recycling, autophagy, and apoptosis (Adler and Bonduriansky, 2014; Bitto et al., 2010). These processes can affect fitness by down-regulating reproduction, growth, and immune response (Gao et al., 2019; Wang and Levine, 2010). Therefore, IGF-1 can influence the critical life-history traits and trade-offs among growth, lifespan, and reproduction (Dantzer and Swanson, 2012, Emlen et al., 2012; Harshman and Zera, 2007). Even though IGF-1 influences many life-history traits, we know less about how IGF-1 acts as a mediator between competing life-history traits in wild animals. Therefore, we aimed to test the role of IGF-1 in life-history decisions in a wild bird.

During the past decades, it became obvious that the relationship between hormones and fitness varies across life-history stages (Crossin et al., 2016; Lattin et al., 2012), and hormones are key mediators of the transition between life-history stages (McMenamin et al., 2022; Tonra et al., 2013). One of the most studied transitions between life-history stages is when organisms enter the “emergency life-history” stage, which helps them to survive unpredictable events. The transition to the “emergency life-history” stage is mediated by many physiological factors, that regulate energy re-allocation from energetically demanding processes (i.e. reproduction) toward survival (Wingfield et al,. 1998). Since the level of IGF-1 is strongly related to catabolic and anabolic processes (i.e. energy allocation), it can have a key role in initiating the “emergency life-history” stage. Unfortunately, our understanding of how IGF-1 initiates the emergency life-history stage, and responds to stressful events remains unclear. Therefore, we aimed to study how different stressors can influence IGF-1 levels (Study 1 and 2). First, in a capture-handling procedure we tested how IGF-1 responded to restraint stress (Study 1). Even though, our main focus was to study IGF-1, we also investigated corticosterone (cort: the main glucocorticoid hormone mediating stress response in birds) in two reasons. First, we need to know if the birds perceived the handling as a stressor, and for that, we can compare the cort levels before and after the handling, as cort is highly responsive to stressors in many species. Second, cort can influence the response of other physiological factors to stress via its downstream effect. Therefore, to investigate the role of IGF-1 in stress response, we need to know whether IGF-1 is independent from cort. Therefore, we tested whether IGF-1 is an independent regulator of the stress response (Study 1). IGF-1 levels are known to change dynamically in response to changes in food availability (i.e. nutritional condition) in humans and agricultural animals, however, our knowledge how IGF-1 react to food availability in animals from natural populations are scarce. Food shortages are one of the main stressors that almost all organisms need to cope with. Therefore, one of the aims of the current dissertation was to study how IGF-1 reacts to food availability in Bearded reedlings (Study 2).

IGF-1 is negatively related to lifespan (Holzenberger et al., 2003; Lewin et al., 2017), but has a positive effect on growth, sexual maturation, and reproduction (Crain

et al., 1995; Flatt et al., 2008; Lewin et al., 2017; Lodjak et al., 2014; Pine et al., 2006; Sparkman et al., 2010; Swanson and Dantzer, 2014; Yakar et al., 1999). For example, the igf1 KO mice (mice where the igf1 gene was knocked out) lived longer than normal mice, but igf1 KO mice were infertile (Dantzer and Swanson, 2012). Higher IGF-1 levels are associated with a shorter lifespan in birds and mammals (Lewin et al., 2017; Lodjak et al., 2018), and we also know that higher IGF-1 levels are associated with higher reproductive rates in birds, mammals and reptiles (Lewin et al., 2017; Lodjak et al., 2018; Sparkman et al., 2009). Although IGF-1 may have the opposite effect on lifespan, and growth/reproduction, we do not know the exact mechanism of how IGF-1 control this trade-off. According to one of the hypothesis, IGF-1 has a negative effect on lifespan, because it interacts with the organism's anti-oxidant defense mechanisms and thus high concentrations of IGF-1 may increase the presence of the reactive oxygen species (ROS) in the blood and cause oxidative damage (Dantzer and Swanson, 2012). Therefore, one of the questions we aimed to study is: whether IGF-1 increases oxidative damage and whether it has a life-shortening effect in bearded reedlings (Study 3).

Higher IGF-1 levels are associated with a faster growth rate but shorter lifespan within a species (Greer et al., 2011), and among species (Swanson and Dantzer, 2014). One of the main roles of IGF-1 is to mediate growth and development, but most of the studies focused on post-natal growth (Lewin et al., 2017; Lodjak et al., 2014,2017; Reinecke et al., 2005; Sparkman et al., 2010). The term ‘growth’ often refers to growth during embryonic and postnatal development. However, many organisms maintain growth throughout their lifetime (e.g. fish, amphibians and reptiles) and, even in animals that have reached their final adult body size, somatic growth events frequently occur. One particularly interesting example of such processes is moulting/shedding. IGF-1 induces the proliferation of hair follicles and elongates their active phase, furthermore, IGF-1 increases the rate of hair growth (Ahn et al., 2012; Li et al., 2014; Weger and Schalke, 2005). It seems that IGF-1 has an important role in integument development in rodents and human tissue cultures. Still, we have scarce knowledge on the role of IGF-1 in moulting and development of feather ornaments. It was shown previously that IGF-1 is higher in shedding (term for moulting in reptiles) compared with non-shedding garter snakes (*Tamnophis elegans*) (Sparkman et al., 2009). Furthermore, it was shown that the stress-induced moult was associated with increased IGF-1 levels in broiler chickens (Mazzuco et al., 2005). Since, moulting is a crucial life-history stage in birds, and the feathers have a role in insulation, flight and even in mate choice, studying how IGF-1 influences moult and the development of feather ornaments can give us insights the role of IGF-1 in life-history decisions. Therefore, in the second part of the recent dissertation, we aimed to study how IGF-1 influences feather growth rate, feather quality (Study 4), and ornamental traits (Study 5) in Bearded reedlings.

1.2. General methods

1.2.1. Study species

The Bearded reedling (*Panurus biarmicus* Linnaeus, 1758) is a small (~14g), resident passerine. Their average life expectancy is 2-3 years with high juvenile mortality, but the oldest male was recaptured 6 years after its initial ringing as nestling (Hořák et al., 2003). Beraded reedling has an extraordinarily fast life history within passerines (Lendvai, 2023). When the population size and the density is low, the pairs may start building the next nest when their nestlings are 3-10 days old, leading to the pairs having overlapping nests in time (Stępniewski and Halupka, 2018). The females are starting to produce the new clutch and they incubate only the new eggs, till males take care of the nestlings of the first nest and help the female with the duties of the new nest and clutch (Stępniewski and Halupka, 2018). They also start to breed very early, we saw the earliest fledglings on the 3rd of April in 2016 (personal observation). They start to breed early in the year, and their average breeding cycle is 15-21 days, which makes it possible to produce up to 4 clutches within one breeding season (Stępniewski and Halupka, 2018).

Both sexes provide care, therefore, both sex are choosy for their partners. Both sexes have sexually selected traits. Both sexes have elongated tail feathers, which play an essential role in mate choice (Romero-Pujante, 2002). Females prefer males with longer tails, but males not always discriminate between long- or short-tailed females (Romero-Pujante, 2002). Males have another sexually selected ornament, the black beard under their eyes (Hoi and Griggio, 2008, 2010). Hoi and Griggio (2008) showed in a mate choice experiment that the females prefer males with experimentally elongated beard feathers. Since Bearded reedlings have a fast life history, and fitness-related sexually selected traits, it is an excellent model system to study how different hormones influence their life-history decisions.

1.2.2. Housing protocol

During my PhD work, it was necessary to keep the birds in captivity for several experiments. We always transferred the birds to one of the outdoor aviaries located in the Botanical Garden of the University of Debrecen, except in case of Study 5, in which the birds were kept in the outdoor aviary of Konrad Lorenz Institute of Ethology, University of Veterinary Medicine, Vienna, Austria. In those aviaries, we provided the birds with reed bundles, a $1m^2$ of water surface to mimic natural conditions, and small artificial nesting sites (e.g. nestboxes or small brackets). During the acclimation period, we provided *ad libitum* food, and water for the birds. According to the study protocol, we slightly modified the housing of the birds; details are described in the “Objectives, methods and major results” section. In Study 3 and 4, we worked on the same flock of birds. We injected them with IGF-1 and tested how IGF-1 influenced mortality (Study 3) and moulting (Study 4).

1.2.3. Blood sampling

We took baseline blood samples from every individual in all studies included in the recent dissertation. In case of “Study 1”, we took a second blood sample 15 minutes later as stress-induced samples, and in the second part of “Study 1” we took only one blood sample after 15 minutes of cort manipulation. During sampling, we always respected international standards, and the health of the experimental birds was always considered a priority (Owen, 2010).

1.2.4. Corticosterone manipulation

To manipulate the cort level individually, we used oral cort manipulation. We fed the birds with cort filled mealworms: (1) control, no cort (only 20 µl peanut oil); (2) low cort, 0.2 mg/ml (4 µg cort + 16 µl peanut oil); and (3) high cort, 0.5 mg/ml cort concentration (10 µg cort + 10 µl peanut oil). The cort concentrations were calculated based on previous studies and the general body mass of the studied species (Breuner et al., 1998; Lõhmus et al., 2006; Spencer and Verhulst, 2008).

1.2.5. IGF-1 manipulation

We used dissolved poly-(lactic-co-glycolid acid) microspheres loaded with IGF-1 in a dispersion medium IGF-1 as a treatment and only the dispersion medium as a control. We injected 100 µl solution subcutaneously between the shoulders of each birds, and the treated birds received a total of 600 ng IGF-1 per injection. See the description the microspheres in Luginbuehl et al., 2013, and the details of the adaptation of this method on bearded reedlings in Lendvai et al. 2021.

1.2.6. Physiological assays

We used a competitive enzyme-linked immunoassay (ELISA) to measure plasma IGF-1 levels from the samples developed in our laboratory at the University of Debrecen. We measured optical density (OD) at 450 nm (reference at 620 nm) using a Tecan microplate reader. Detailed methods of the assay validated for Bearded reedling are described in Mahr et al. (2020).

We measured plasma cort levels with direct radioimmunoassay (RIA). We counted the radioactivity of the bound fraction in a liquid scintillation counter (QuantaSmart) see details in Lendvai, Bókony and Chastel, 2011. We measured plasma MDA (malondialdehyde, which is a marker to measure oxidative damage caused by ROS) levels at Babes-Bolyai University (Cluj-Napoca, Romania) with High-Performance Liquid Chromatography (HPLC) with UV detection at 254 nm. Detailed methods of the MDA assay are described in Bókony et al. (2014).

1.3. Objectives, methods and major results

For a detailed description of methods, including study design and statistical methods, see the Appendix.

Study 1.

(i) Objectives

The possible hormonal regulators of trade-offs need to collect information about resource availability and forward a signal to the relevant parts of the organism to regulate energy expenditure in the face of environmental variation (Harshman and Zera, 2007). One possibility to explore is whether the given hormonal pathway may have this life-history regulatory function to expose individuals to stressors. Stressors create a potentially life-threatening situation, requiring a fundamental reallocation of energy-consuming processes. Stressors can be easily standardized, and the response to them is ecologically relevant because to maximise fitness, all organisms must be able to cope with environmental changes, such as predator attacks, inclement weather, or food shortages (Hawlena and Schmitz, 2010; Romero et al., 2000; Wingfield et al., 1998). Therefore, understanding the response to stressors of the given hormone can provide great insight into its possible role in shaping life-history strategies.

IGF-1 levels are expected to change under handling and nutritional stress. For instance, in response to restraint stress, circulating IGF-1 levels and other components of the IIS pathways decreased in pigs *Sus scrofa domesticus* (Farmer et al., 1991; Wirthgen et al., 2017), and fish (Davis and Peterson, 2006; Wilkinson et al., 2006) suggesting that IGF-1 is a relevant hormone of the stress response. However, it is not clear whether the change in IGF-1 levels is triggered by the stress-induced change in glucocorticoids (GCs; known as “stress hormones”) or it is the direct consequence of the stressor (Davis and Peterson, 2006; Dell et al., 1999; Unterman et al., 1993). Previously it was shown that GC administration decreased IGF-1 levels in chicken (Leili and Scanes, 1998), fish (Kajimura et al., 2003; Peterson and Small, 2005), and rats (Gayan-Ramirez et al., 1999), but this effect exist only at high GC concentrations or prolonged GC exposure (Bossis and Porter, 2003; Davis and Peterson, 2006; Kajimura et al., 2003).

Until recently, the effects of stressors on IGF-1 levels or the mechanistic link between HPA (Hypothalamic-Pituitary-Adrenal axis) and HPS axis in any free-living organism was not studied. Therefore, we aimed to study **whether (Q1) an external stressor affects plasma levels of IGF-1 and whether (Q2) cort (the main GC stress hormone in birds) directly affects IGF-1 levels**.

(ii) Methods

In this study, we used two different methods to test the IGF-1 stress response. First, we captured 17 wintering free-living Bearded reedlings between September 2015

and January 2016. We used a standard capture-handling-restraint protocol to trigger a physiological stress response (Wingfield, 1994). We took the first blood sample as soon as the bird was captured, then placed the birds in a cloth bag until the following blood sampling. We took the second blood sample 15 minutes after the initial capture.

Second, we captured 21 wintering Bearded reedlings between 18th October and 16th November 2016. We transferred them to an outdoor aviary, where they spent four months. At the onset of the study, we moved the birds to individual cages and manipulated their cort level using a non-invasive, oral manipulation via mealworms filled with: no cort (control), low (0.2mg/ml), or high cort dose (0.5mg/ml). All birds were assigned to one of the treatment groups, we gave to the birds through the backdoor of their cage a mealworm filled with one of the cort concentrations or just solvent according to their treatment group and we took blood samples 15 minutes after the birds consumed the mealworms. We repeated the procedure again, where every bird was assigned to a different treatment group.

(iii) Results and conclusions

In the first part of the study, we found that cort levels were significantly higher, while IGF-1 levels were significantly lower after the 15-minute restraint stress. Sex and body mass did not influence cort levels, while IGF-1 levels were higher in males than in females, but body mass did not affect IGF-1 levels. Our results are consistent with previous studies showing that cort increases in response to stress, and suggest that our handling was stressful for the birds (Remage-Healey & Romero, 2001; Buehler et al., 2008). However, our result, namely IGF-1 decreasing due to handling stress is in line with previous studies on captive fish (Davis and Peterson, 2006; Wilkinson et al., 2006; Wirthgen et al., 2017), in other bird studies, they showed that IGF-1 level is unrelated to handling time in nestlings (Lodjak, Mägi and Tilgar, 2014). The effect of stressors can be age-specific, as shown previously in fish and chicken (Perrot et al., 1999; Yun et al., 2005), which can explain why IGF-1 decreases in adult birds, while it does not change in nestlings. IGF-1 regulates the anabolic processes and hinders the catabolic effects of cort. Therefore the decrease of IGF-1 due to handling stress is consistent with the allostatic concept of the stress response (McEwen and Wingfield, 2003) i.e. organisms need to suppress energetically costly anabolic processes (such as immune function) and reallocate energy into those physiological and behavioural processes that promote survival (Wingfield et al., 1998).

Due to our results and the role of IGF-1 in shifting metabolism from anabolic to catabolic processes, we expected that a higher cort stress response would be related to a more significant decrease in IGF-1 levels. But we did not find such a relationship between IGF-1 and cort at the baseline level, and the change in cort and IGF-1 levels was also unrelated. In the second half of the study, we found that cort manipulation increased cort levels, but did not influence IGF-1 levels. These results suggest that the HPA and the HPS axes are independent of each other at the endpoint of their hormonal cascade. However, Lodjak, Tilgar, and Mägi (2016) showed that the relationship

between IGF-1 and cort in nestlings may depend on the individual's nutritional status. The relationship between IGF-1 and cort was positive in nestlings with better nutritional conditions, while this was negative in birds with the worst nutritional conditions. In our study the birds were kept on ad libitum food availability, therefore, our captive birds were in good condition (good nutritional condition) and they may be able to maintain both IGF-1 and cort levels as well. This idea was supported by a medical study that showed that normally fed individuals can diminish the protein catabolic effect of glucocorticoids by maintaining their IGF-1 levels compared with starved individuals (Botfield, Ross, and Hinds, 1997). However, this hypothesis remained untested in natural populations, and we intend to test the IGF-1 response to food restriction see details in Study 2.

Study 2.

(i) Objectives

If resources are limited, the organisms need to decide which life-history traits they invest more energy into (van Noordwijk and de Jong, 1986). One of the primary resources that determine life-history strategies is food. Therefore, the physiological mechanisms that play a role in nutrient sensing could have a key role in mediating energy allocation between competing life-history traits. Because food availability is often unpredictable and food shortages generally happen in nature, the organisms had to develop physiological adaptations to cope with fluctuations in food availability (Groscolas and Robin, 2001; Harshman and Zera, 2007; Killen et al., 2011). However, we know that IGF-1 responds to changes in food availability (Berryman et al., 2008; O'Sullivan et al., 1989; Rahmani et al., 2019), and our knowledge of IGF-1's role in facilitating an appropriate response to changes in food availability in wild animals are limited. As far as we know, only a handful of studies examined the effect of food restriction on IGF-1. Duncan et al. (2015) showed that the IGF-1 level decreased in *Sceloporus undulatus* when they had limited access to food. Furthermore, IGF-1 levels differed between the two ecotypes of garter snakes (*Thamnophis elegans*), and they showed that IGF-1 was higher in those ecotypes (lakeshore snakes) which have continuous access to food, compared to those ecotypes (meadow snakes) where food availability is varying annually (Sparkman et al., 2009). **Therefore, we aimed to study how IGF-1 responds to fluctuations in food availability in a wild-caught captive population of Bearded reedlings**.

(ii) Methods

We caught 24 juvenile Bearded reedlings at Hortobágy-Halastó, in July 2017. After the capture and the morphometric measurements, we transferred the birds into an outdoor aviary. The birds spent 7 months in the aviary before the experimental procedure to acclimate to the captive conditions. During this period, *ad libitum* food and water were provided to the birds. We captured the birds two weeks before the experiment; we weighed and transferred them into individual cages located in the

outdoor aviary. All birds were provided with ad libitum food and water. We established two dietary regimes: control (110% of individual daily food consumption) and restricted diet (70% of individual daily food consumption), and we exposed the individuals to both dietary regimes twice in a row. One dietary regime lasted for 3 days; then the birds were fed *ad libitum* for one day before they got the other dietary regime for 3days. We let the birds recover for another 3 days on *ad libitum* food, then we repeated the experiment. That means all birds were assigned twice to control and twice to a restricted diet. We measured body mass and circulating IGF-1 levels after each dietary treatment. Due to the repeated experimental design, we could disentangle among- and within individual hormonal variations.

(iii) Results and conclusions

Food restriction decreased all individuals' body mass, indicating an effective and physiologically challenging treatment. IGF-1 was positively associated with body mass, corroborating previous findings in different taxa such as fish, reptiles, and mammals (Cameron et al., 2007; Crain et al., 1995; Sparkman et al., 2009). Males had higher IGF-1 levels than females, but if we controlled for body mass, the sex difference in IGF-1 levels disappeared. These findings suggest that the sex differences originate from the sex-dependent body mass differences rather than the sex itself, but this relationship needs to be tested in future studies.

We found that overall, food restriction increased IGF-1 levels. Still, at the individual level, we found high among-individual variance in response to food restriction, i.e. some individuals decreased, others showed little response or increased their IGF-1 levels, and the within-individual response was highly repeatable. This result contradicts previous findings, where they found a consistent decrease in IGF-1 levels due to food restriction (Morishita et al., 1993; Schew et al., 1996). In our study, the response of IGF-1 to food restriction was also affected by the individual's body mass, namely, individuals larger than the average were more likely to decrease their IGF-1 levels, while birds lighter than the average were more likely to increase their IGF-1 levels. IGF-1 influences energy metabolism by controlling glucose uptake via suppressing insulin activity (Aguirre et al., 2016). Also, IGF-1 regulates preadipocyte differentiation and increases lipogenesis to facilitate the formation of fat reserves (Scavo et al., 2004; Smith et al., 1988), which allows organisms to build up energy storage to survive unpredictable environmental events. After the differentiation of preadipocytes, the adipose tissues stop producing IGF-1 receptors, and only a high concentration of IGF-1 can effectively prevent lipolysis, and stimulate glucose transport (DiGirolamo et al., 1986). The IGF-1 role in metabolism and anabolic processes and our results suggest that the birds in different conditions may have different coping mechanisms against food restriction. **Lighter individuals** may increase their IGF-1 level in response to food restriction to maintain their energy homeostasis and compensate for the noxious effect of protein degradation and apoptosis, such as muscle atrophy (Musaró et al., 1999; Timmer et al., 2018) and slow

down weight loss (O'Sullivan et al., 1989). On the other hand, **heavier individuals** decrease their IGF-1 levels in response to food restriction to increase their blood glucose levels via gluconeogenesis and suppress insulin activity (Yakar et al., 2004). The amount of released energy may be enough to survive the fluctuation in food availability. However, this hypothesis needs to be tested.

Study 3.

(i) Objectives

The insulin/insulin-like signalling pathway (IIS) has been suggested as a key physiological mechanism regulating lifespan and ageing (Berryman et al., 2008; Dantzer and Swanson, 2012; Holzenberger et al., 2003; Kappeler et al., 2008; Piper et al., 2008). IGF-1, as the primary hormone of the IIS pathway in vertebrates, at high levels, stimulates growth, and reproduction, but increases mortality (Dantzer and Swanson, 2012; Kenyon, 2010). Higher IGF-1 levels are associated with a shorter lifespan (Lewin et al., 2017; Lodjak et al., 2018). However, the exact mechanism of how IGF-1 regulates lifespan is not clear. In vertebrates, one hypothesis suggests that reduced IIS signalling increases resistance to oxidative stress (OS) (Dantzer and Swanson, 2012; Holzenberger et al., 2003; Kenyon, 2010). OS is an imbalance between reactive oxidative species (ROS) and the organism's ability to detoxify (antioxidative defense) or repair the damage caused by ROS (Haussmann and Treidel, 2015). ROS, such as peroxides or free radicals, can damage proteins, lipids, or even the DNA itself, causing the improper functioning of the cells and leading to reduced lifespan (Barja, 2013; Finkel and Holbrook, 2000; Gershman et al., 1994; Harman, 1956; Vágási et al., 2018). It was shown that the reduced activity of insulin-like signalling (via reduced IGF-1 receptor gene activity) increased lifespan and the resistance against OS in mice (Holzenberger et al., 2003). Vágási et al., 2020 showed in House sparrows (*Passer domesticus*) that IGF-1 is positively related to MDA. However, IGF-1 influences lifespan, and we know that IGF-1 signalling has a potential role in OS resistance, we do not know how IGF-1 and OS are interconnected in the regulation of lifespan in wild animals. Therefore, **we aimed to test (i) the effect of IGF-1 on oxidative damage** (expressed through malondialdehyde [MDA] levels) and (ii) **whether IGF-1 could have a long-term effect on lifespan**. Previously was shown that mortality is sex dependent in birds (Schindler et al., 2015; Székely et al., 2014), and *Igf1r* mutant female mice show higher lifespan expansion (by 33%) than *Igf1r* mutant male mice (by 15.9%) compared with their wild-type littermates (Holzenberger et al., 2003). Since sex-dependent mortality influences the population structure (e.g. adult sex ratio) and demography and *Igf1r* have also a sex-specific effect on lifespan, we included sex in our models.

(ii) Methods

To test our questions, we captured 16 female and 25 male juveniles (hatched in the year of capture) Bearded reedlings between the 28th and 30th July 2017. After the capture and the morphometric measurements, we transferred the birds into an outdoor aviary, where we kept groups of either three or four individuals in cages. The birds were randomly assigned to one of the treatment groups: control or treated (272 ng/mg IGF-1). We took blood samples right before the treatment (day 0) to measure baseline hormone levels, then we took blood samples 24 (day 1) and 96 hours (day 4) after the treatment. After the experiment, we released the birds into two outdoor aviaries. We captured the birds once again three months after the treatment to test the long-term repeatability of IGF-1 levels, then we released them back to the aviary for an additional 13 months to follow the mortality of our study population.

(iii) Results and conclusions

IGF-1 levels did not differ between the groups before treatment on day 0, but it was higher in the treatment than in the control group on day 1. However, the difference between the treatment groups disappeared by day 4. IGF-1 level was higher in males than females, and IGF-1 levels showed high within-individual repeatability.

Males had higher pre-treatment MDA levels than females. Here, for the first time, we showed experimental support for the hypothesis that an elevation of circulating IGF-1 levels may cause sex-specific oxidative damage in the short-term. We found that IGF-1 injection significantly increased MDA levels in males, but tended to decreased it (albeit non-significantly) in females on day 1 of the experiment. These differences disappeared by day 4. Vágási et al., 2020 showed in a previous study on House sparrows that IGF-1 is positively related to MDA level (Vágási et al., 2020), which is consistent with our results in case of male Bearded reedlings. Treatment (day 1) did not influence mortality over the 16 months of the study. However, the baseline IGF-1 level (pre-treatment) has a positive relationship with survival in males, the MDA level did not have a significant effect. On day 4 (post-treatment) higher MDA levels were associated with increased mortality in males, while did not influence mortality in females. We did not find any effect of day 4 IGF-1 on mortality.

Overall, males had higher IGF-1 and MDA levels than females and were more sensitive to IGF-1-induced oxidative damage. There is an opposite pattern in mammals, and females are more sensitive to variations in IGF-1 levels (Holzenberger et al., 2003). Compared with mammals, birds show a reversed sex-specific mortality pattern, where males have longer lifespan than females (Bronikowski et al., 2022). Therefore, our study raises the possibility that IGF-1-caused oxidative damage may contribute to sex-specific mortality patterns.

Study 4.

(i) Objectives

Growth is an energetically highly demanding process, therefore, trade-offs can occur between growth and other life-history traits because of the limited resources (Werner and Anholt 1993; Yearsley et al., 2004). In general, individuals with a higher growth rate have a shorter lifespan than their slow-growing conspecifics (Metcalfe and Monaghan, 2003; Monaghan and Ozanne, 2018; Werner and Anholt 1993). The term "growth" means not just the growth during postnatal development till reaches the adult body size but all the somatic growth processes which occur during the organism's lifetime, such as moulting. Birds are particularly interesting in the studies of moulting because the quality of their feathers directly affects their survival and fitness (Jenni and Winkler, 2020a; Serra et al., 2007). The moulting of the birds is under tight control and optimized for the given environment and the life history of the species (Barta et al., 2008; De La Hera et al., 2009; Kiat and Sapir, 2017). However, we know that moulting is one of the major life-history stages of the birds, but we have surprisingly little knowledge of its hormonal regulation (Jenni and Winkler, 2020a). Furthermore, the role of IGF-1 in the moulting of birds is overlooked. We know of only one study showing that stress-induced moult was related to IGF-1 levels in broiler chickens (Mazzuco et al., 2015). Therefore, **we aimed to study whether the experimentally elevated IGF-1 influenced feather growth rate, moult intensity, and the quality of the feathers.**

(ii) Methods

To test our questions, we captured 41 juvenile (hatched in the year of capture) Bearded reedlings between 28th and 30th July 2017. We quantified the moulting stage of the bird by Jenni and Winkler (2020b), and we transferred only moulting juveniles into an outdoor aviary, where we kept groups of three or four individuals in cages. We used the same birds and protocol for IGF-1 manipulation described in Study 3. We calculated the moult index as the sum of the moulting scores of the individual feathers (see details in Vágási et al., 2010) on day 0 (pre-treatment) and day 15 (post-treatment). We took blood samples on days 0, 1, and 4. We measured body mass and the length of growing feathers on days 0, 1, 4, and 15. When the birds finished their moult, we captured them again and measured their wing, tail length, and the length of the males' beards (these are modified long body feathers). We plucked the longest tail feather (Ta1) and established its quality by length, mass, rachis diameter, and the number of fault bars (Jovani and Rohwer, 2017; Pap et al., 2008).

(iii) Results and conclusions

Our study was the first to experimentally test how IGF-1 influences feather growth in wild birds. We found that the IGF-1 manipulation increased IGF-1 levels on day 1, but this effect disappeared by day 4. Previously it was shown that the artificial

elevation of IGF-1 increases the hair growth rate in human organ cultures and mice (Ahn et al., 2012; Su et al., 1999). Therefore, we expected that IGF-1 supplementation would increase the feather growth rate. However, we did not find this effect, even though Mahr et al., 2020 showed that IGF-1 is associated with the natural variation in tail feather length in the same species. However, we found that the treated birds displayed a higher number of actively growing feathers than control birds, i.e. the intensity of moult was higher in the treatment group than in the control group on day 15. This result is consistent with Li et al. (2014), who showed that IGF-1 manipulation increased the number of growing hair follicles in wild-type mice. However, IGF-1 did not affect the growth rate of individual feathers. We found that IGF-1 can increase the intensity of moult, and as a consequence, the moulting period can be shorter. Even though rapid feather re-growth has many advantages (e.g. shortened period of reduced flight capability), the increased moulting intensity can be associated with reduced feather quality (Dawson et al., 2000; Vágási et al., 2010). We did not show any adverse effect of IGF-1 manipulation on feather quality, IGF-1 influenced neither the rachis diameter nor feather mass. Furthermore, IGF-1 improved the feather quality, because the IGF-1-treated group had significantly fewer fault bars (malformation of the feather often caused by stress or disease) in their feathers compared with the control group.

Study 5.

(i) Objectives

In birds, feathers are important not just for locomotion and insulation but play a crucial role in camouflage and mate choice, and they underlie sexual selection (Groscolas and Cherel, 1992; Hill and McGraw, 2006). Developing these plumage ornaments is costly, which may result in suppressed immunity or even increased oxidative stress. Therefore, there is a trade-off between the development of ornaments and self-maintenance (Flatt and Heyland, 2011; Hau, 2007; Ketterson and Nolan, 1999; Nolan et al., 1992). The development of plumage ornaments and the growth of feathers require a lot of energy and major changes in metabolism, such as increased cell differentiation, proliferation rate, and protein synthesis (Kuenzel, 2003). Since IGF-1 has a role in regulating these processes, which are tightly linked to moulting, it might also influence the development of plumage ornaments. However, IGF-1 has a well-documented role in growth and development, we did not find any study investigating the role of IGF-1 in plumage ornamentation. Bearded reedlings express multiple ornaments simultaneously, such as the males' black beard (elaborated melanin-based feathers/ornament), which underlies inter- and intrasexual selection (Hoi and Griggio, 2008). Previously, it was shown that tail length is a sexually selected trait in both females and males (Romero-Pujante et al., 2002, Griggio, 2016). Also, the structural plumage components, characterized by reflection in the UV range, such as the males' blue head and pink flank, and the achromatic bright chin in both males and females, are

highly sensitive to the individual condition during moult. Therefore, **we aimed to study the relationship between plumage traits and IGF-1.**

(ii) Methods

To test our questions, we captured 42 moulting Bearded reedlings (which have moulted more than 2/3 of their plumage) in September and October 2016 in Lake Fertő. After the capture, the morphometric measurements, and blood sampling, we transferred the birds into a semi-natural, outdoor aviary located at Konrad Lorenz Institute of Ethology, University of Veterinary Medicine, Vienna, Austria. All captive birds finished the moulting under the same conditions.

When the birds finished their moult, we characterized their plumage traits. We measured UV chroma (the proportion of reflectance in the UV range compared to the total reflectance) and brightness using a spectrometer with a deuterium halogen lamp. We measured the UV chroma three times in each of the following regions of the body: in males, we measured the head, flank, back, and chin; in females, we measured the chin, cheek, and back. In addition, we measured the length of the tails in both sexes and the beard of the males. In the following step, we tested the relationship between plasma IGF-1 level and plumage ornaments.

(iii) Results and conclusions

We were able to show for the first time that IGF-1 influences the development of sexually selected plumage traits in birds. We found a significant positive relationship between tail length and IGF-1 levels in males, but not in females. IGF-1 was not associated with the beard length of the males. We found a positive relationship between IGF-1 levels and UV chroma in males. In females, we found a more complex picture. IGF-1 was associated with darker colouration in females with lower body mass, while in heavier females, IGF-1 was associated with brighter colouration. Our results suggest that the effect of IGF-1 on plumage is influenced by sex and condition. It is well known that moulting is an energy-demanding process that requires major physiological changes to increase cell proliferation, growth, and differentiation (Kuenzel 2003). Therefore, individual condition can determine the quality of the developing feathers (Jovani and Blas, 2004; Murphy et al., 1988; Pap et al., 2008). In several bird species, it was shown that malnutrition had a negative effect on the quality of feathers (Murphy et al. 1988; Pap et al. 2008). IGF-1 is a nutrient-sensing hormone that can influence how energy is divided between major physiological processes that influence feather growth (Blumenthal et al. 2011; Dantzer and Swanson 2012; Tighe et al. 2016). Therefore IGF-1 may be capable of adjusting energy expenditure to external and internal environments and influencing plumage development through these processes.

1.4. General discussion and conclusions

We found that IGF-1 levels decrease due to a standardized stressor (Study 1) but show a high among-individual variance in response to food restriction (Study 2). Also, we found that the IGF-1 stress response is independent of the glucocorticoid stress response (Study 1). We found that IGF-1 has a positive relationship with MDA levels, and birds with higher IGF-1 levels were more likely to survive (Study 3). We found that IGF-1 does not influence the growth rate of feathers but has a positive relationship with moult intensity, i.e. higher IGF-1 levels were associated with more simultaneously growing feathers (Study 4). Also, birds with higher IGF-1 had better quality feathers (Study 4), and we found that IGF-1 plays a role in the development of plumage in both sexes (Study 5). Still, the relationship between IGF-1 and plumage ornament in females depends on body condition (Study 5). In all of our studies, we found that baseline IGF-1 levels show high variance among individuals.

Previously, it was shown in many studies on vertebrates that glucocorticoid levels are raising rapidly after stress exposure, and only the samples taken in 3 minutes can be used as baseline samples (Romero and Reed, 2005). To be sure that our handling was stressful for our birds and triggered physiological stress responses, we measured cort as well from our samples. Cort significantly increased, while IGF-1 significantly decreased after 15 minutes of handling stress (Study 1). Vágási et al. (2020) showed in house sparrows that the 30 minutes of handling stress also decreased the level of IGF-1 (Vágási et al., 2020). These results are in line with previous studies on non-avian species such as fish (Davis and Peterson, 2006; Wilkinson et al., 2006) and pigs (Farmer et al., 1991; Wirthgen et al., 2017), where the confinement stress also significantly decreased IGF-1 levels. These studies, along with our results suggest that IGF-1 is a good candidate to establish an appropriate physiological response to the changing environment.

However, to understand how animals cope with stressful situations, we need to explore how different physiological mechanisms interact with each other (Vágási et al., 2020). To test how IGF-1 interacts with other physiological pathways, it is inevitable to investigate the relationship between the HPA and HPS axes. We examined the relationships between cort and IGF-1 in both baseline and stress-induced samples. While the two axes may be interconnected (Bossis and Porter, 2003; Dell et al., 1999; Kajimura et al., 2003; Peterson and Small, 2005; Reindl and Sheridan, 2012), previous studies have shown that only the prolonged and/or pharmacological increase of glucocorticoids could influence IGF-1 levels (Davis and Peterson, 2006; Kajimura et al., 2003). Firstly, we investigated the relationship between cort and IGF-1 at the baseline level. We did not find any relationship between cort and IGF-1 in wintering Bearded reedlings (Study 1), but Vágási et al. (2020) showed in breeding house sparrows that cort had a positive relationship with IGF-1 levels. It was shown that IGF-1 has a positive effect on reproduction via elevating the rate of steroidogenesis of reproductive hormones and the development of specific reproductive structures

(Giudice, 1999; Demeestere et al., 2004; Wang and Hardy, 2004; Weinzimer and Cohen, 1999). Since IGF-1 is necessary for the appropriate functioning of the reproductive system in both sexes, IGF-1 is probably higher during the breeding season and the nature of the relationship between IGF-1 and cort is changing among different life-history stages. This hypothesis, namely that the relationship between IGF-1 and cort is context-dependent, can be supported by Vágási et al. (2020). Vágási et al. (2020) showed that the relationship between cort and IGF-1 changed when the individuals stepped into the so-called "emergency" life-history stage. Due to stressors, the studied physiological network was reorganized, the positive association of cort with IGF-1 disappeared, and they became independent regulators. Finally, we artificially elevated cort levels in wintering Bearded reedling. While the treatment induced increased cort levels, the manipulation did not alter IGF-1 levels.

On the other hand, Lodjak et al. (2016), found a context-dependent relationship between IGF-1 and cort in Great tit nestlings. IGF-1 was positively related to cort in decreased broods, while they found a negative relationship between IGF-1 and cort in enlarged broods. Based on previous studies which showed that brood size manipulation has an effect on provisioning rate per capita compared with control broods (Pettifor et al., 2001; Sanz and Tinbergen, 1999), Lodjak et al. (2016) hypothesised the relationship between cort and IGF-1 depends on the individuals' physiological condition. They hypothesised the existence of a physiological turning point where the relationship between cort and IGF-1 can change. Our findings (Study 2), that heavier birds decreased, while light birds increased their IGF-1 levels, may support the idea of such a physiological turning point. But it should be taken into account that we found that food restriction increased IGF-1 levels, while Lodjak et al (2016) found that IGF-1 was lower in groups with poorer food availability. This difference can happen because the nestlings are in an active growth period and higher IGF-1 is needed for faster growth and earlier fledging, while adult birds can express different strategies to cope with environmental challenges. We found a high among-individual variance between individual IGF-1 responses to food restriction (Study 2); heavier birds decreased while lighter birds increased their IGF-1 levels. For lighter birds, the very fast weight loss can be fatal. Therefore, they may increase their IGF-1 to slow down protein degradation and use energy reserves (which can manifest in slower weight loss). This hypothesis corroborates a previous study on mice, where the IGF-1-injected individuals lost less weight than the control individuals during starvation (O'Sullivan et al., 1989). On the other hand, heavier birds with decreased IGF-1 levels may suppress insulin activity to elevate blood glucose levels, and this amount of glucose may be enough to keep their life processes normal during food restriction. However, Vágási et al. (2020) showed in house sparrows that after handling stress, the higher the IGF-1 level is, the higher the blood glucose level, which contradicts our hypothesis that heavier birds with decreased IGF-1 levels can increase blood glucose levels. The differences can come from that IGF-1 may express different response patterns to different stressors.

IGF-1 interacts not just with the HPA axis, but other physiological traits that influence fitness as well. It was shown that high IGF-1 concentration is associated with a shorter lifespan in reptiles, passerines, and mammals (Holzenberger et al., 2003; Lewin et al., 2017; Lodjak et al., 2018; Sparkman et al., 2009). The exact mechanism of how IGF-1 can influence longevity is still far from clear, but we tested a major hypothesis, namely that IGF-1 mediates longevity across oxidative stress (Dantzer and Swanson, 2012; Holzenberger et al., 2003; Kenyon, 2010). We showed for the first time that experimentally elevated IGF-1 levels may induce oxidative damage (MDA level) in Bearded reedlings (Study 3). Our results are in line with previous correlational results, where plasma IGF-1 levels were positively related to MDA levels in adult House sparrows (Vágási et al. 2020). Our study experimentally increased circulating IGF-1 levels, resulting in sex-specific differences in oxidative damage. One day after the manipulation, MDA levels were higher in IGF-1-treated individuals than in controls in males, while the treatment had no effect in females. However, we did not show that the IGF-1-induced MDA level influenced survivors, males that had relatively higher post-treatment MDA levels were less likely to survive, while females showed the opposite pattern. We found that males had overall higher IGF-1 and MDA levels than females and were more sensitive to IGF-1-induced oxidative damage. Previous studies showed the opposite pattern in mammals: females were more susceptible to variation in IGF-1 levels (Elis et al., 2011; Van Heemst et al., 2005; Holzenberger et al., 2003). In mammals the females, while in birds the males are the homogametic sex, which brings up the possibility that the sex-specific effect of IGF-1 on lifespan depends on genetic sex determination (Bronikowski et al., 2022).

One of the major life-history stages of adult birds is moult, which is an energetically demanding process. Moult, i.e. the renewal of feathers is highly important to birds because feathers are crucial for foraging (i.e. moving to the feeding ground, or hunting in case of birds of prey), insulation, also feathers play a key role in mate choice in certain species. During our studies, we found a controversial effect of IGF-1 on feather length. We showed that neither IGF-1 treatment nor the natural variation in IGF-1 levels influences the length of the feathers in juvenile Bearded reedlings (Study 4), but we found a positive relationship between tail feather length and IGF-1 only in adult males in the same species (Study 5). Based on these results, we suggest that IGF-1 could have an age-specific effect on feather length. The lack of the IGF-1 effect on wing feathers is not entirely surprising, because the wing feathers are crucial for flying, and the birds may benefit from disentangling the control of flight feather growth from a hormone which highly sensitive to environmental changes (Study 1 and 2). However, IGF-1 did not influence feather growth rate, it seems IGF-1 increases the number of simultaneously moulted feathers. This result is in line with previous studies on mammals, which showed that IGF-1 positively influences the number of hair follicles (Ahn et al., 2012; Lie et al., 2014). The feather growth rate seems to have a physiological limit (Rohwer et al., 2009). Accelerated moult is associated with lower feather quality, including feather mass (Dawson, 2004), the number of fault bars

(Vágási et al., 2012), also colouration (Griggio et al., 2009; Serra et al., 2007). Although previous studies showed that accelerated moult is associated with worse feather quality, we found that IGF-1 treatment increased moult intensity, and increased feather quality by decreased fault bars (Study 4). Feather quality can be assessed not only by the macrostructure of the feather, but by the nanostructure (structural plumage) as well. We found that IGF-1 has a positive effect on the feathers' structural plumage, causing higher UV chroma (proportion of reflectance in the UV range/full reflectance) in male Bearded reedlings (Study 5). On the other hand, we found that the relationship between IGF-1 and brightness (the degree of melanisation) is condition-dependent in female Bearded reedlings, in females with low body mass IGF-1 has a negative relationship with brightness causing darker colour, while in heavy birds IGF-1 has a positive effect on brightness causing lighter colour (Study 5).

Despite that there is a trade-off between moulting and the other life-history stage and/or trait, we know very little about the physiological control of moult including feather growth, moult intensity, and plumage ornamentation. Moult and feather quality are highly dependent on environmental changes, and it was shown that physiological stress and nutritional status have a negative effect on feather quality and influence the length of moult (Griggio et al., 2009; Hudson and Wilcoxen, 2018; Svensson and Merilä, 1996). Considering that IGF-1 mediates physiological processes necessary for moulting (e.g. cell differentiation and proliferation or protein synthesis), and is sensitive to environmental changes (Study 1 and 2) can influence feather quality and plumage ornamentation via regulating the process of moult (Study 4 and 5).

1.5.4. Conclusions

We found large inter-individual variance in the baseline levels of IGF-1 in all studies. We also showed consistently high individual variance in the plastic response of IGF-1 (reaction norms) to different stressors. Therefore, the natural variation of IGF-1 may be the result of individual optimization - i.e. Optimal Endocrine Phenotype Hypothesis (Bonier and Cox, 2020) to mediate the trade-offs between life-history traits and stages. We studied how IGF-1 influences important life-history traits such as survival and growth (as feather growth), and how IGF-1 can modulate important life-history stages such as moulting and the “emergency” life-history stage. In the present dissertation, I showed that IGF-1 positively affects feather quality but increases oxidative damage that can be related to a shorter lifespan. These results suggest that IGF-1 may stand at the crossroads of life-history trade-offs and can be one of the key physiological factors to establish the optimal or near-optimal endocrine phenotypes in response to the environment to maximise fitness.

1.5. New results to science

- IGF-1 levels decrease due to restraint stressor (Study 1).
- An experimental increase in glucocorticoid levels does not induce a decrease in circulating IGF-1 levels (Study 1).
- IGF-1 levels show high among-individual variance in response to food restriction (Study 2).
- Experimentally elevated IGF-1 levels induce transient increase in MDA levels in males, but not in females, and birds with higher IGF-1 levels are more likely to survive (Study 3).
- IGF-1 does not influence the growth rate of feathers but has a positive relationship with moult intensity, indicating that higher IGF-1 levels are associated with more simultaneously growing feathers. Birds with higher IGF-1 have better quality feathers (Study 4).
- IGF-1 levels have positive association with tail length and UV chroma in males (Study 5).
- IGF-1 levels have negative association with UV chroma in light females, while positive association in heavy females (Study 5).
- All studies showed that the baseline IGF-1 level shows high variance among individuals.

1.6. Summary

Life-history theory is one of the branches of evolutionary biology, which aims to explain the causes and consequences of variation in life cycles and the trade-offs between competing life-history traits (Flatt and Heyland, 2011; Stearns, 1993). One of the most robust patterns is that fecundity is inversely related to lifespan. This antagonistic relationship between survival and reproduction is a central tenet in life-history theory and is explained by the "costs of reproduction" (Williams, 1966). Because resources are often limited in nature, the idea that life-history trade-offs are the result of resource constraints became a fundamental concept of life-history theory (Roff and Fairbairn, 2007). Traditionally, it has been thought that the cause of these trade-offs is the limited internal energy reserves. However, later studies showed that the trade-offs might not be the cause of limited resources but rather the molecular signal originating from the gonads (Hsin and Kenyon, 1999). Therefore, another view has emerged, which states that the "pleiotropic" effects of regulators cause the trade-off between competing life-history traits (Leroi, 2001). Because hormones can simultaneously regulate many traits (hormonal pleiotropy), they are key mediators of life-history trade-offs between reproduction and lifespan (Martin et al., 2011; Wingfield et al., 1998). Hormones integrate information from both external (i.e. ecological or demographic processes) and internal (i.e. nutritional condition, age-specific survival) environments and act as a mediator to shape the best response to the given circumstances (Zera and Harshman, 2001). Since major traits of the phenotype are under endocrine control, hormones are one of the major determinants of pleiotropy, life history correlations, and trade-offs. Although many signalling pathways shape phenotype, in this thesis, I focused on the role of insulin-like growth factor-1 (IGF-1) in determining life-history strategies in a common passerine, the Bearded reedling (*Panurus biarmicus*).

Study 1.

One possibility to explore is whether the given hormonal pathway may have this life-history regulatory function to expose individuals to stressors. Stressors can be easily standardized, and the response to them is ecologically relevant because to maximise fitness, all organisms must be able to cope with environmental changes (Hawlena and Schmitz, 2010; Romero et al., 2000; Wingfield et al., 1998). Therefore, understanding the response to stressors of the given hormone can provide great insight into its possible role in shaping life-history strategies. IGF-1 levels are expected to change under handling and nutritional stress (Davis and Peterson, 2006; Wirthgen et al., 2017) suggesting that IGF-1 is a relevant hormone of the stress response. However, it is not clear whether the change in IGF-1 levels is triggered by the stress-induced change in glucocorticoids (GCs; known as "stress hormones") or it is the direct consequence of the stressor (Davis and Peterson, 2006). Until recently, the effects of stressors on IGF-1 levels or the mechanistic link between HPA (Hypothalamic-

Pituitary-Adrenal axis) and HPS axis in any free-living organism was not studied. Therefore, we aimed to study whether (Q1) an external stressor affects plasma levels of IGF-1 and whether (Q2) cort (the main GC stress hormone in birds) directly affects IGF-1 levels. We found that cort levels were significantly higher, while IGF-1 levels were significantly lower after the 15-minute handling stress. Our results are consistent with previous studies showing that cort increases in response to stress, and suggest that our handling was stressful for the birds (Remage-Healey & Romero, 2001; Buehler et al., 2008). However, our result, namely IGF-1 decreasing due to handling stress is in line with previous studies on captive fish (Davis and Peterson, 2006; Wilkinson et al., 2006; Wirthgen et al., 2017), in other bird studies, they showed that IGF-1 level is unrelated to handling time in nestlings (Lodjak, Mägi and Tilgar, 2014). The effect of stressors can be age-specific as it was shown previously in fish and chicken (Perrot et al., 1999; Yun et al., 2005), which can explain why we found that IGF-1 decreases in adult birds, while it does not change in nestlings. We did not find a relationship between IGF-1 and cort at the baseline level, and the change in cort and IGF-1 levels was also unrelated. Also, we found that cort manipulation increased cort levels, but did not influence IGF-1 levels. These results suggest that the HPA and the HPS axes are independent of each other at the endpoint of their hormonal cascade.

Study 2.

Food shortages are one of the main stressors that almost all organisms need to cope with. Therefore, the physiological mechanisms that play a role in nutrient sensing could have a key role in mediating energy allocation between competing life-history traits. Our knowledge of IGF-1's role in facilitating an appropriate response to changes in food availability in wild animals are limited (Duncan et al., 2015; Sparkman et al., 2010). Therefore, we aimed to study how IGF-1 responds to fluctuation in food availability in a wild-caught captive population of Bearded reedlings. In general, IGF-1 was positively associated with body mass, which is consistent with previous findings in different taxa such as fish, reptiles, and mammals (Cameron et al., 2007; Crain et al., 1995; Sparkman et al., 2009). Males had higher IGF-1 levels compared with females, but if we controlled for body mass the sex difference in IGF-1 levels disappeared. These findings suggest that the sex differences originate from the sex-dependent body mass differences rather than the sex itself. We found that overall, food restriction increased IGF-1 levels, but at the individual level, we found high among-individual variance in response to food restriction i.e. some individuals decreased, others showed little response or increased their IGF-1 levels, and the within-individual response was highly repeatable. In our study, the response of IGF-1 to food restriction was also affected by the individual’s body mass, namely, individuals larger than the average were more likely to decrease their IGF-1 levels, while birds lighter than the average were more likely to increase their IGF-1 levels. Our results suggest that the birds in different conditions may have different coping mechanisms against food restriction.

Study 3.

The insulin/insulin-like signalling pathway (IIS) has been suggested as a key physiological mechanism regulating lifespan and ageing (Dantzer and Swanson, 2012; Holzenberger et al., 2003; Kappeler et al., 2008). Higher IGF-1 levels are associated with a shorter lifespan (Lewin et al., 2017; Lodjak et al., 2018). However, the exact mechanism of how IGF-1 regulates lifespan is not clear. In vertebrates, one of the hypothesis suggests that reduced IIS signalling increase resistance to oxidative stress (OS) (Dantzer and Swanson, 2012; Holzenberger et al., 2003; Kenyon, 2010). However, IGF-1 influences lifespan, and we know that IGF-1 signalling has a potential role in OS resistance, we do not know how IGF-1 and OS are interconnected in the regulation of lifespan in wild animals. Therefore, we aimed to test (Q1) the effect of IGF-1 on oxidative damage (expressed through malondialdehyde [MDA] levels) and (Q2) whether IGF-1 could have a long-term effect on lifespan. We manipulated the IGF-1 levels, then we took blood samples before (day 0), 24 hours (day1) and 96 (day 4) hours after the manipulation. IGF-1 levels did not differ between the treatment groups before treatment, but it was higher in the treatment than in the control group on day 1. However, the difference between the treatment groups disappeared by day 4. Here, for the first time, we showed experimental support for the hypothesis that an elevation of circulating IGF-1 levels may cause sex-specific oxidative damage in the short-term. We found that IGF-1 injection significantly increased MDA levels in males, but non-significantly decreased in females on day 1 of the experiment. These differences disappeared by day 4. Vágási et al., 2020 showed in a previous study on House sparrows that IGF-1 is positively related to MDA level (Vágási et al., 2020), which is consistent with our results in case of male Bearded reedlings. Treatment (day 1) did not influence mortality over the 16 months of the study. However, the baseline IGF-1 level (day 0) has a positive relationship with survival in males, the MDA level did not have a significant effect. Day 4 MDA levels were associated with increased mortality in males, while did not influence mortality in females. We did not find any effect of day 4 IGF-1 on mortality. Overall, males had higher IGF-1 and MDA levels than females and were more sensitive to IGF-1-induced oxidative damage. In mammals, there is an opposite pattern, and females are more sensitive to variations in IGF-1 levels (Holzenberger et al., 2003). Compared with mammals, birds show a reversed sex-specific mortality pattern, where males have longer lifespan than females (Bronikowski et al., 2022). Therefore, our study raises the possibility that IGF-1-caused oxidative damage may contribute to sex-specific mortality patterns.

Study 4.

Growth is an energetically highly demanding process, therefore, trade-offs can occur between growth and other life-history traits (Werner and Anholt 1993; Yearsley et al., 2004). Moulting as a growth process, is also one of the most important life-history stages of birds. However, we know that moulting is one of the major life-history stages of the birds, the role of IGF-1 in the moulting of birds is overlooked.

Therefore, we aimed to study whether the experimentally elevated IGF-1 influenced feather growth rate, moult intensity, and the quality of the feathers (Study 4). IGF-1 supplementation did not increase the feather growth rate. But we found that the treated birds displayed a higher number of actively growing feathers than control birds, i.e. the intensity of moult was higher in the treatment group than in the control group on day 15. This result is consistent with Li et al. (2014), who showed that IGF-1 manipulation increased the number of growing hair follicles in wild-type mice. However, IGF-1 did not affect the growth rate of individual feathers, we found that IGF-1 can increase the intensity of moult, and as a consequence, the moulting period can be shorter. Even though rapid feather re-growth has many advantages (e.g. shortened period of reduced flight capability), the increased moulting intensity can be associated with reduced feather quality (Dawson et al., 2000; Vágási et al., 2010). We did not show any adverse effect of IGF-1 manipulation on feather quality, IGF-1 influenced neither the rachis diameter nor feather mass. Furthermore, IGF-1 improved the feather quality, because the IGF-1-treated group had significantly fewer fault bars (malformation of the feather often caused by stress or disease) in their feathers compared with the control group.

Study 5.

In birds, feathers are important not just for locomotion and insulation but play a crucial role in camouflage and mate choice, and they underlie sexual selection (Groscolas and Cherel, 1992; Hill and McGraw, 2006). Since IGF-1 has a role in regulating those physiological processes, which are tightly linked to moulting, it might also influence the development of plumage ornaments. Therefore, we aimed to study the relationship between plumage traits and IGF-1 (Study 5). We found a significant positive relationship between tail length and IGF-1 levels in males, but not in females. IGF-1 was not associated with the beard length of the males. We found a positive relationship between IGF-1 levels and UV chroma in males. In females, we found a more complex picture. In lighter females, IGF-1 was associated with darker colouration, while in heavier females IGF-1 was associated with brighter colouration. Our results suggest that the effect of IGF-1 on plumage is influenced by sex and condition. It is well known that moulting is an energy-demanding process that requires major physiological changes to increase cell proliferation, growth, and differentiation (Kuenzel 2003). Therefore, individual condition can determine the quality of the developing feathers (Jovani and Blas, 2004; Murphy et al., 1988; Pap et al., 2008). In several bird species, it was shown that malnutrition had negative effect on the quality of feathers (Murphy et al. 1988; Pap et al. 2008). IGF-1 is a nutrient-sensing hormone that can influence how energy is divided between major physiological processes that influence feather growth (Blumenthal et al. 2011; Dantzer and Swanson 2012; Tighe et al. 2016). Therefore IGF-1 may be capable of adjusting energy expenditure to external and internal environments and influencing plumage development through these processes.

To conclude, we found large inter-individual variance in the baseline level of IGF-1 in all studies, also we showed high individual variance in reaction norms to different stressors. Therefore, the natural variation of IGF-1 may be the result of individual optimization - i.e. Optimal Endocrine Phenotype Hypothesis (Bonier and Cox, 2020). We studied how IGF-1 influences important life-history traits such as longevity and growth (as feather growth), and how IGF-1 can modulate important life-history stages such as moulting and the “emergency” life-history stage. In the present dissertation, I showed that IGF-1 has a positive effect on feather quality, but increases oxidative damage that can be related to shorter lifespan. These results suggest that IGF-1 may stand at the crossroads of life-history trade-offs, and can be one of the key physiological factors to establish the optimal or near-optimal endocrine phenotypes in response to the environment to maximise fitness.

1.7. Összefoglalás

Az életmenet evolúció az evolúcióbiológia egyik területe, melynek célja az életmenetben található nagymértékű változatosság kialakulásának okainak és ennek következményeinek vizsgálata (Flatt és Heyland, 2011; Stearns, 1993). Az életemenet elmélet azokat a geno- és fenotípusos tulajdonságokat vizsgálja, melyek meghatározzák az élőlények túlélését és szaporodási sikerét (Roff, 2002). Életmenet tulajdonságok alatt azokat a változókat értjük, melyek meghatározzák az élőlények életútját és fitneszét. A leggyakrabban vizsgált életmenet tulajdonságok a következőek: születéskori méret, a szexuális érettség kora, az utódok száma, mérete és ivararánya, élethossz.

Az evolúció során ezekben a tulajdonságokban nagy változatosság alakult ki nem csak a fajok között, hanem fajon belül is, annak érdekében, hogy adott környezeti körülmények között minél magasabb fitneszt (relatív szaporodási siker) érjenek el (Stearns, 2000). Habár a legjobb stratégia az lenne, ha az élőlények egyaránt képesek lennének növelni az élethosszukat és az utódaik számát, a források korlátozott elérhetősége miatt ez nem lehetséges. A források korlátozott elérhetősége úgy nevezett forrás felosztási dilemmához vezet a jelenlegi- és a jövőben szaporodás, illetve a túlélés között. Ennek következtében kialakul egy energetikai csereviszony az életmenet tulajdonságok között, ami sokszor negatív fenotípusos kapcsolathoz vezet (Roff és Fairbairn, 2007; Stearns, 1989; Zera és Harshman, 2001). Habár, az életmenet stratégiákban található változatosság akár véges is lehetne, azt látjuk, hogy az életemenet stratégiákat egy úgy nevezett “lassú-gyors” tengely mentén tudjuk elhelyezni (Stearns, 1983). A viszonylag gyors életmenettel jellemezhető fajok/egyedek több energiát fektetnek a szaporodásba és kevesebbet a túlélésbe/élethosszba, vagyis rövidebb ideig élnek és több utódot nevelnek, mint a lassú életmenettel rendelkező fajok/egyedek (Bauwens és Diaz-Uriarte, 1997; Blackburn, 1991; Saether, 1988). A életmenet stratégiák között található különbségeket az élőlények fiziológiai folyamatai határozzák meg.

A hormonok olyan kémiai hírvivő anyagok, melyek a fenotípus minden aspektusát befolyásolják, mint például az anyagcserét, a morfológiát vagy akár az életmenet tulajdonságokat. A hormonok a külső-, és belső környezetből érkező ingerek hatására alakítják ki a leghatékonyabb válaszreakciót a fennálló környezeti körülményekre (Zera és Harshman, 2001). Mivel a hormonok egyszerre több tulajdonságot is befolyásolnak (pleiotrópia), ezért az életmenet tulajdonságok (pl.: élethossz és szaporodás) közötti csere-viszonyok kulcsfontosságú szabályozói (Martin és mtsai., 2011; Wingfield és mtsai., 1998). Az egyik leggyakrabban vizsgált hormoncsalád az életmenet stratégiák szabályozásában a glükokortikoidok. A glükokortikoidok szintje nagy mértékű változatosságot mutat fajok, életmenet állapotok és életmenet stratégiák között (Bókony és mtsai., 2009; Palacios és mtsai., 2012; Schoenle és mtsai., 2021; Schultner és mtsai., 2013; Vitousek és mtsai., 2018). A glükokortikoidok befolyásolják az alapanyagcserét (Bonier és mtsai., 2009a, 2009b),

de váratlan környezeti események hatására részt vesznek az energia újrafelosztásának a szabályozásában is (Boonstra, 2013; Ramenofsky és Wingfield, 2017), vagyis szerepük van az úgynevezett “vészhelyzeti életmenet állapot” kialakításában.

Habár a glükokortikoidok egyike a legtöbbet vizsgált hormoncsaládoknak az életmenet stratégiák kialakulásának szabályozásában, egy filogenetikailag viszonylag új hormoncsalád, mely a gerinceseknél jelenik meg először. Az inzulin/inzulinszerű jelátviteli út (IIS) ezzel szemben az összes *Metazoa*-ban (valódi szövetes állatok) jelen van, emellett befolyásolja a forrásfelosztást és szerepe van az életmenet stratégiák kialakításában is (Broughton és mtsai., 2005; Dantzer és Swanson, 2012; Harshman és Zera, 2007; Lodjak és mtsai., 2018). Korábbi vizsgálatokban kimutatták, hogy az IIS befolyásolja az élethosszt és a szaporodást (Dantzer és Swanson, 2012; Lodjak és mtsai., 2018). Emellett kimutatták, hogy az IIS közvetlenül befolyásolja az élőlények tápanyag érzékelő folyamatait is (Taguchi és White, 2008). Evégett, az IIS-ben meg van az az evolúciós potenciál, hogy limitált forrás ellátottság esetén, az életmenet tulajdonságok közötti csere-viszonyok központi szabályozója legyen (Dantzer és Swanson, 2012; Emlen és mtsai., 2012; Harshman és Zera, 2007). A gerinctelenekkel ellentétben, a gerincesek csak 3 inzulin-szerű hormonnal rendelkeznek: az inzulin, az inzulin-szerű növekedési faktor 1 és 2 (IGF-1 és IGF-2)(Brogiolo és mtsai., 2001; Butler és LeRoith, 2001; Jones és Clemmons, 1995; Leevers, 2001). Az inzulin a glükóz homeosztázist szabályozza, az IGF-2 az embrió és a magzat, míg az IGF-1 pedig a megszületett/kikelt egyedek növekedését és fejlődését határozza meg. Gerincesekben az IIS a szomatotróp jelátviteli tengely (hipotalamusz → agyalapi mirigy → szomatortóp tengely) tagja, ahol a növekedési hormon serkenti az IGF-1 termelődését elsősorban a májban (Roith, Scavo, és Butler, 2001). Az IGF-1 az egyik legfontosabb hormon, amely a gerincesek anyagcseréjét és fejlődését, azok tápanyagellátottságát figyelembe véve szabályozza (Dantzer és Swanson, 2012; Lodjak és mtsai., 2016; Taguchi és White, 2008). Ha a tápanyag ellátottság korlátozott, akkor a különböző élőlényeknek dönteniük kell arról, hogy a rendelkezésükre álló forrásokat hogyan használják fel, vagyis melyik életmenet tulajdonságba fektetnek több energiát (van Noordwijk és de Jong, 1986). Ezért a tápanyagérzékelésben szerepet játszó IGF-1 kulcsszerepet játszhat az életmenet tulajdonságok közötti forrás felosztásban. Mezőgazdasági és laboratóriumi állatokkal végzett vizsgálatokból tudjuk, hogy az IGF-1 csökken a táplálék megvonás/csökkentés hatására (Berryman és mtsai., 2008; O’sullivan és mtsai., 1989; Rahmani és mtsai., 2019), ezzel ellentétben viszonylag kevés vizsgálat foglalkozott az IGF-1 táplálékelérhetőség megváltozására adott válaszával természetes populációkban. Duncan és munkatársai (2015) kimutatták, hogy az IGF-1 csökken a *Sceloporus undulatus* nevű gyíkfajban abban az esetben, ha a táplálékelérhetőség limitált. Emellett kimutatták, hogy az IGF-1 szint különbözik a *Thamnophis elegans* két öktotípusa között. Az IGF-1 abban az ökotípusban volt magasabb, ahol a táplálék ellátottság folyamatos, összehasonlítva az éves szinten változó táplálék elérhetőséggel rendelkező ökotípussal (Sparkman és mtsai., 2009). Saját vizsgálatainkban kimutattuk, hogy az IGF-1 egy szabadon élő madárfaj a

barkóscinege (*Panurus biarmicus*) esetén is reagál a táplálék ellátottságra (Tóth és mtsai., 2022; 2. Tanulmány). Azonban a korábbi vizsgálatokkal ellentétben, mi nem találtunk egyértelmű csökkenést az IGF-1 szintben a táplálék mennyiség csökkenésének hatására. Az egyes egyedek IGF-1 szintje nőtt, csökkent vagy nem változott, azonban az IGF-1 változás egyeden belül ismételhető volt, mely változás összefüggött az egyedek kondíciójával. Hargitai és munkatársai (2022) kanárikon (*Serinus canarius*) kimutatták, hogy az IGF-1 szintje áplálék csökkentés hatására nő. Továbbá kimutatták, hogy meglepő módon az IGF-1 szint negatív kapcsolatban van a tojásrakás idejével, vagyis minél alacsonyabb az IGF-1 szint annál korábban raknak tojást a vizsgálatban résztvevő egyedek. A fent említett vizsgálatok alapján arra következtethetünk, hogy az IGF-1 táplálék csökkenés hatására adott válasza függ a fajtól, hiszen a gyíkfajokban alacsonyabb volt az IGF-1 szint a rossz táplálék ellátottságú területeken, továbbá az IGF-1 szint csökkent, míg a madárfajokban az IGF-1 szint nőtt vagy rendkívül változatos választ mutatott táplálék csökkenés hatására. Továbbá, az IGF-1 választ jelentős mértékben befolyásolhatja az életmenet állapot, hiszen míg a kanárik már szaporodásra készültek, addig a barkóscinegék még téli nyugalmi állapotukban voltak.

Az egyik leggyakrabban vizsgált életmenet állapot az úgynevezett “vészhelyzeti életmenet állapot”, melyet ragadozó támadás vagy akár hirtelen időjárás változások is kiválthatnak (Wingfield és mtsai., 1998). Az ilyen “stresszes” szituációknak a túlélésére az élőlények különböző fiziológiai és viselkedésbeli változásokkal reagálnak (Wingfield és mtsai., 1998). A fentebb említett glükokortikoidoknak szerepe van az energia újrafelosztásában ezekben a helyzetekben, vagyis a rendelkezésre álló energiát az energiaigényes folyamtok felől (pl.: szaporodás) a túlélés irányába öszpontosítják. Több vizsgálat is kimutatta, hogy a glükokortikoidok szintje gyorsan emelkedik stresszhatás után (Cockrem és mtsai., 2002, Romero és Reed, 2005; Schoech és mtsai., 2007). Azonban jóval kevesebb információ áll a rendelkezésünkre arról, milyen szerepe van az IGF-1-nek a stresszválasz kialakításában. Korábban kimutatták, hogy az IGF-1 szintje csökken fogvatartási stressz esetén sertésekben (Farmer és mtsai., 1991; Wirthgen és mtsai., 2017) és halakban (Davis és Peterson, 2006; Wilkinson és mtsai., 2006), ami arra utal, hogy az IGF-1 fontos szabályozója a stressz válasz kialakításának. Azonban az nem egyértelmű, hogy az IGF-1 szintjének változását a glükokortikoidok stresszor által megváltoztatott szintje (downstream hatás) vagy a maga a stresszor közvetlenül okozza (Davis és Peterson, 2006; Dell és mtsai., 1999; Unterman és mtsai., 1993). A mesterségesen megemelt glükokortikoid szint csökkenti az IGF-1 szintet baromfiban (Leili és Scanes, 1998), halakban (Kajimura és mtsai., 2003; Peterson és Small, 2005) és patkányokban (Gayan-Ramirez és mtsai., 1999), de ez a hatás csak magas glükokortikoid koncentráció vagy hosszan tartó glükokortikoid expozíció esetén mutatható ki (Bossis és Porter, 2003; Davis és Peterson, 2006; Kajimura és mtsai., 2003). Annak megállapítására, hogyan reagál az IGF-1 a fogvatartási stresszre és hogy az IGF-1 stresszválaszban betöltött szerepe független-e a glükokortikoidoktól

természetes populációkban, több vizsgálatot is végeztünk. Először is egy terepi vizsgálatban megállapítottuk, hogy az IGF-1 szintje csökken, míg a kortikoszteron (glükokortikoid hormon és a fő “stressz” hormon madarakban) szintje nőtt 15 perces fogvatartás hatására, továbbá a két hormon válasz reakciója egymástól független volt barkóscinegékben (Tóth és mtsai., 2018; 1. tanulmány). Mivel ebben az esetben még nem lehettünk abban biztosak, hogy a kortikoszteron nincs hatással az IGF-1 szintjére, ezért egy következő vizsgálatban mesterségesen megemeltük a kortikoszteron szintet. Míg a madarak kortikoszteron szintje erőteljesen magasabb volt a kortikoszteronnal kezelt csoportokban (alacsony vagy magas kortikoszteron koncentráció) mint a kontroll csoportban, addig az IGF-1 szint nem különbözött a csoportok között. Vagyis, az IGF-1 a kortikoszterontól függetlenül befolyásolja a fiziológiai stressz választ barkóscinegékben (Tóth és mtsai., 2018; 1. tanulmány). Ezt alátámasztja Vágási és mtsai (2020) tanulmánya, melyben azt vizsgálták, hogy a fiziológiai hálózat átalakul-e stressz hatására házi verebek szaporodó populációjában. Vizsgálatukban kimutatták, hogy alap állapotban (stresszor hatása nélkül) pozitív kapcsolat van a kortikoszteron és IGF-1 között, azonban stresszor hatására ez a kapcsolat eltűnik és a két hormon egymástól függetlenné válik. Mivel az IGF-1 képes integrálni a külső (pl.: táplálék elérhetőség, fogvatartás) és belső (pl.: rendelkezésre álló energia mennyiség, kondíció) környezetből érkező ingereket, ezért az életmenet tulajdonságok között fennálló energetikai csereviszony egy potenciális irányítója lehet.

Az IGF-1 negatív kapcsolatban áll az élethosszal (Holzenberger és mtsai., 2003; Lewin és mtsai., 2017), de pozitív hatással van a növekedésre, és a szaporodásra, továbbá előrébb hozza a szexuális érettség idejét is (Crain és mtsai., 1995; Flatt és mtsai., 2008; Lewin és mtsai., 2017; Lodjak és mtsai., 2014; Pine és mtsai., 2006; Sparkman és mtsai., 2010; Swanson és Dantzer, 2014; Yakar és mtsai., 1999). Annak ellenére, hogy tudjuk az IGF-1 ellentétes hatást fejt ki a különböző életemenet tulajdonságokra (pl.: az élethosszra és a szaporodásra), nem ismerjük a pontos mechanizmusát annak, hogy az IGF-1 hogyan szabályozza ezt a csereviszonyt. Egy hipotézis szerint, az IGF-1 megnöveli a reaktív oxigén gyökök mennyiségét a vérben és ezáltal oxidatív károsodást okoz, melynek negatív hatása van a maximális élethosszra (Dantzer és Swanson, 2012, Vágási és mtsai., 2019). Ennek az elméletnek a tesztelésére megnöveltük a barkóscinegék IGF-1 szintjét. Az IGF-1 kezelés sikeresen megnövelte a plazma IGF-1 szintjét a következő 24 órában mindkét ivarban. A lipidek oxidatív károsodása (plazma malondialdehid szintje - MDA) jelentős ivari különbségeket mutatott: a hímekben nőtt, míg a tojókban csökkent az MDA szintje az IGF-1 kezelés hatására. 4 nappal a kezelés után az IGF-1 és az MDA plazma koncentrációja visszatért a kezelés előtti szintre. Habár a kezelési csoportok között nem volt különbség a túlélés tekintetében, a kezelés előtti IGF-1 szint negatív, míg a kezelés utáni MDA szint pozitív hatással volt a mortalitás valószínűségére hímekben. Ezek az eredmények arra utalnak, hogy a hímek érzékenyebbek lehetnek az IGF-1 által megnövelt oxidatív stresszre, mint a tojók. Azonban egereken és embereken végzett vizsgálatokban ellenkező mintát találtak, vagyis a nőstények tűntek érzékenyebbnek az

IGF-1 szintjének változására (Holzenberger és mtsai., 2003; van Heemst és mtsai., 2005). Ez azért érdekes, mert az emlősökkel összehasonlítva, a madaraknak fordított ivar-specifikus halálozási mintázata van, vagyis a hímek általában tovább élnek. Így felvetődik annak a lehetősége, hogy az IGF-1 és az IGF-1 okozta oxidatív károsodás hozzájárul az ivar-specifikus halálozási minták kialakulásához. Emellett meglepő módon a kezelés előtt mért magasabb IGF-1 szint csökkentette a mortalitás valószínűségét a hímekben. Ez az eredmény arra utal, hogy az IGF-1 szintben megfigyelhető változatosság az egyed szintű optimalizáció eredménye lehet (amit Optimális Endokrín Fenotípus Hipotézisnek neveztek el)(Bonier és Cox 2020). Ebben az összefüggésben, a jó minőségű egyedek képesek megfizetni a magas IGF-1 szint árát (pl.: az oxidatív károsodás tekintetében)(Higashi és mtsai., 2010), miközben hozzájárulnak a fitneszük növekedéséhez (pl.: a szaporodás vagy a gyulladáscsökkentő válaszok fokozása), ahogy az a természetes szelekciónak kitett fajoknál várható.

A vedlés, mint növekedési folyamat, egy kulcsfontosságú életmenet állapot számos állatcsoport esetén, mint például a hüllők és a madarak. A vedlés egy energia igényes folyamat, mely madarak esetén kiemelkedően fontos, mivel a tollaknak kulcsfontosságú szerepe van a repülésben, a szigetelésben és az egyedek közötti kommunikációban is. Egyetlen olyan vizsgálatot találtunk, ami az IGF-1 vedlésre gyakorolt hatását vizsgálja természetes populációkban. Ebben kimutatták, hogy az IGF-1 szintje magasabb a vedlésben lévő *Tamnophis elegans* siklófaj esetén (Sparkman és mtsai., 2009). Továbbá, az egyetlen madarakon (broiler tyúkokon) végzett vizsgálat azt mutatta, hogy azoknak az egyedeknek, melyek stressz által beindított vedlési állapotban vannak, magasabb az IGF-1 szintje, mint a nem vedlő társaiké (Mazzuco és mtsai., 2005). Habár az IGF-1-nek látszólag fontos szerepe van a vedlés szabályozásában, az IGF-1 tollnövekedésben betöltött szerepe ismeretlen. Ezért az IGF-1 tollnövekedésében, vedlés intenzitásában, és tollminőség kialakításában betöltött szerepét barkóscinegén vizsgáltuk. A tanulmány során 41 fiatal (az adott évben kikelt fiókát) egyedet fogtunk be és osztottunk két kezelési csoportba: kontroll és kezelt (IGF-1-el manipulált). Az IGF-1 szint jelentős mértékben volt magasabb a kezelt csoportban, mint a kontroll csoportban. Habár, a tollnövekedés sebességében nem találtunk különbséget a két csoport között, a vedlés intenzitása erősebb (egyszerre növesztett tollak száma) és a tollminőség (kevesebb növekedési hiba a tollban) jobb volt az IGF-1-el kezelt csoportban, mint a kontroll csoportban. Eredményeink arra utalnak, hogy az IGF-1-nek nincs hatása a tollnövekedés sebességére, de a vedlés intenzitásának megnövelésével lerövidítheti a vedlés teljes időtartalmát. Habár az IGF-1 által lerövidített vedlési időszaknak jelentős hatása lehet a pillanatnyi túlélésre (pl.: röpképtelen időszak lerövidülése récefélék és akár barkóscinegék esetén), az IGF-1 által okozott oxidatív stressz negatív hatással lehet az élethosszra (3. tanulmány). A vedlés, azonban nem csak a repüléshez és a szigeteléshez szükséges tollak lecserélődéséhez szükséges, hanem a párválasztásban fontos színezet kialakulásához is. Ezért vizsgálatainkban arra kerestük a választ, hogy az IGF-1 kapcsolatban áll-e a színezet és a dísztollak kialakulásával barkóscinegékben. A madarak befogása során

vért vettünk minden egyedtől, majd a befogott madarakat a vedlés befejezéséig kültéri, fél-természetes röpdékben tartottuk fogva. A vedlés befejeztével megmértük a hímek “barkóját”, melyek módosult, melanin alapú tollak. Továbbá lemértük a szárny, és farokhosszat, és lemértük a színezeti jellegek UV chroma-ját (az UV-tartományban lévő fényvisszaverő képesség/teljes fényvisszaverő képesség aránya) és világosságát (a melanizáltság mértéke). Az IGF-1 pozitív hatással volt a farokhosszra hímkenél, míg a tojók esetén nem befolyásolta a farokhosszt. Továbbá az IGF-1 nem befolyásolta a barkó hosszát hímeknél. Emellett azt találtuk, hogy IGF-1 pozitív kapcsolatban volt az UV chroma-val hímeknél. A tojók esetén a fényesség kondíció függő kapcsolatban volt az IGF-1-el. A könnyebb egyedekben az IGF-1 negatív (a sötétebb színezet), míg a nehezebb egyedekben pozitív (világos színezet) kapcsolatban volt a fényességgel.

Összefoglalva, az IGF-1 egy olyan hormon, ami gyorsan reagál a környezeti körülmények megváltozására és stressz hatásokra, mint például táplálékhiány és a befogás (1. és 2. tanulmány). Az IGF-1 jelentős egyedek közötti különbséget (1-3. tanulmány), de egyeden belül nagymértékű ismételhetőséget (2-3. tanulmány) mutat. Ez arra utal, hogy az IGF-1 plazmából mérhető szintje egy egyedi fenotípusos jelleg, mely az egyed szintű optimalizáció eredménye lehet. Kimutattuk, hogy az IGF-1 válasza és kifejtett hatása több esetben is kondíciófüggő, és akár különböző színezet kialakulásához is vezethet, melyen keresztül közvetetten befolyásolhatja a párválasztást és a fitneszt. A magas IGF-1 szint pozitívan befolyásolja a vedlés intenzitását (4. tanulmány), melynek közvetett pozitív hatása lehet a lerövidült röpképtelen perióduson, vagy a párválasztáshoz szükséges színezet korábbi kialakításán keresztül (4. és 5. tanulmány). Azonban a magas IGF-1 szint magas oxidatív károsodáshoz vezethet, mely az élethossz lerövidülését okozhatja. Így az IGF-1 az intenzív vedlés és az élethossz közötti csere-viszony egyik szabályozó mechanizmusa lehet. Meglepő módon kimutattuk, hogy a magas IGF-1 szint pozitív hatással van az élethosszra, ami ellentmond a korábbi szakirodalmi adatoknak, miszerint a magas IGF-1 szint negatívan befolyásolja az élethosszt. Mivel az IGF-1 szintje összefügg a testtömeggel és kondícióval, ezért azt feltételezhetjük, hogy a jó kondíciójú egyedek képesek megbírkózni az IGF-1 által okozott oxidatív károsodással. Azonban az, hogy a magas IGF-1 szinttel rendelkező egyedek kihívást jelentő körülmények között (pl.: hirtelen időjárás változás) jobban teljesítenek-e, vagy magasabb fitnesszel rendelkeznek-e az alacsony IGF-1 szinttel rendelkező társaikhoz képest, további vizsgálatok tárgyát kell képezze. Saját vizsgálatainkból kitűnik, hogy az IGF-1 több életmenet tulajdonságot és életmenet állapotot is befolyásol, azonban a pontos szerepének megismerése további vizsgálatokat igényel.

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Part 2 - Appendix

In this chapter I list published articles presented in my dissertation and additional publications as well. Furthermore, I include the published papers relevant for my dissertation.

2.1. Publications included in the dissertation

Study 1 - Tóth Z., Ouyang J.Q., Lendvai Á.Z., 2018. Exploring the mechanistic link between corticosterone and insulin-like growth factor-1 in a wild passerine bird. *PeerJ* 6:e5936

Study 2 - Tóth Z., Mahr K., Ölveczki G., Őri L., Lendvai Á.Z., 2022. Food restriction reveals individual differences in Insulin-like Growth Factor-1 reaction norms. *Frontiers in Ecology and Evolution,* 10:826968

Study 3 - Lendvai, Á.Z., Tóth, Z., Mahr, K., Pénzes, J., Vogel-Kindgen, S., Gander, B.A., Vágási, C.I. IGF-1 induces sex-specific oxidative damage and mortality. (under review in Oecologia)

Study 4 - Lendvai, Á.Z., Tóth, Z., Mahr, K., Osváth, G., Vogel-Kindgen, S., Gander, B.A., 2021. Effects of experimental increase in insulin-like growth factor 1 on feather growth rate, moult intensity and feather quality in a passerine bird. *Journal of Experimental Biology* 224(14) jeb242481

Study 5 - Mahr, K., Vincze, O., Tóth, Z., Hoi, H., Lendvai, Á.Z., 2020. Insulin-like growth factor 1 is related to the expression of plumage traits in a passerine species. *Behavioral Ecology and Sociobiology* 74, 39.

Contribution to the studies included in the dissertation

Study 1

ZT, JQO, ÁZL conceived and designed the experiments, ZT and ÁZL performed the experiments and analyzed the data, ZT prepared figures and/or tables, ZT, JQO, ÁZL authored or reviewed drafts of the paper and approved the final draft, ÁZL contributed reagents/materials/analysis tools.

Study 2

ZT and ÁZL designed the experimental protocol and conducted the statistical analyses. ZT, GÖ, LŐ, and ÁZL performed the experimental procedures with contribution from KM. ZT and ÁZL performed the lab analyses. ZT, KM, and ÁZL wrote the manuscript with contributions from GÖ and LŐ. All authors contributed to the article and approved the submitted version.

Study 3

ÁZL, KM and ZT conceived and conducted the experiment, ÁZL, KM and ZT collected the samples and the data, ÁZL, ZT, JP and CIV measured the samples, SMK and BAG contributed reagents, ÁZL analysed the data, ÁZL and CIV wrote the article, all authors approved the final version.

Study 4

Conceptualization: ÁZL, ZT, KM; Methodology: ÁZL, GO, SV, BAG; Formal analysis: ÁZL; Investigation: ÁZL, ZT, KM, GO; Resources: SV, BAG; Writing - original draft: ÁZL.; Writing - review and editing: ÁZL, ZT, KM, GO, SV, BAG; Project administration: ÁZL, ZT; Funding acquisition: ÁZL, KM.

Study 5

ÁZL, HH, OV, and KM designed the study; OV, KM, and ZT conducted the experiment and took samples and measurements; ÁZL and ZT conducted the lab analysis; KM and ÁZL conducted statistical analyses; and KM and ÁZL wrote the manuscript with significant contributions from OV, ZT, and HH. All of the authors read and approved the final manuscript.

2.2. Additional publications

1. Rádai, Z., Kiss, J., Nagy, N.A., Somogyi, A.Á., Fülöp, A., Tóth, Z., Babits, M.D., Németh, Z., 2022. State and physiology behind personality in arthropods: a review. *Behavioral Ecology and Sociobiology* 76, 150.

2. Montoya, B., Tóth, Z., Lendvai, Á.Z., Stier, A., Criscuolo, F., Zahn, S., Bize, P., 2022. Does IGF-1 Shape Life-History Trade-Offs? Opposite Associations of IGF-1 With Telomere Length and Body Size in a Free-Living Bird. *Frontiers in Ecology and Evolution*, 10:853674.

3. Hargitai, R., Boross, N., Tóth, Z., Lendvai, Á.Z., 2022. Food restriction delays breeding and affects insulin-like growth factor-1, oxidative damage and haematocrit value before egg-laying in female canaries. *Journal of Avian Biology* e02866.

4. Huber, N., Mahr, K., Tóth, Z., Szarka, E.Z., Çınar, Y.U., Salmón, P., Lendvai, Á.Z., 2021. The stressed bird in the hand: Influence of sampling design on the physiological stress response in a free-living songbird. *Physiology & Behavior* 238, 113488.

5. Vágási, C.I., Tóth, Z., Pénzes, J., Pap, PL., Ouyang, J.Q., Lendvai, Á.Z., 2020. The Relationship between Hormones, Glucose and Oxidative Damage is Condition and Stress-dependent in a Free-living Passerine Bird. *Physiological and Biochemical Zoology* 93, 466-476.

6. Szöllősi, E., Tóth, Z., Mahr, K., Hoi, H., & Lendvai, Á., 2020. Extremely low malaria prevalence in a wetland specialist passerine. *Parasitology*, 147, 87-95.

7. Bókony, V., Móricz, Á.M., Tóth, Z., Gál, Z., Kurali, A., Mikó, Z., Pásztor, K., Szederkényi, M., Tóth, Z., Ujszegi, J., Üveges, B., Krüzselyi, D., Capon, R.J., Hoi, H., Hettyey, A., 2016. Variation in Chemical Defense Among Natural Populations of Common Toad, Bufo bufo, Tadpoles: the Role of Environmental Factors. *Journal of Chemical Ecology* 42, 329–338.

Study 1

Tóth Z., Ouyang J.Q., Lendvai Á.Z. 2018. Exploring the mechanistic link between corticosterone and insulin-like growth factor-1 in a wild passerine bird. PeerJ 6:e5936

Exploring the mechanistic link between corticosterone and insulin-like growth factor-1 in a wild passerine bird

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ABSTRACT

Background. Physiological regulators of life history trade-offs need to be responsive to sudden changes of resource availability. When homeostasis is challenged by unpredictable stressors, vertebrates respond through a set of physiological reactions, which can promote organismal survival. Glucocorticoids have been traditionally recognized as one of the main regulators of the physiological stress response, but the role of an evolutionarily more conserved pathway, the hypothalamic-pituitary-somatotropic (HPS) axis producing insulin-like growth factor-1 (IGF-1) has received much less attention. Although IGF-1 is known to affect several life history traits, little is known about its role in the physiological stress response and it has never been studied directly in adult wild animals.

Methods. In this study, we combined field observations with a controlled experiment to investigate how circulating levels of IGF-1 change in response to stress and whether this change is due to concomitant change in glucocorticoids in a free-living songbird, the bearded reedling *Panurus biarmicus*. We used a standard capture-restraint protocol in field observation, in which we took first and second (stress induced: 15 minutes later) samples. In a follow-up experiment, we used a minimally invasive oral corticosterone manipulation.

Results. We showed that corticosterone levels significantly increased while IGF-1 levels significantly decreased during capture and handling stress. However, change in corticosterone levels were not related to change in IGF-1 levels. We found that experimentally elevated corticosterone levels did not affect IGF-1 levels.

Discussion. Our results are the first to highlight that circulating IGF-1 levels are responsive to stress independently from glucocorticoids and suggest that the HPS axis is an autonomous physiological pathway that may play an important role as regulator of life-history decisions.

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Additional Information and Declarations can be found on page 14

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INTRODUCTION

Resource allocation trade-offs are central to the evolution of life-histories. Physiological mediators of such trade-offs need to monitor resource availability and transmit a signal

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to relevant parts of the organism to adjust energy expenditure in face of environmental variation. Possible candidates of such key life-history regulatory mechanisms must therefore integrate information from both the external and internal environment and be responsive to changes in resource availability (*Harshman & Zera, 2007*). One way to investigate whether a given physiological mechanism could have such life-history regulatory functions is to assess whether it fulfils these requirements of information processing, integration and responsiveness. A useful framework to analyse these questions is when individuals are exposed to stressors. This approach is biologically and ecologically relevant because in order to successfully reproduce and survive, all organisms must be able to cope with environmental challenges. An organism can display a stress response when challenged by unpredictable, noxious changes in the environment, such as the attack of a predator, an infection, inclement weather, or food shortage (*Wingfield et al., 1998*; *Kitaysky, Wingfield & Piatt, 1999*; *Romero, Reed & Wingfield, 2000*; *Hawlena & Schmitz, 2010*). Mounting an appropriate stress response requires a dramatic reorganization of resource allocation. Understanding this response provides great insight into life-history decisions and resource allocation mechanisms.

An important regulator of the stress response is the endocrine system, which plays a central role in regulating the adjustment of morphology, physiology and behaviour to deal with current conditions (*Ricklefs & Wikelski, 2002*; *Flatt & Heyland, 2011*; *Wingfield & Boonstra, 2013*). Hormones function as integrators that process information from the environment and orchestrate multiple processes simultaneously to maximise survival and reproduction under the given circumstances (*Wingfield et al., 1998*; *Martin et al., 2011*). Although some pathways have been clearly represented in the study of the stress response (e.g., HPA axis—hypothalamic-pituitary-adrenal axis), other, more evolutionarily conserved pathways are understudied even though they can play a key role in the evolution of the endocrine system.

The HPA cascade begins at the brain that perceives the stressor and launches a series of downstream hormonal changes. At the endpoint of this axis, the adrenal cortex secrete increased amounts of glucocorticoids into the bloodstream, which stimulate energy production via gluconeogenesis. However, this pathway appeared during the evolution of vertebrates, by which period robust regulation of homeostasis and resource allocation had already evolved (*Stoks, 2001*; *Baker Michael, 2003*; *Bijlsma & Loeschcke, 2005*; *Pauwels, Stoks & De Meester, 2005*; *Hawlena & Schmitz, 2010*). A more conserved endocrine pathway is the insulin/insulin-like signalling pathway (the IIS pathway), which is present in all animals and regulates resource allocation and stress resistance (*Broughton et al., 2005*; *Harshman & Zera, 2007*; *Dantzer & Swanson, 2012*). In vertebrates, the IIS pathway is integrated into the Hypothalamic-Pituitary-Somatotropic (HPS) axis. As part of this hormonal cascade, growth hormone (GH) stimulates the secretion of insulin-like growth factor-1 (IGF-1), primarily from the liver (*Roith, Scavo & Butler, 2001*).

IGF-1 is an evolutionarily highly conserved nutrient sensing hormone that has pleiotropic effects influencing key life-history traits and major life-history trade-offs among growth, reproduction and lifespan (*Dantzer & Swanson, 2012*). IGF-1 is negatively related to lifespan (*Holzenberger et al., 2003*; *Lewin et al., 2016*), but has a positive effect on

growth, sexual maturation and reproduction (*Crain et al., 1995*; *Yakar et al., 1999*; *Pine et al., 2006*; *Flatt et al., 2008*; *Sparkman, Vleck & Bronikowski, 2009*; *Lewin et al., 2016*; *Lodjak, Mägi & Tilgar, 2014*; *Swanson & Dantzer, 2014*). IGF-1 therefore may mediate the trade-off between longevity and reproduction because reduced IGF-1 signalling has been shown to increase lifespan and the expression of genes involved in stress resistance while increased IGF-1 signalling is necessary for reproduction (*Holzenberger et al., 2003*; *Harshman & Zera, 2007*; *Dantzer & Swanson, 2012*; *Lewin et al., 2016*).

Although the role of IGF-1 has been established in resource allocation, its role in coping with stressful situations and the crosstalk between the HPA and the HPS axes remain poorly understood. It has been shown that IGF-1 levels change under nutritional and handling stress. For instance, in response to 5 min of restraint, circulating IGF-1 levels decreased by 21% within 60 min in Yorkshire pigs *Sus crofa domesticus* and remained suppressed for up to 150 min (*Farmer et al., 1991*). A more recent study in pigs also found that restraint stress caused a drop in circulating IGF-1 levels and affected other components of the IIS pathway, suggesting that the IGF-system represents a physiologically relevant biomarker of stress response (*Wirthgen et al., 2017*). Similarly, IGF-1 levels significantly decreased due to short-term (15 min) and long term (24 h) confinement stress in sunshine bass (hybrid of *Morone saxatilis* and *M. chrysops*), in Atlantic salmon *Salmo salar* and in rainbow trout *Oncorhynchus mykiss* (*Wilkinson et al., 2006*; *Davis & Peterson, 2006*).

Whether the decrease in IGF-1 levels is a direct consequence of exposure to stressors on the HPS axis or is due to the stress-induced activity of the HPA axis is controversial (*Unterman et al., 1993*; *Dell et al., 1999*; *Davis & Peterson, 2006*). On the one hand, it is well established that glucocorticoids initiate and orchestrate the emergency life-history stage within minutes to hours from the appearance of the stressor in temperate bird species (*Breuner, Greenberg & Wingfield, 1998*; *Wingfield et al., 1998*; *Romero & Remage-Healey, 2000*; *Löhmus, Sundström & Moore, 2006*), and we also know that glucocorticoids are interconnected with the somatotropic axis (*Dell et al., 1999*). For instance, glucocorticoids play a role in the embryonic development of the HPS axis by affecting GH gene expression, IGF-1 transcription (*Dell et al., 1999*; *Bossis & Porter, 2003*; *Reindl & Sheridan, 2012*). Experimental studies have also shown that exogenous glucocorticoids cause lower IGF-1 levels in rats (*Gayan-Ramirez et al., 1999*), chicken (*Leili & Scanes, 1998*) and fish (*Kajimura et al., 2003*; *Peterson & Small, 2005*). On the other hand, these effects seem to operate only at high glucocorticoid concentrations and/or at a prolonged exposure. For example, although *Bossis & Porter (2003)* found that glucocorticoids mediate the embryonic development of the HPS axis, hormone treatment for at least 8 h was necessary to detect a significant increase in GH gene expression. Similarly, while a pharmacological increase in cortisol resulted in a decrease of IGF-1 levels in tilapia *Oreochromis mossambicus* 24–48 h post injection (*Kajimura et al., 2003*), a more moderate, short-term elevation in cortisol did not affect IGF-1 levels in sunshine bass (*Davis & Peterson, 2006*).

While some of these laboratory, aquacultural and agricultural studies suggest a relationship between stress, glucocorticoids and IGF-1, our knowledge remains very limited about how stressors affect IGF-1 levels in free-living organisms. Importantly, we do not know how the HPA and HPS axes are linked mechanistically. Birds are particularly

interesting model systems for studying the IIS, because their metabolism is faster yet their lifespan is longer than similar-sized mammals (*Costantini, 2008*), and differences in how the IIS is regulated compared to the most studied mammalian models might provide a deeper understanding of the evolution of this pathway (*Holmes & Ottinger, 2003*; *Dantzer & Swanson, 2012*). Although the IGF-system has been extensively studied in poultry (reviewed in *McMurtry, 1998*) almost nothing is known about its regulation in free-living birds (notable exceptions are *Lodjak, Mägi & Tilgar, 2014*; *Lodjak, Tilgar & Mägi, 2016*; *Lodjak et al., 2017*)

While interest in the ecological and evolutionary relevance of IGF-1 has recently increased (*Sparkman, Vleck & Bronikowski, 2009*; *Sparkman et al., 2010*; *Palacios, Sparkman & Bronikowski, 2012*; *Lodjak, Mägi & Tilgar, 2014*; *Reding et al., 2016*; *Lodjak, Tilgar & Mägi, 2016*; *Lodjak et al., 2017*), to the best of our knowledge no study has directly tested the effects of stressors on the activity of the HPS axis or investigated the mechanistic link between the HPA and HPS axes in any free-living organisms. Therefore, we aimed at answering whether: (1) acute stress affects plasma levels of IGF-1 and whether (2) glucocorticoids directly affect circulating IGF-1 levels. First, we hypothesized that acute stress will affect the activity of the HPS axis; therefore, we predicted that circulating IGF-1 levels will decrease in response to short-term acute stress. Second, to investigate the crosstalk between the HPA and the HPS axes, we experimentally increased corticosterone levels using a minimally invasive technique. We predicted that if the HPS axis receives direct input from glucocorticoids, then increased glucocorticoid levels will down-regulate IGF-1 levels. Alternatively, if the HPS axis responds to external stressors directly, then we predicted that short-term elevation of circulating glucocorticoids will not affect IGF-1 levels. To answer these questions, we studied free-living bearded reedlings *Panurus biarmicus*, a small (~14 g), sexually dimorphic, resident songbird common throughout wetlands of Eurasia.

MATERIAL AND METHODS

Study animal and field study

We captured 17 wintering free-living bearded reedlings *Panurus biarmicus* (Linnaeus, 1758) in Hungary at Virágoskúti-halastó (N47.6518, E21.3589) between September 2015 and January 2016. We caught the birds with continuously observed mist-nets and subjected them to a standardized capture-handling-restraint protocol (*Wingfield, 1994*). The first blood sample (50~100 µl) was taken as soon as possible after the bird hit the net (mean handling time: 4:58 min; range: 2–10 min). The initial handling times contained one high outlier, inclusion or removal of this point did not affect our results qualitatively. The handling time was not detectably related to either corticosterone levels ($t = 1.276$, $p = 0.223$) or IGF-1 levels ($t = -0.785$ $p = 0.445$). The bird was then placed into an opaque cloth bag and the collection of a subsequent blood sample (50–100 µl) was started 15 min after the initial capture (completed mean time: 17:51 min; range: 15–19 min). We chose to take the second blood sample after 15 min because we were interested in the short-term acute stress-response, and we wanted to minimize the possible downstream effects of corticosterone on IGF-1 levels. The total volume of the two blood samples was

under 140 µl that met the recommendation of *Owen (2011)*. The study was approved by the Institutional Animal Care and Use Committee at the University of Debrecen (DEMAB/19-6/2015) and the regional government agency (HBB/17/00870-3/2015).

Experimental study

Housing protocol

Twenty-one wintering bearded reedlings were captured with mist-nets in Hungary at Hortobágy-Halastó (N47.6211, E21.0757) between 18 of October and 16 of November in 2016. After capture, they were housed in an outdoor aviary at the Botanical Garden of the University of Debrecen where they were kept for 4 months and acclimated to captivity. Four weeks prior to the experiment, the birds were transferred to individual cages measuring 25 × 25 × 25 cm. All sides of the cages (except for the front) were made of a non-transparent board (OSB); therefore, birds were kept in visual but not acoustic isolation. Individual cages were separated by a non-transparent removable divider. Because bearded reedlings are very social, live in flocks and maintain strong pair bonds throughout the year (*Lovász, Fenyvesi & Gyurácz, 2017*; *Griggio & Hoi, 2011*), long-term individual separation may be perceived as stressful for the birds (as found in other social species, e.g., *Remage-Healey, Adkins-Regan & Romero, 2003*). To avoid such additional stressors, before the experiment, birds were kept in pairs, by removing the divider between adjacent cages. Food (a mixture of apple, carrot, quark, cracked dried fish, dried *Gammarus* sp., cracked corn, cracked dry cat food, a commercial soft food mixture for birds and mealworms) and water were available *ad libitum* at all times.

Experimental design

Corticosterone levels were manipulated orally using a minimally invasive technique described by *Breuner, Greenberg & Wingfield (1998)*, in which corticosterone dissolved in peanut oil was injected into mealworms *Tenebrio molitor*. We used a randomized block design with two doses of exogenous corticosterone and a control manipulation for each block.

The day before experimental day, we removed the food from the birds 1.5 h before sunset and the mobile dividers were inserted into the cages to keep the birds individually. Water was still available *ad libitum*. The next morning (between 8:00–9:00, to avoid daily variation in hormone levels and to standardize the duration of the food removal), the experimenter quietly entered the room (the cages were oriented in a way that the birds could not see the door) and gave one mealworm to the selected bird through a small hole covered by a semi-transparent layer at the back of the cages, so that the bird could not see the experimenter, but we could observe the birds and record the time when they consumed the mealworm (mean time of ingestion was 39 s). We paid particular attention to avoid any visual or acoustic contacts with the birds. Fifteen minutes after the bird consumed the mealworm, the bird was captured through a backdoor at the cages and a single blood sample (~70 µl) was taken as soon as possible. After blood sampling, body mass (to the nearest 0.1 g) of the sampled individuals was also recorded and they were released back to their cage. The exact times when we entered the room, when the mealworm was given, when the bird ate the mealworm, when we caught the birds in the cages and when blood

sampling was completed were all recorded. Handling time was defined as the time between when we opened the door of individual cages and blood sample collected. Total procedural time was defined as the time elapsed between the experimenter entering the room and when blood sampling was completed. Treatments were carried out in blocks, so that 3 birds in a block got the mealworms subsequently (with approximately 1 min staggering). Two blocks were sampled in a morning. After the treatments and sampling, the birds received the usual ad libitum bird chow and fresh water and were left undisturbed for the rest of the day. After we processed all birds, the experiment was repeated one week later, in which each individual received a different treatment than in the first trial, hence all birds received two different treatments. The treatments for the second trial were randomized again within blocks and birds were randomly assigned to the blocks.

Mealworm injection

Mealworms were injected with 20 µl of peanut oil (VWR catalogue number: ACRO416855000) containing one of the following concentrations of corticosterone (Sigma catalogue number: NET399250UC): (1) control, no corticosterone; (2) low corticosterone, 0.2 mg/ml (4 µg corticosterone + 16 µl peanut oil); and (3) 0.5 mg/ml corticosterone concentration (10 µg corticosterone + 10 µl peanut oil). We made a stock solution for every concentration before we started the experiment. Hereafter we used those stock solution for injection. After thoroughly vortexing the solution, we injected it into mealworms with a 1 ml syringe using a 26G-needle. The chosen corticosterone concentrations were based on previous studies and were calculated as dose per body mass. In red-eyed vireos *Vireo olivaceus* (12–16 g), plasma corticosterone concentrations were elevated with a 0.2 mg/ml concentration corticosterone solution (which corresponds to 0.28 µg corticosterone per 1g of body mass) (*Löhmus, Sundström & Moore, 2006*). In Gambel's white crowned sparrows *Zonotrichia leucophrys gambelii* (25–28 g) a low (0.2 mg/ml–0.15 µg/g) and a high (1 mg/ml–0.7 µg/g) dose were used, but the low dose did not elevate significantly the plasma corticosterone levels (*Breuner, Greenberg & Wingfield, 1998*). Nestling Zebra-finches *Taeniopygia guttata* (6–15 g) received a dose of 0.25 mg/ml (1.19 µg/g) corticosterone, which resulted in a significant increase in circulating corticosterone levels (*Spencer & Verhulst, 2008*). Therefore, our manipulations correspond to a dose of 0.25 µg/g (low) and 0.62 µg/g (high).

Blood sampling

We took blood samples by puncturing the brachial vein with a 26G-needle and collecting blood in heparinized capillary tubes. Samples were kept on ice until transferring them to the lab (1–7 h in the field and max. 1 h in the experiment). Samples were centrifuged at 2,200 g for 10 min and the plasma was removed with a Hamilton syringe. We divided the plasma into 2 aliquots, one for IGF-1 (15 µl) and one for corticosterone (15 µl). We stored the samples at −20 °C until assayed for corticosterone by radioimmunoassay (RIA) and assayed for IGF-1 by enzyme-linked immunosorbent assay (ELISA).

Hormone assays

Plasma IGF-1 levels were measured in duplicates by a commercial avian ELISA kit (catalogue: cIGF1ELISA, lot number D00035) from IBT GmbH, Germany. The assay was developed to measure chicken IGF-1, and the amino acid sequence of IGF-1 is identical in chicken and in *P. biarmicus* (ÁZ Lendvai et al., 2018, unpublished data), therefore this assay was expected to perform well in our study species. Serial dilutions of a plasma pool of *P. biarmicus* were parallel of the standard curve. IGF-1 was separated from its binding proteins using an acidic extraction in accordance with the manufacturer's instructions. The final concentrations were determined colourimetrically measuring the absorbance at 450 nm using a Tecan F50 microplate reader. In this assay, we also included chicken *Gallus gallus* plasma samples as a reference, and the obtained IGF-1 concentrations for the chicken samples (375.2 ± 69.4 SE ng/ml) were an order of magnitude higher than what we had expected based on the literature (30–50 ng/ml, *Ballard et al., 1990*). Therefore we measured known concentrations of an international IGF-1 gold standard (WHO/NIBSC 02/254, a product used in different laboratories to calibrate IGF-1 values; *Chanson et al., 2016*) and recalibrated all concentrations against this standard. IGF-1 concentrations of the recalibrated chicken reference samples (53.0 ng/ml) were similar to published results and were in agreement with an in-house ELISA developed in our laboratory (ÁZ Lendvai et al., 2018, unpublished data). Therefore, we used the recalibrated values in our analyses. Note however, that the recalibration only affects the absolute values reported, since the concentrations obtained originally would yield the same results, albeit on a different scale. Minimal detection limit was 1.5 ng/ml, and none of the samples fell below this limit. Intra-assay coefficient of variation was 3.9%, inter-assay CV was 5.7%.

Total corticosterone from plasma samples was quantified through direct radioimmunoassay (*Lendvai, Bókony & Chastel, 2011*). We extracted corticosterone from plasma using diethyl-ether, and extracts were reconstituted in phosphate-buffered saline. We let the samples incubate overnight at 4 °C. We added ~10 K dpm of 3H-Cort (Catalogue number: NET399250UC, lot number: B00025; Perkin Elmer, Waltham, MA, USA), antiserum (Sigma C8784-100ST, lot number: 092M4784) and phosphate-buffered saline. We let the solution sit overnight at 4 °C. Dextran-coated charcoal was added to separate corticosterone bound to antibodies. After centrifugation, the radioactivity of the bound fraction was counted in a liquid scintillation counter (QuantaSmart). All samples were processed in one assay (intra-assay CV: 3.5%).

Statistical analyses

We analysed our data in R statistical environment, R version 3.3.2 (*R Core Team, 2017*). We fitted linear mixed models with function 'lmer' from package lme4 (*Bates et al., 2015*; version: 1.1-13), and we used stepwise backward model selection to find the best fitting model. Degrees of freedom for linear mixed models were calculated using the Satterthwaite approximation and corresponding p-values were obtained using the package lmerTest (*Kunetsova, Brockhoff & Christensen, 2016*; version: 2.0-33).

To test the effects of handling stress on IGF-1 and corticosterone, we used generalized linear mixed models with individual as a random intercept. In the initial model, IGF-1 was

the dependent variable, handling (first or second sample) and its two-way interaction with body mass and sex were the explanatory variables, and ring number (as individual identity) was the random factor. The same initial model structure was used to model corticosterone levels. We also analysed the relationship between IGF-1 levels and corticosterone levels in the first sample with a linear model controlling for body mass. Next, we calculated the stress-induced change in both corticosterone and IGF-1 levels (values from the first sample subtracted from the second samples), and analysed whether the magnitude of change between these two hormones were related in a linear model. In this analysis, the dependent variable was the change in IGF-1 levels and the explanatory variable was the change in corticosterone levels, while controlling for body mass and sex.

The experimental data were analysed using linear mixed models. Here, we considered treatment as a three-level factor (high corticosterone, low corticosterone and control). In the initial model, IGF-1 or corticosterone was the dependent variable, treatment and its two-way interaction with sex and body mass were the explanatory variables. The treatment blocks and the weeks of the experiment were also included as fixed effects. In both models we controlled for the handling time and total procedural time.

RESULTS

Field samples

The 15 min capture-restraint stress induced a significant increase in corticosterone levels (Fig. 1A, Table 1), and body mass was positively related to corticosterone levels in the first sample (Table 1). Sex and the two-way interaction between stress and sex or body mass did not affect corticosterone levels (Table 1). Handling stress caused a significant decrease in IGF-1 levels (Fig. 1B, Table 2), and IGF-1 levels were higher in males (Table 2). Body mass was not related to IGF-1 levels in the first sample. Body mass and the two way interaction between stress and sex or body mass did not affect the IGF-1 levels (Table 2).

We did not find a significant relationship between IGF-1 and corticosterone levels in the first samples ($t = 0.786$, $p = 0.450$). The changes in IGF-1 and in corticosterone levels induced by the handling stress were also unrelated, even after controlling for body mass or sex (p's >0.9). One bird showed an unusual pattern in the corticosterone data, in which corticosterone decreased in response to handling stress. Removing this data point did not alter any of our conclusions.

Corticosterone manipulation

The body mass of the captive birds did not change during the two experimental weeks, and IGF-1 levels, corticosterone levels, block or sex were all unrelated to body mass (p's >0.5). The experimental week or the treatment blocks did not affect corticosterone or IGF-1 levels (p's >0.2).

Both low and high dose of treatment significantly increased corticosterone levels compared to the control treatment (Fig. 2A, Table 3). Other effects, including sex, mass and the two-way interaction of treatment with sex and mass, handling time and total procedural time did not influence corticosterone levels (Table 3). Although the manipulation was successful in creating differences in circulating corticosterone levels, the

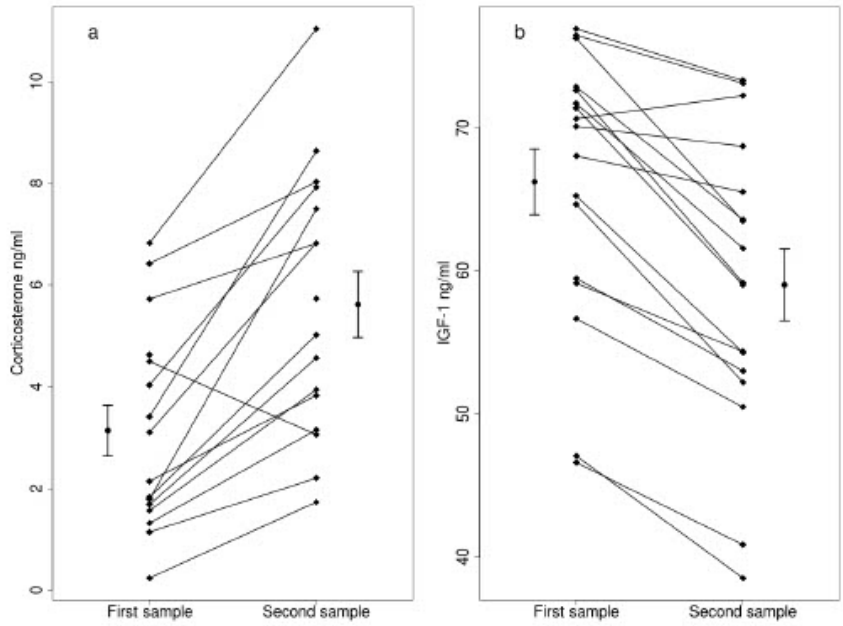

Figure 1 Effects of capture handling stress on corticosterone (A) and IGF-1 (B) levels. Capture handling stress causes (A) a significant increase in circulating corticosterone levels ($n = 16$) and (B) a significant decrease in circulating IGF-1 levels ($n = 17$) in free-living bearded reedlings (*Panurus biarmicus*). Squares denote the individual IGF-1 and corticosterone concentrations, and lines between the squares connect the first and second samples from the same individual. The dots and arrows beside the individual points represent the mean and standard error values of corticosterone (A) or IGF-1 (B) levels.
Full-size DOI: 10.7717/peerj.5936/fig-1

treatment did not affect IGF-1 levels (Fig. 2B, Table 4). Furthermore, sex, mass and the two-way interaction of treatment with sex and mass, handling time and total procedural time did not affect IGF-1 levels during the experiment (Table 4).

DISCUSSION

We explored the mechanistic link between the HPA and HPS axes using a free-living songbird, resulting in two key findings. First, we found that in response to a standardized stressor, circulating IGF-1 levels decreased in wild bearded reedlings within 15 min. To our knowledge, this is the first study that reports such an effect for any free-living species. Second, we found that experimentally elevated corticosterone levels did not result in a decrease in IGF-1 levels during the same time frame as we found in the field. This result suggests that the somatotropic axis may respond to environmental stimuli independently from the HPA axis and may be part of the adaptive physiological coping mechanisms used to maintain or restore homeostasis in stressful situations.

Table 1 Results from the final linear mixed model after backward elimination for corticosterone in the field study. Parameter estimates of variables affecting circulating corticosterone levels in free-living bearded reedlings (*Panurus biarmicus*). Results are from the final linear mixed-effects model after stepwise backward elimination of non-significant effects. The initial model structure was: Corticosterone ~Handling × (Sex + Mass). The terms excluded during model selection with the associated *p*-values in the model before elimination are shown below the table.

	Estimate	Std. Error	*df*	*t*-value	*p*-value
Intercept	−22.207	7.701	10.843	−2.884	0.015
Handling (stress)	2.619	0.570	10.664	4.593	<0.001
Mass	1.678	0.500	10.833	3.356	0.006

Notes.
Terms excluded: Handling*Sex $p = 0.458$, Sex $p = 0.401$, Handling*Mass $p = 0.096$.

Table 2 Results from the final linear mixed model after the backward elimination for IGF-1 in the field study. Parameter estimates of variables affecting circulating corticosterone levels in free-living bearded reedlings (*Panurus biarmicus*). Results are from the final linear mixed-effects model after stepwise backward elimination of non-significant effects. The initial model structure was: IGF-1 ~Handling × (Sex + Mass). The terms excluded during model selection with the associated *p*-values in the model before elimination are shown below the table.

	Estimate	Std. Error	*df*	*t*-value	*p*-value
Intercept	55.666	3.217	13.809	17.306	<0.001
Handling (stress)	−6.698	1.173	13.235	−5.709	<0.001
Sex (males)	14.181	3.880	13.001	3.655	0.003

Notes.
Terms excluded: Handling*Mass $p = 0.644$, Handling*Sex $p = 0.507$, Mass $p = 0.313$.

Increases in plasma corticosterone levels in response to stress have been previously reported in many species. Our data obtained in bearded reedlings in the field is consistent with these finding, showing that corticosterone levels increase in response to capture/restraint stress and suggest that the birds perceived the procedure as stressful. Although many studies collect the stress-induced blood sample at 30 min (*Wingfield, Vleck & Moore, 1992*; *Remage-Healey & Romero, 2001*; *Buehler et al., 2008*), we chose to reduce the restraint period to study the effects of short-term acute stress on IGF-1 levels, while minimizing the potential confounding downstream effects of corticosterone. The effects of glucocorticoids are mainly genomic, which typically act over an hour, but require at least 15 min (reviewed in *Haller, Mikics & Makara, 2008*). Some short-term direct effects of corticosterone have been also demonstrated, such as effects on RNA synthesis (reviewed in *Haller, Mikics & Makara, 2008*). Therefore, by choosing a shorter restraint period, we aimed at decreasing the time during which the organism may have been exposed to the physiological effects of elevated glucocorticoids (*Buehler et al., 2008*). Although the effects of stress on IGF-1 levels in free-living organisms have not been reported before, this finding is consistent with previous studies in captive animals (*Farmer et al., 1991*; *Wilkinson et al., 2006*; *Davis & Peterson, 2006*; *Wirthgen et al., 2017*). However, our findings do not support the conclusion of earlier avian studies (*Lodjak, Mägi & Tilgar, 2014*; *Lodjak, Tilgar & Mägi, 2016*; *Lodjak et al., 2017*), in which handling time was reported to be unrelated to IGF-1 levels, although in nestlings. Such effects may be age-specific. For example,

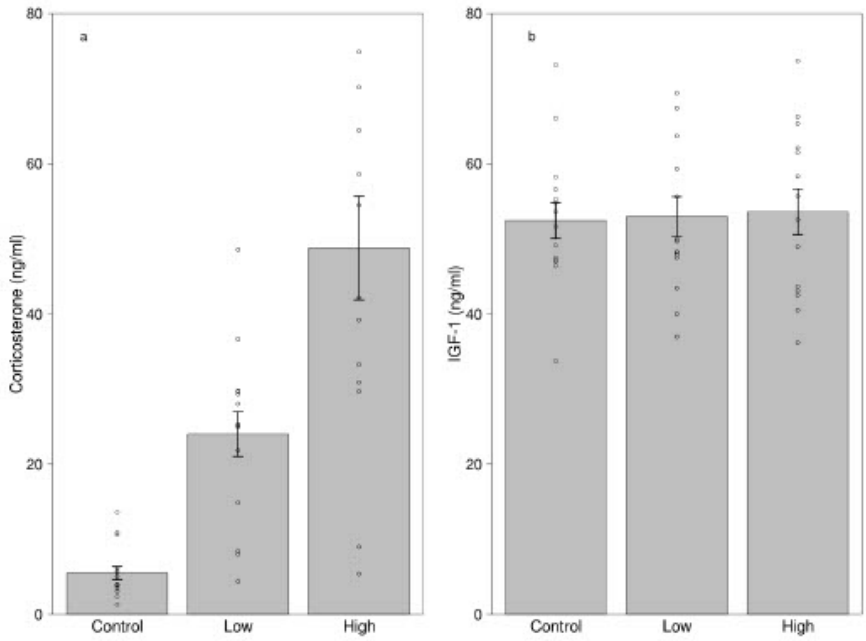

Figure 2 Effects of dietary corticosterone treatment on circulating (A) corticosterone ($n = 42$) and (B) IGF-1 levels ($n = 42$). Corticosterone levels were significantly higher in the low corticosterone ($n = 14$) and the high corticosterone ($n = 14$) group compared with the control group ($n = 14$), although the IGF-1 levels did not differ between the treatment groups. We used 21 individuals in the experiment, and every bird received two different treatments.

Full-size DOI: 10.7717/peerj.5936/fig-2

gilthead seabream *Sparus aurata* differed in expression of IGF-1 and IGF-1R mRNA levels during ontogeny (*Perrot et al., 1999*). In a study on Korean native ogol chicken, circulating IGF-1 levels gradually increased during the post-hatching period (*Yun et al., 2005*). The stress-responsiveness of IGF-1 may also vary with development, and may explain why *Lodjak, Mägi & Tilgar (2014)* and *Lodjak, Tilgar & Mägi (2016)* did not find that IGF-1 was affected by handling. Furthermore, stress-responsiveness of IGF-1 may also be species-specific, although we also found that capture-restraint stress caused a decrease in IGF-1 levels in adult free-living house sparrows *Passer domesticus* (CI Vágási et al., 2018, unpublished data). Therefore, we suggest that further studies of IGF-1 levels should take into account the potentially confounding effect of additional stressors.

The decrease in IGF-1 under stress is consistent with the allostatic concept of the stress response (*McEwen & Wingfield, 2003*). Under stressful situations, the organism has to re-establish homeostasis and in order to do so, it has to suppress energetically costly anabolic processes and reinforce those behavioural and physiological processes that promote immediate survival (*Wingfield et al., 1998*). IGF-1 is a prime regulator of anabolic processes and antagonistic of the catabolic effects of glucocorticoids, therefore the decrease

Table 3 Results from the final linear mixed model on the effects of experimental treatment on corticosterone. Parameter estimates of variables affecting circulating corticosterone levels after oral administration of corticosterone in bearded reedlings (*Panurus biarmicus*). Results are from the final linear mixed-effects model after stepwise backward elimination of non-significant effects. The initial model structure was: Corticosterone ~Handling time + Procedural time + Treatment × (Sex + Mass). The terms excluded during model selection with the associated *p*-values in the model before elimination are shown below the table.

	Estimate	Std. Error	*df*	*t*-value	*p*-value
Intercept	5.660	4.550	28.750	1.244	0.223
Treatment (low)	18.260	6.100	33.790	2.993	0.005
Treatment (high)	43.100	6.100	33.790	7.006	<0.001

Notes.
Terms excluded: Treatment (Low)*Mass $p = 0.685$, Treatment (High)*Mass $p = 0.958$, Treatment (Low)*Sex $p = 0.543$, Treatment (High)*Sex $p = 0.578$, Sex $p = 0.530$, Total procedural time $p = 0.267$, Handling time $p = 0.338$, Mass $p = 0.249$.

Table 4 Results from the final linear mixed model testing the effects of experimental treatment on IGF-1 levels. Parameter estimates of variables affecting circulating IGF-1 levels after oral administration of corticosterone in bearded reedlings (*Panurus biarmicus*). Results are from the final linear mixed-effects model after stepwise backward elimination of non-significant effects. Treatment was part of the experimental design, so were kept in the final model, despite being not-significant. The initial model structure was: IGF-1 ~Handling time + Procedural time + Treatment × (Sex + Mass). The terms excluded during model selection with the associated *p*-values in the model before elimination are shown below the table.

	Estimate	Std. Error	*df*	*t*-value	*p*-value
Intercept	52.865	2.900	5.670	18.228	<0.001
Treatment (low)	−0.932	3.862	38.000	−0.241	0.810
Treatment (high)	0.709	3.862	38.000	0.184	0.855

Notes.
Terms excluded: Handling time $p = 0.934$, Treatment (Low)*Mass $p = 0.911$, Treatment (High)*Mass $p = 0.729$, Mass $p = 0.620$, Total procedural time $p = 0.502$, Treatment (Low)*Sex $p = 0.795$, Treatment (High)*Sex $p = 0.052$, Sex $p = 0.561$.

of IGF-1 under acute stress is consistent with its role as one of the physiological mechanisms responsible for maintaining homeostasis. For instance, in a previous study in mice, IGF-1 levels decreased markedly in food restricted animals and the individuals started to lose weight (*O'Sullivan et al., 1989*). However, experimental IGF-1 administration during starvation reduced the rate of weight loss through the inhibition of the catabolic processes (*O'Sullivan et al., 1989*). The sudden drop of IGF-1 levels in response to the stressor suggests that this physiological change prepares the animal for the metabolic challenges faced by slowing down anabolic processes and permitting the catabolic effects of glucocorticoids.

In light of these results, we expected that higher glucocorticoid stress responses would be associated with the largest decrease in IGF-1 levels. However, despite the opposite direction of change in the two hormones, neither levels in the first sample nor the magnitude of corticosterone and IGF-1 stress responses were related in the field study at the individual level: birds with the strongest corticosterone increase were not the ones that decreased their IGF-1 levels the most, and vice versa. Furthermore, we did not find any relationship between those parameters if we controlled for body mass. In order to test the relationship between the two hormones more thoroughly, we carried out an experiment in which we manipulated corticosterone in a minimally invasive manner.

Our dietary hormone treatment increased the circulating levels of corticosterone, while control birds did not show a marked increase in corticosterone levels over the course of the study. These results suggest that similarly to previous studies (*Breuner, Greenberg & Wingfield, 1998*; *Lõhmus, Sundström & Moore, 2006*; *Spencer & Verhulst, 2008*), our oral hormone treatment was successful. Corticosterone concentrations after ingesting the mealworm were significantly higher in both the low and the high dose group compared to the controls, albeit with large individual variation. Despite this rapid increase in corticosterone levels in the absence of a physical stressor, IGF-1 levels in our treated birds remained at the level of the controls, with minimal effect sizes; therefore, we can be confident that the treatment did not affect IGF-1 secretion. Our results are similar to those reported in the sunshine bass, in which confinement stress for 15 min resulted in a decrease in IGF-1 levels, but the dietary hormone treatment did not affect plasma IGF-1 concentrations (*Davis & Peterson, 2006*).

These results suggest that the HPA and HPS axes are not linked downstream at the endpoint of these hormonal cascades, but the crosstalk between these pathways happens at the hypothalamic-pituitary level. We argue that this is the reason behind the discrepancy between the conclusions of studies using physiological and pharmacological doses (see above). Dexamethasone, a powerful glucocorticoid agonist, which is known for having a strong negative feedback on the HPA axis has also been shown to decrease plasma IGF-1 concentration in chickens (*Leili & Scanes, 1998*), which supports the notion that integration of the HPA and HPS axes is operating at higher regulatory levels.

According to a recent study performed on great tit nestlings, the relationship between corticosterone and IGF-1 varies with the nutritional condition of the individuals. *Lodjak, Tilgar & Mägi (2016)* found that pre-fledging plasma IGF-1 levels of nestlings in good condition (from broods that were experimentally reduced) were positively related to feather corticosterone (an integrated measure of corticosterone over several days during the development), whereas the association between IGF-1 and feather corticosterone levels was negative in nestlings with lower nutritional condition (from enlarged broods). In control broods however, there were no association between the two hormones. In our captive study, the birds had *ad libitum* food availability and they were all in good condition; therefore, one possible explanation for the absence of the relationship between IGF-1 and corticosterone may be that birds in good condition can afford to tolerate higher glucocorticoid concentrations without decreasing IGF-1 levels. In line with this possibility, biomedical studies have shown that IGF-1 diminishes the protein catabolic effects of glucocorticoids only in normally fed but not in starved subjects (*Botfield, Ross & Hinds, 1997*).

CONCLUSIONS

In this study, we showed for the first time that IGF-1 levels decrease in response to stress in a free-living songbird, and that the magnitude of this response is not related to the glucocorticoid stress response. Furthermore, an experimental increase in corticosterone did not affect circulating IGF-1 levels. While glucocorticoids may still have non-linear or

permissive effects on IGF-1 regulation, our results suggest that the HPA and HPS axes are both stress responsive and are not tightly co-regulated at their downstream endpoints. These results raise the possibility that the interaction between IGF-1 and corticosterone may modulate the adaptive response of organisms in stressful situations. Investigations of the relationship between glucocorticoids and fitness remain equivocal, with some studies showing positive, negative and also no relationship (reviewed in *Bonier et al., 2009*). The lack of a general glucocorticoid-fitness relationship has been suggested to be a result of the flexibility and environmentally context dependent nature of glucocorticoids (*Bonier & Martin, 2016*). Our results showing that IGF-1 levels are responsive to stress independently from glucocorticoids suggest that the HPS axis is an autonomous physiological cascade that may be also involved in the mediation of life history decisions and affect fitness components (*Harshman & Zera, 2007*; *Dantzer & Swanson, 2012*; *Lewin et al., 2016*). IGF-1 is an evolutionary ancient regulatory hormone with a primary role to provide an organism-wide internal signal about resource availability, and may alter the function of glucocorticoids. If overall resource availability is high (as was the case in our captive study), then IGF-1 levels can act as a physiological buffer against the adverse effects of increased corticosterone levels. However, when the central nervous system receives input from the environment challenging the organism, it may require the reallocation of resources, which is reflected in decreased IGF-1 levels. Therefore, circulating IGF-1 levels can be an important biological indicator of individual internal state and a useful parameter to investigate in the study of life-history decisions.

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Zsófia Tóth conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Jenny Q. Ouyang conceived and designed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Ádám Z. Lendvai conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

Regional government agency (licence no HBB/17/00870-3/2015).

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

Field experiments were approved by regional government agency (licence no HBB/17/00870-3/2015).

Data Availability

The following information was supplied regarding data availability:

The raw data are provided in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.5936#supplemental-information.

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Study 2

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Food Restriction Reveals Individual Differences in Insulin-Like Growth Factor-1 Reaction Norms

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Most organisms have to cope with unpredictable environmental challenges such as fluctuations in nutritional resources. Insulin-like growth factor-1 (IGF-1) is an evolutionarily conserved hormone that is highly sensitive to the individual nutritional status and regulates major life-history traits including lifespan and reproduction across vertebrates. We investigated the role of IGF-1 during periods of food shortages by altering between two feeding regimes (110 and 70% of daily food intake) after a period of *ad libitum* feeding in captive bearded reedlings (*Panurus biarmicus*). Each dietary treatment was repeated twice. Birds lost mass under food restriction, but the magnitude of mass change depended on the preceding dietary conditions. Moreover, bearded reedlings showed large, repeatable individual differences in their IGF-1 reaction norms with some individuals increasing IGF-1 levels in response to a restricted diet, whereas others showed no responses or decreased IGF-1 levels. This variation was explained by differences in average body mass: heavier individuals had higher IGF-1 levels during the control treatment and were more likely to decrease IGF-1 levels in response to the dietary restriction than did lighter ones. This result uncovers an individual by environment interaction (I × E) and may have important implications for the evolution of IGF-1 related hormonal phenotypes in this species.

Keywords: insulin-like growth factor-1 (IGF-1), stress, *Panurus biarmicus*, body mass, endocrinology, nutrition

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INTRODUCTION

Unpredictable fluctuations in the availability of food (e.g., because of droughts, cold winters) are ubiquitous and organisms have developed an array of morphological, physiological and behavioral adaptations to cope with such environmental challenges (Groscolas and Robin, 2001; Harshman and Zera, 2007; Killen et al., 2011). The role of the endocrine system is particularly interesting in this context, because hormones are highly plastic traits that integrate external (environmental) and internal (e.g., nutritional status) information to respond to stochastic environments (Harshman and Zera, 2007; Regan et al., 2020). Across their responsiveness, hormones show a reaction norm (i.e., differential phenotypic expression of a given genotype due to changing environments), which is highly variable within and among individuals (Pigliucci, 2001;

Cockrem, 2013). The among-individual variation in reaction norms (individual by environment interaction, I × E) shape phenotypically plastic responses to resource availability in a given environment (Williams, 2008; Baugh et al., 2014; Lendvai et al., 2014; Hau and Goymann, 2015; Madliger and Love, 2016; Vitousek et al., 2018; Houslay et al., 2019). The degree of plasticity influences not only the individual but also the ability of the populations to respond to small immediate changes, such as food shortages or predator attacks, and to long-term effects, such as climate change (Reed et al., 2006; Visser, 2008).

One of the hormonal systems that evolved to respond to the variation in food availability is the insulin/insulin-like signaling pathway (IIS) (Regan et al., 2020). IIS is known to have a prominent role in regulating energy metabolism and is directly integrated with nutrient-sensing cellular mechanisms (Saltiel and Kahn, 2001). In vertebrates, the main ligand of this system is insulin-like growth factor-1 (IGF-1), one of the key factors regulating the organism's metabolism and development in relation to its nutritional status (Dantzer and Swanson, 2012; Lodjak and Mägi, 2017). These effects might be tightly linked to how IGF-1 determines the transition from the catabolic to the anabolic state. Low nutrient/energy availability is suggested to decrease IGF-1 production and secretion, which leads to increased cell recycling, autophagy, and apoptosis (Bitto et al., 2010; Adler and Bonduriansky, 2014). These processes can have direct and indirect fitness effects by down-regulating reproduction, growth, and the immune response (Wang and Levine, 2010; Gao et al., 2019). On the other hand, up-regulation of IGF-1 can delay muscle atrophy (i.e., an excessive amount of apoptosis of cells) during food restriction and reduce overall weight loss (O'Sullivan et al., 1989; Cleveland et al., 2009; Abe et al., 2019).

Even though fluctuations in food availability frequently occur in nature and IGF-1 might facilitate adaptations toward these conditions (O'Sullivan et al., 1989), our knowledge about the role of IGF-1 in shaping responses to such events in wild animals is limited. The majority of literature presents the findings of medical and agricultural sciences, focusing on humans and laboratory or farmed animals (Robinson et al., 2006; Berryman et al., 2008; Valente et al., 2013; Mauch et al., 2016; Rahmani et al., 2019). There are only a few examples from wild, free-living animals, such as a study in Eastern fence lizard (*Sceloporus undulatus*) which shows that food-restriction decreased plasma IGF-1 levels (Duncan et al., 2015). Another study on nestlings of a passerine species discusses the possibility that the growth-enhancing effects of IGF-1 during early development might be affected by parental food supply (Lodjak et al., 2014). Hence, to understand the variation in IGF-1 responses to current food availability and its contribution to shaping physiological phenotypes, it is important to aim for experimental studies on wild-type animals originating from natural populations.

To explore the role of the IGF-1 response to variation in food availability in a wild animal, we conducted a food-restriction experiment, using captive adult bearded reedlings (*Panurus biarmicus*). We repeatedly exposed individuals to changing dietary regimes, by alternating between control (110% of the daily intake) and restricted diet (70% of the daily intake). After each dietary treatment, we measured body mass and circulating IGF-1 levels of the birds. Our experimental design allowed us to disentangle among- and within-individual hormonal variation and to make predictions at both levels. Considering that IGF-1 levels are regarded to reflect the individual nutritional status, we made two predictions. First, we predicted that among individuals, larger (heavier) birds would have higher circulating IGF-1 levels. Second, we predicted that within individuals, circulating IGF-1 levels would decrease in response to the restricted dietary regime and this decrease would be proportional to body mass loss under the restricted diet.

MATERIALS AND METHODS

General Methods

In July 2017, we caught 24 Bearded reedlings (*Panurus biarmicus*) at Hortobágy-Halastó, (47°38'13.7 N and 21°04'42.8 E, Hungary) using mist-nets. At the time of capture, all birds were juveniles (yearlings). The age and sex were determined by examining plumage and bill coloration (Svensson, 1992). All birds were ringed with a standard aluminum ring, and we measured tarsus length (to the nearest 0.01 mm) and body mass (to the nearest 0.1 g).

Immediately after the capture and the subsequent measurements, the birds were transferred into an outdoor aviary (3.65 × 3.35 × 2.75 [L × W × H] m) located at the University of Debrecen (47°33'32.9 N and 21°37'14.6 E). The aviary was furnished with reed bundles, branches, and a pool (1 m^2 water surface). Food (a mixture of grated apple, carrot, quark, a commercial soft food mixture for insectivorous birds, ground dry cat food as a protein supplement and live mealworms) and water were provided *ad libitum*. The birds remained for seven months in the aviary before the onset of the experiment (5 February 2018) to habituate to the captive conditions. By the time the experiment started, all individuals had completed their post-juvenile molt and the reproductive period had not yet begun.

We followed all applicable international, national, and institutional guidelines for the use of animals. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution and approved by the Regional government agency (license no HBB/17/00870-3/2015).

Experimental Protocol

The experimental protocol was based on Lendvai et al. (2014), with modifications. Two weeks prior to the experiment, the birds were captured, weighed and transferred into individual cages (measuring 30 × 26 × 32 [L × W × H] cm), located in the outdoor housing facilities.

During the acclimation period, individuals received *ad libitum* food (as described above) and water. Mealworms were not provided, because live mealworms can leave the feeder, which could have affected the dietary treatments (see below). After 2 weeks of acclimation in the individual cages, we measured the daily food intake (DFI) for each individual for five consecutive days. We fed the birds every morning between 09:00 and 10:00

by filling their feeder with 38 g of food. A 24 h later, we removed the bottom tray and the feeder from each cage and measured the weight of the remaining, and spilled food with a digital scale. The DFI was calculated as the difference between the initial weight in the feeder and the weight of remaining food plus spillage. This procedure was performed twice over 2 weeks, and the average daily food intake (ADFI) was calculated as the average of 10-day DFI values for each individual.

After measuring the food intake, birds were kept under *ad libitum* food regime for one additional week. After this period, individuals were randomly assigned to one of the treatment groups: food-restricted (70% of their individual ADFI) or control (110% of their individual ADFI). The treatment was based on previous studies showing that a 30% reduction in food is sufficient to trigger changes in hormone levels (e.g., Valle et al., 2015, 2020). The *ad libitum* diet differs from the control diet in that it is available in large quantities without any restriction throughout the day. In contrast, the control diet is only slightly more than the individuals' daily food requirement measured under plentiful food conditions (note that food consumption was measured on an *ad libitum* diet). Therefore, the control diet (i.e., 110% of ADFI) can still be considered as limited dietary regime if an individual's daily energy requirement is increased (e.g. for strategic fattening or regaining mass; Gosler, 1996). We used a randomized block design during the experiment, with each block containing one food-restricted and one control individual. We tested four blocks (i.e., 8 birds) per day, to minimize the bleeding time. Each treatment lasted for three consecutive days (trial; **Figure 1**).

The order of treatments was randomized so that 12 birds received the control and 12 birds the food-restricted treatment during the first trial. After the first trial, each individual received the opposite treatment for three consecutive days (trial 2). After two trials, all birds received the resting diet (*ad libitum* food enriched with mealworms) for 3 days before trials 3 and 4, for which we reversed the order of the treatments for each individual to separate the effect of the treatment sequence from a group effect. At the end of each trial, we measured the body mass, and took a blood sample (into a heparinized capillary tube) for hormone analyses. We did not sample the birds at the end of the resting period to minimize the number of blood samplings while focusing on the hormonal responses to the restricted vs. the control diet. We refer to the first part of the experiment (trials 1-2) as "week 1" and the second part of the experiment (trials 3-4) as "week 2" (**Figure 1**).

On each sampling day, we randomized the sampling order for individuals. Blood sampling was always carried out by two experimenters and two assistants. The assistants caught the particular birds following the previously randomized sampling order and gave them to the experimenters, who were blind to the treatment. The assistants also recorded the bleeding time and morphometric variables measured by the experimenters. Bleeding was carried out by puncturing the brachial vein with a 26G sterile needle. We took a maximum of 140 µl blood from each individual, which corresponds with the international ethical standards in blood sampling (Owen, 2010). The bleeding of the first birds always started at 09:00. We recorded when the first person entered the housing facilities and when the blood sample was collected (bleeding time average: 5 min 36 s [min: 1 min 7 s, max: 11 min 48 s]). Preliminary analyses showed that IGF-1 levels were not related to bleeding time, and adding bleeding time never improved the model fit, therefore, this variable was discarded from subsequent analyses. All birds were fed immediately after blood sampling. Blood was kept on ice and centrifuged within one hour to separate plasma from erythrocytes. We stored the samples at −20°C until further processing.

To minimize observer bias, all measurements (during the experiment and hormone assays) were performed blindly for the individuals' treatment.

IGF-1 Assay

Plasma IGF-1 levels were measured in a competitive ELISA developed in our laboratory at the University of Debrecen, which was validated for Bearded reedlings and described previously in Mahr et al. (2020). Briefly, 96-well microplates were coated overnight at 4°C with 100 µl of an antibody raised against IGF-1 in rabbits. The capture antibody was incubated for 2 h at room temperature (24°C) with 20 µl known concentrations of synthetic chicken IGF-1 in serial dilutions starting at 500 ng/ml or 20 µl of sample and 100 µl biotinylated IGF-1. After incubation,

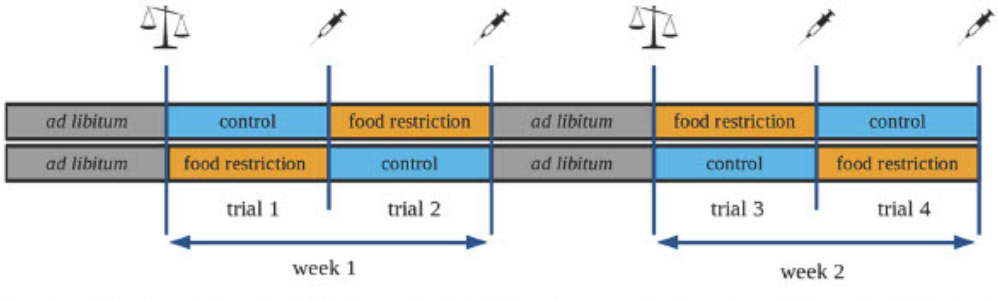

FIGURE 1 | Schematic illustration of the experimental design. Every treatment lasted for three consecutive days, and we called these periods "trials". Every bird received the control and the food-restricted treatment twice. We refer to the first replicate as "week 1" and to the second replicate as "week 2". The syringe icon denotes blood sampling and body mass measurements, whereas the balance icon indicates only body mass measurements.

the microplate was washed three times with 250 µl of PBS buffer containing 0.025% Tween 20, and 100 µl of streptavidin-horseradish peroxidase conjugate was added to all wells and incubated at room temperature for 30 min. After washing, 100 µl of tetra-methyl-benzidine was added to the wells and incubated at room temperature for 30 min. The enzymatic reaction was stopped by adding 100 µl of 1 M H_2SO_4, and optical density (OD) was measured at 450 nm (reference at 620 nm) using a Tecan F50 microplate reader. We used chicken plasma in quadruplicates to determine intra- and inter-assay coefficient of variation (6.8 and 10.88%, respectively). We were not able to calculate the concentration from three samples of IGF-1 because of insufficient plasma volume.

Statistical Analysis

We analyzed our data in a Bayesian framework, using R version 3.6.2 (R core team, 2019) and the package "MCMCglmm" (Hadfield, 2010). First, we analyzed how the treatment affected the body mass of the birds. We fitted a trivariate mixed-effects model, where body mass measured after each treatment period (*ad libitum*, control, or food-restricted) was used as response variable. The experimental week and the order of treatments (food restriction followed by control "RC" or control followed by food restriction "CR") and sex were used as fixed effects. Random intercepts were included for individual identity. This trivariate model allowed us to estimate the individual variance in body mass for *ad libitum*, control, and food-restricted diets separately, and the covariance between these terms. Based on these values, we calculated the among-individual cross-context (cross-treatment) correlations, which indicate intra-individual variation in the response given to the treatment (Dingemanse and Dochtermann, 2013; Houslay and Wilson, 2017). A high cross-context correlation (r close to 1) indicates consistent differences between individuals, e.g., a heavy bird under an *ad libitum* diet is likely to remain the heaviest (albeit with lower body mass) in the control and food-restricted diet as well. However, a lower r-value indicates that individuals react to different conditions differently (i.e., reaction norms cross over).

Second, we analyzed how the treatment affected IGF-1 concentrations. We used a univariate mixed-effects model with IGF-1 as the response variable, individual identity as the random intercept, and experimental week, treatment order group, treatment, and body mass as fixed effects. However, because body mass varies both among- and within-individuals, in this model, we partitioned the variance explained by body mass into among-individual and within-individual components (van de Pol and Wright, 2009). Among-individual body mass specifies the individual-specific average body mass, which explains variance due to consistent differences among individuals (e.g., a generally heavy vs. a light bird). On the other hand, the within-individual body mass component expresses the changes in body mass due to the specific dietary treatments.

Finally, we analyzed the covariation between IGF-1 and body mass. To do so, we built a multivariate model containing standardized body mass and IGF-1 (i.e., zero-centered and divided by the standard deviation), each included as a separate variable at each treatment level. Because blood samples were only collected after food-restricted and control (but not *ad libitum*) diets, this model contained four response variables (food-restricted and control treatment levels for both body mass and IGF-1). Individual identity was included as a random intercept. Experimental week, treatment order group, and sex were added as fixed factors. As above, in the trivariate model for body mass, this model also assumed a multivariate normal distribution full variance-covariance matrix that estimated all specific level variances and covariances. We also built alternative models with identical random and fixed structures, where specific parts of the variance-covariance matrix were constrained to zero. The model fit of these alternative models was compared using the Deviance Information Criterion, the Bayesian alternative of the Akaike Information Criterion. In this multivariate model, we also calculated the conditional among-individual variance for treatment-induced IGF-1 levels as variance in IGF-1 levels minus the square of the body mass~IGF-1 covariance divided by the variance in body mass, for each food treatment level. This value represents the among-individual variance in IGF-1 that is not accounted for by variation in body mass due to the food restriction (Dingemanse and Dochtermann, 2013). When credible intervals of the estimate do not overlap zero, it can be interpreted as a "significant" variation among individuals. To facilitate interpretation, we also provide Bayesian p-values for fixed effects (Hadfield, 2010). As for the body mass model, we also estimated the cross-context correlation for the IGF-1 response, which indicates whether individual reaction norms cross over (if lower than 1).

RESULTS

Body Mass

Males were heavier than females, but sex did not interact with any other variables. The dietary treatment affected body mass in a complex manner, where body mass differed between treatments, the week, the order of treatments and the interaction between order and treatment (**Table 1**). Experimental food restriction had a strong effect: body mass declined in all individuals (**Figure 2** and **Supplementary Figure 1**). However, this body mass loss during food restriction was stronger when it followed the *ad libitum* diet than when it followed the control diet. This difference in the severity of body mass loss depending on the food availability in the preceding period was similar in both groups that differed in the order they received the treatments (**Figure 2** and **Supplementary Figure 1**). The dietary conditions that the birds experienced in the preceding period also affected body mass change during the control treatment. Despite having access to 110% of their daily food requirement, birds lost body mass during control treatment if it followed the *ad libitum* diet. However, if the control treatment followed the restricted (70%) diet, birds regained mass during this period (**Supplementary Figure 1**). These effects resulted in a pattern that when birds received the control treatment after *ad libitum* (order "CR"), then body mass after control treatment was mid-way between *ad libitum* and restricted diet. However, when the control treatment followed the food restriction (order "RC"), the difference between body mass measured after control and restricted diet was more pronounced (**Figure 2**). Also,

TABLE 1 | The trivariate mixed-effects model showed that the experimental food restriction strongly affected the birds' body mass.

	estimate	95% CI (lower, upper)	pMCMC
Mass (AL)	0.215	−0.247, 0.662	0.340
Mass (C)	−0.355	−0.791, 0.073	0.109
Mass (FR)	−1.115	−1.533, −0.698	<0.001
Sex (M)	0.509	0.030, 0.982	0.037
Week	0.456	0.193, 0.721	0.002
Group (RC)	−0.170	−0.440, 0.100	0.206
Mass (C) × Week	−0.108	−0.420, 0.192	0.482
Mass (FR) × Week	−0.004	−0.283, 0.285	0.964
Mass (C) × Group (RC)	0.329	0.019, 0.649	0.043
Mass (FR) × Group (RC)	−0.350	−0.645, −0.073	0.020

All birds lost weight due to food restriction. Furthermore, body mass loss was stronger if it happened after an ad libitum period. Mass (AL) is the body mass during ad libitum period, Mass (C) is the body mass during control diet, Mass (FR) is the body mass during the restricted diet. Group (RC) is when control diet followed the restricted diet and Group (CR) is the opposite. Body mass was standardized (mean = 0, SD = 1) before the analyses. CI denotes 95% Bayesian credible intervals, pMCMC denotes the Bayesian p-value.

during the mid-experiment recovery (*ad libitum*) phase, birds increased their body mass so much that they became heavier by the beginning of week 2 compared to the beginning of the experiment (week 1). The latter effect was especially pronounced in the group that finished week 1 with the restricted diet (**Supplementary Table 1**; **Figure 2**; **Supplementary Figure 1**). These results were corroborated by univariate Bayesian analyses (**Supplementary Table 1**).

While individuals differed in their average body mass, the change in body mass was mostly parallel across feeding regimes among individuals (**Supplementary Figure 2**). Individual correlation of body mass across treatments was very high (*ad libitum* – control: 0.95, control - food-restricted: 0.94), indicating that consistent differences in body mass between the individuals remained unchanged despite the experimentally induced changes in body mass (**Supplementary Figure 2**).

IGF-1

Univariate analyses of IGF-1 showed that IGF-1 levels were higher during the food-restricted diet than during the control (4.40 [0.39; 8.22], $p = 0.02$) once we controlled for variation in body mass (**Figure 3**). To investigate this effect further, we divided body mass into two variance components: among-individual body mass (reflecting average mass differences among birds) and within-individual changes in body mass (reflecting experimentally induced loss and regain of body mass). This model showed that while among-individual body mass was positively related to IGF-1 (4.95 [0.75; 9.01], $p = 0.02$), within-individual changes in body mass tended to be in the opposite direction: i.e., body mass loss was associated with an increase in IGF-1 levels (resulting in a negative slope: −29.85 [−63.21; 2.05], $p = 0.06$), and this effect was the strongest in birds with low average body mass (1.81 [−0.27; 3.80], $p = 0.07$, **Figure 4**). However, the effects of within- and among individual body mass on IGF-1 had wide credible intervals that slightly encompassed 0 and were considered statistically marginally non-significant.

Males tended to have higher IGF-1 levels than females (5.92 [−0.25; 12.15], $p = 0.062$). Since males were also heavier than females, we repeated the above model by including sex as an additional variable. Controlling for sex did not improve model fit (ΔDIC = −0.2), and sex did not explain significant variation in IGF-1 levels (4.17 [−2.6; 10.57], $p = 0.182$). At the same time, the other estimates remained similar, indicating that the effects of sex were driven mainly by the higher body mass of males. This was further supported by a model showing that individuals heavier than the median body mass of their sex were more likely to decrease their IGF-1 levels in response to the treatment (−6.87 [−12.12; −1.95], $p = 0.009$), whereas individuals lighter than the average showed the opposite pattern and were more likely to increase their IGF-1 levels (5.09 [1.66; 8.52], $p = 0.003$), (**Figure 4**).

When body mass and IGF-1 were analyzed together in a multivariate model (**Table 2**), we found significant positive covariance between body mass and IGF-1 under the control diet, corroborating results from the univariate analysis that birds with higher average body mass also had higher IGF-1 levels. The model that estimated covariance between body mass and IGF-1 had better support than the one where body mass was not allowed to covary with IGF-1 levels (ΔDIC = 5.2). IGF-1 levels after food restriction tended to be related to treatment-induced body mass, although this effect remained marginally non-significant (0.19 [−0.06; 0.49]). Furthermore, we found that individuals differed significantly in their IGF-1 levels in response to the food restriction. Still, this difference in individual IGF-1 response was independent of the body mass change caused by food restriction (conditional among-individual variance = 0.36 [0.01; 0.94], repeatability = 0.44). The analysis of change in IGF-1 levels from control to food restriction revealed significant individual variation and crossing reaction norms (**Supplementary Figure 3**), indicating that some individuals decreased, whereas others increased IGF-1 in response to the treatment (cross-context correlation: $r = 0.70$, this model is considerably better supported than the model, where the cross-context correlation was fixed to 1: ΔDIC = 19.6).

DISCUSSION

Our experiment revealed that in response to food restriction, bearded reedlings showed marked individual differences in their IGF-1 reaction norms. While, as predicted, in some individuals, IGF-1 levels decreased in response to a restricted diet, the majority of the birds showed no response or even an increase. We also showed that heavier individuals had higher overall IGF-1 levels, and were more likely to decrease IGF-1 in response to the food-restriction. These results uncover the presence of an individual by environment interaction (I × E) and may have important implications for the evolution of IGF-1-related hormonal phenotypes in this species.

All birds lost weight during the restricted dietary regime, demonstrating that the experimental treatment was sufficient to simulate low food availability. Furthermore, during the mid-experiment *ad libitum* diet, birds increased their body mass to a higher level than their initial body mass at the beginning of

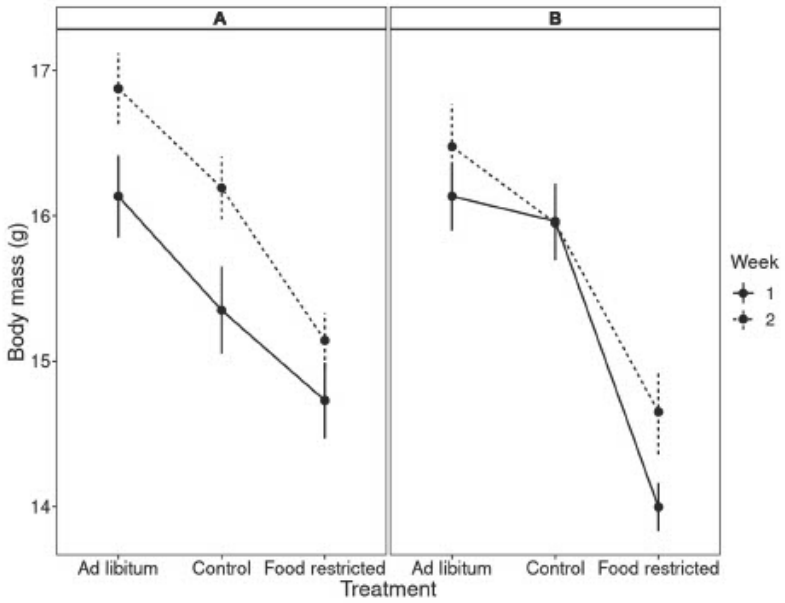

FIGURE 2 | Mean ± SE body mass in bearded reedlings after different dietary treatments. The *x*-axis shows the type of dietary treatments while the two panels show in which order the birds received these treatments (see also **Supplementary Figure 1**). **(A)** Indicates control diet followed by restricted diet, and **(B)** means restricted diet followed by control diet. Solid and dashed lines indicate week 1 and 2 of the experiment, respectively. Note that body mass variation was affected by treatment, week, the order of treatments and the treatment × order interaction.

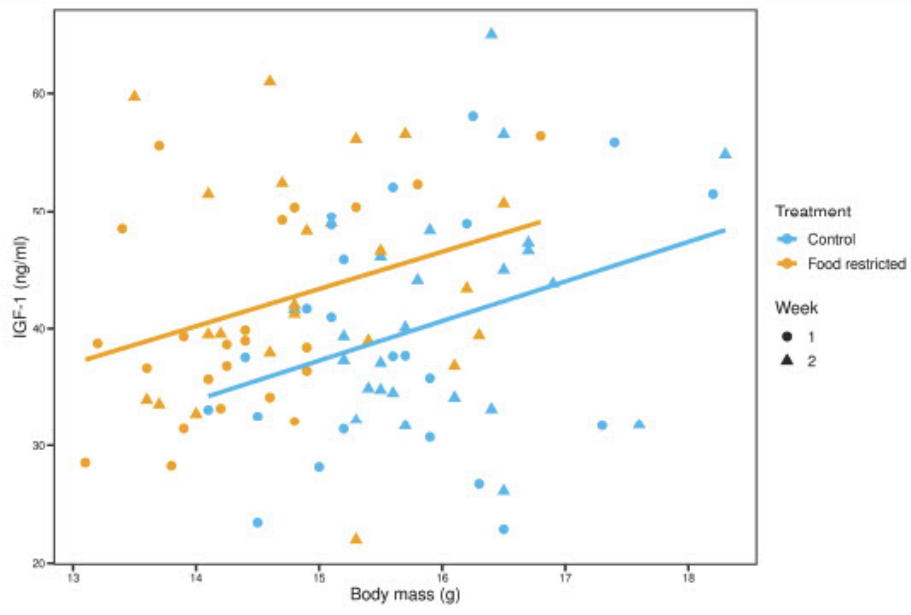

FIGURE 3 | Birds with higher average body mass have higher IGF-1 levels. Blue and orange solid lines show the among-individual relationships for control and food-restricted treatment, respectively.

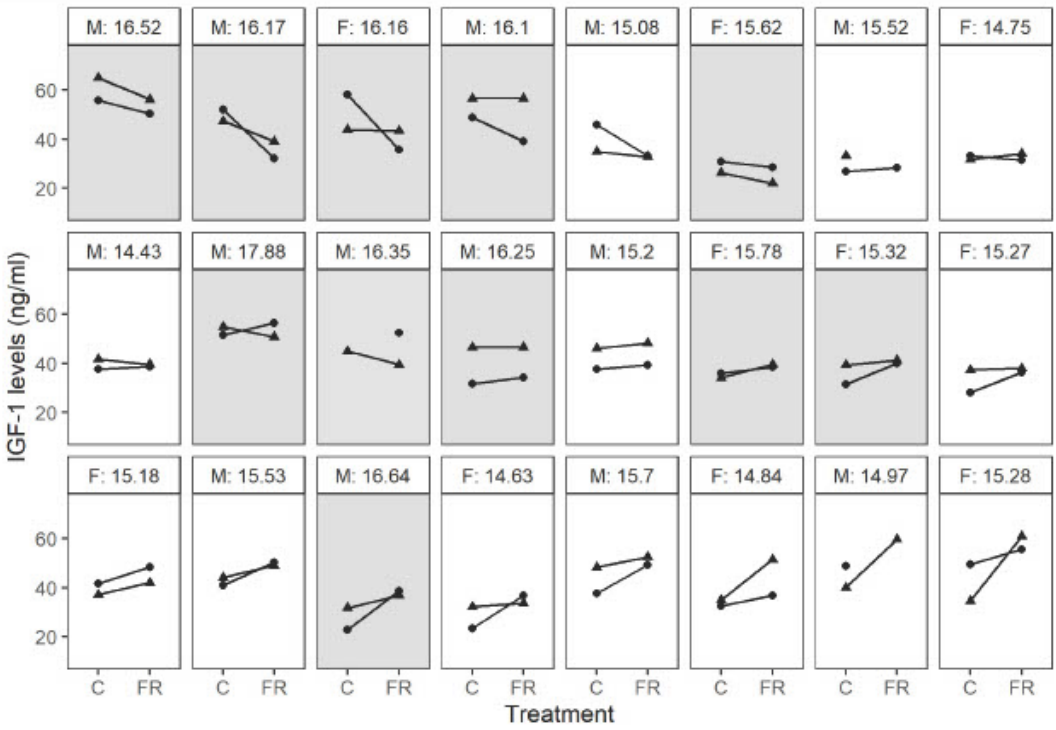

FIGURE 4 | IGF-1 reaction norms in response to food restriction show large among-individual variation. Each panel represents an individual; dots or triangles connected by a line denote IGF-1 levels measured after control (C) or food-restricted (FR) treatment during the first and the second half of the experiment (week 1: dots, week 2: triangles). The text above the panel show the sex (M: males, F: females) and the average body mass (g) for each individual. Light and dark panels indicate individuals lighter and heavier than the median mass of their sex, respectively. For three individuals, one of the samples are missing.

TABLE 2 | Variance-covariance matrix from the multivariate MCMCglmm model including IGF-1 and body mass under each treatment (control, "C" or food restricted, "FR").

	IGF1 (C)	IGF1 (FR)	Mass (C)	Mass (FR)
IGF1 (Control)	0.72 [0.13; 1.33]			
IGF1 (FR)	**0.40 [0.04; 0.86]**	0.62 [0.04; 1.19]		
Mass (Control)	**0.33 [0.03; 0.71]**	0.17 [−0.11; 0.52]	0.46 [0.09; 0.86]	
Mass (FR)	**0.32 [0.05; 0.67]**	0.19 [−0.06; 0.49]	**0.29 [0.04; 0.55]**	0.32 [0.06; 0.61]

The diagonal shows the variances, while off-diagonal elements correspond to the covariance between the variables. Significant covariance terms are highlighted in bold font.

the experiment. Finally, the decrease in body mass was stronger when the restricted diet followed the *ad libitum* feeding regime than when it followed the control regime (**Figure 2**). Our findings are in line with the adaptive regulation hypothesis, namely that individuals should maintain their current energetic status during low food availability and regain reserves during periods of high food availability to reduce the future risk of starvation (Witter et al., 1995; Fauchald et al., 2004).

The results on body mass resemble findings on House sparrows by Lendvai et al. (2014), even though the duration of the food-restriction period was shorter (3 days vs. 1 week) and the severity of the food restriction was lower in the current study (30 vs. 40% restriction). Our study design allowed us to separate the effects of the treatment sequence from a group effect because, at the midpoint of the experiment, the order of treatments was reversed in each experimental group (**Figure 1**). These results emphasize that individual responses to a standardized food restriction depend on previously experienced resource availability (Acquarone et al., 2002; Cucco et al., 2002; Fokidis et al., 2012). During the transition from *ad libitum* to control diet,

birds were presented with less food in their feeder. Although they could still meet their daily energetic requirements (the control diet consisted of 110% of their individual daily food intake, measured under conditions when food was available in plentiful conditions), the lower amount of food left in the feeder at the end of the day may have been a visual cue for anticipating a deterioration of nutritional conditions. On the contrary, the 110% of daily food requirement may be seen as a significant improvement for birds who switched from the restricted treatment. As a consequence, while birds entering the control treatment from *ad libitum* conditions lost body mass during the control regime, birds previously experiencing food restriction regained body mass during the same treatment (**Supplementary Figure 1**). These responses indicate that birds may anticipate future resource availability based on previous experiences.

The perception of potential shortages in food resources might also determine individual physiological responses that allow individuals to mitigate the costs of low nutrient availability if they anticipate a decrease in nutritional conditions. While these dynamic changes in body mass were strong and consistent among individuals (**Supplementary Figure 2**), we found that changes in IGF-1 levels were markedly different among individuals (**Figure 4** and **Supplementary Figure 3**). First, IGF-1 levels were repeatable within individuals and positively related to the average body mass, i.e., heavier birds had higher IGF-1 levels. Males were heavier than females and tended to have higher IGF-1 levels [as shown before in Tóth et al. (2018)], but once we controlled for body mass, the sex difference in hormone levels disappeared. Our findings on the positive relationship between body mass and IGF-1 are consistent with the available (albeit scarce) literature on fish (Cameron et al., 2007), reptiles (Crain et al., 1995; Sparkman et al., 2009), and mammals (Lewin et al., 2016; Tighe et al., 2016), although our result alone does not imply a causal relationship.

Second, individuals differed in their hormonal response to food restriction. While some individuals decreased their IGF-1 levels when food became scarce, others showed little response or even increased their IGF-1 levels (**Figure 4**). This difference remained after controlling for individual variation in body mass changes. Intriguingly, the variation in reaction norms was associated with the average body mass: relatively lighter birds were more likely to increase IGF-1 levels, while heavier birds were more likely to show a reduction in IGF-1. The restricted dietary regime is expected to decrease IGF-1 expression and secretion across invertebrates and vertebrates (Morishita et al., 1993; Schew et al., 1996). In contrast with our predictions and previous findings, we did not observe a consistent decrease in IGF-1 levels during food restriction, but we found significant among-individual variation (I × E) in the IGF-1 response to food restriction. Therefore, the question arises why food restriction had diverse effects on IGF-1 levels in adult bearded reedlings and whether this physiological response might facilitate survival under conditions of low food availability and constitute a possible coping mechanism to unpredictable environmental cues?

IGF-1 strongly affects energy metabolism including the elevation of glucose uptake without lowering free fatty acid levels (Kastin, 2013; Aguirre et al., 2016). It also promotes the formation of fat reserves via regulation of preadipocyte differentiation and increased lipogenesis (Smith et al., 1988; Scavo et al., 2004), which allows organisms to preserve energy to survive harsh environmental conditions. However, after preadipocytes differentiate, they stop expressing IGF-1 receptors. Therefore, in adipose tissues, only a high concentration of IGF-1 can effectively prevent lipolysis, and stimulate glucose transport (DiGirolamo et al., 1986). Based on our study and the role of IGF-1 in anabolic processes, we suggest that individuals might express different strategies when confronted with reduced food resources based on their initial energy status. Lighter (i.e., lean) individuals might produce more IGF-1 to maintain their energy homeostasis and mitigate the adverse effects of apoptosis and protein degradation, such as muscle atrophy (Musarò et al., 1999; Timmer et al., 2018). Furthermore, increasing IGF-1 during moderate or early stages of fasting might prevent protein degradation and facilitate the maintenance of cell growth and proliferation until energy becomes available (Scavo et al., 2004). On the contrary, heavier (i.e., fat) birds with decreased IGF-1 levels may be able to suppress insulin activity and increase blood glucose levels via gluconeogenesis, which is enough to maintain their normal life processes during harsh conditions and favor survival (Yakar et al., 2004). This hypothesis remains to be tested.

Only a few studies have suggested that food restriction may increase IGF-1. For example, Ayson et al. (2007) observed in rabbitfish that the hepatic IGF-1 mRNA level was higher in the starved (no food for 15 days) group than in the control group, albeit only in the early part of starvation (2^{nd} and 3^{rd} days). During prolonged starvation (15^{th} –18^{th} days), the IGF-1 mRNA level became significantly lower in the starved group compared with the controls. Ayson et al. (2007) suggested that previous studies may have missed the early increase of IGF-1 in response to starvation. However, another study on broiler chicken found that food restriction resulted in higher IGF-1 levels than controls throughout the study (from 15 to 28 weeks of age) (Hocking et al., 2007). We also found that female canaries responded to food restriction by increasing IGF-1 levels during breeding (Hargitai et al., 2022). These studies only analyzed the overall response to food-restriction, but here we show that within a single population, individuals may differ markedly in their physiological response to changes in nutritional conditions.

IGF-1 can also interact with other physiological parameters to modulate phenotypic responses. For example, Lodjak et al. (2016) showed in pied flycatcher nestlings that IGF-1 and glucocorticoid levels are positively related under high food abundance, while this relationship turns negative under low food abundance. They hypothesized the existence of a threshold level in physiological conditions over which the relationship between the two hormones can change. Our finding that heavier birds were more likely to decrease IGF-1 levels under food-restriction, whereas light birds were more likely to increase it may support the existence of such a physiological turning point, which may explain the variance of IGF-1 reaction norm during food-restriction. Accordingly, the relationships between IGF-1 and other physiological traits such as glucose levels, glucocorticoids and a marker of oxidative stress can be reorganized during environmental challenges (Vágási et al., 2020). These results suggest that the relationship between IGF-1

and other physiological factors is context- and condition-dependent and their joint effect on life-history or fitness-related traits is still unexplored in natural populations.

It should also be considered that with few exceptions, most of our knowledge about the effects of food availability on circulating IGF-1 levels comes from experiments conducted on model organisms (e.g., mice) in controlled laboratory settings and/or farm or breeding facilities (Puig and Tjian, 2006; Dantzer and Swanson, 2012). The feeding patterns of these animals (e.g., maintained unlimited access to food in lab condition) may not reflect the natural feeding habits of wild populations. Furthermore, laboratory and farm animals have been artificially selected on specific traits, such as rapid growth and an early onset of the reproduction, and therefore display different life histories and physiological phenotypes (e.g., metabolism) from wild animals (Petersson et al., 1996; Leili et al., 1997; Geiser et al., 2007; Auer et al., 2016; Bolstad et al., 2017). Considering that these traits correlate positively with IGF-1 levels (Frystyk et al., 1999), the measured physiological responses to low food availability may not reflect those of natural populations. Even though we conducted our experiments in captive animals, our study animals came from a natural population, therefore, it can represent the natural variation in IGF-1 response to food restriction.

Here, we uncovered large individual variation in the IGF-1 reaction norms, and the next step is to identify how these phenotypic differences are related to fitness. Dietary restriction is the most robust intervention that extends lifespan and delays ageing in various organisms, and it has been suggested that adaptive plasticity in the insulin/insulin-like signaling pathway underpins the physiological basis of this effect (Regan et al., 2020). Indeed, variation in IGF-1 levels has been connected to various life-history traits in vertebrates (Dantzer and Swanson, 2012). While IGF-1 is most often studied during post-natal growth, there is growing evidence that variation in IGF-1 is connected to fitness-related traits, such as lifespan and fecundity (Lodjak and Mägi, 2017), oxidative stress (Vágási et al., 2020) and ornament expression (Mahr et al., 2020; Lendvai et al., 2021) in adult birds. Given these relationships and that after an initial challenge IGF-1 levels do not return immediately to the baseline level (Gabillard et al., 2006), we expect that the way individuals react to variation in food availability may have longer-term, adaptive consequences. Therefore, the temporary lack of food could have a prolonged effect on reproduction via condition dependent IGF-1 regulation.

In conclusion, our study provides novel information on the existence of repeatable individual variation and multiple reaction norms in IGF-1 levels in response to food restriction. The variability of reaction norms can contribute to maintaining the genetic diversity within populations. This has a major ecological and evolutionary consequence because high genetic diversity reduces bottlenecks caused by environmental challenges and also involves the possibility of fast adaptation to the new circumstances (Fisher, 1930; Bouzat, 2010). IGF-1 showed a highly plastic response to one of the major environmental challenges (food deprivation). Therefore, we propose that IGF-1 might hold a prominent role in shaping adaptive responses to environmental changes, among other physiological variables, which remains to be tested.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Környezetvédelmi és Természetvédelmi Főosztály, Hajdú-Bihar Megyei Kormányhivatal.

AUTHOR CONTRIBUTIONS

ZT and ÁZL designed the experimental protocol and conducted the statistical analyses. ZT, GÖ, LŐ, and ÁZL performed the experimental procedures with contribution from KM. ZT and ÁZL performed the lab analyses. ZT, KM, and ÁZL wrote the manuscript with contributions from GÖ and LŐ. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.826968/full#supplementary-material

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Study 3

Lendvai, Á.Z., **Tóth, Z.**, Mahr, K., Pénzes, J., Vogel-Kindgen, S., Gander, B.A., Vágási, C.I. IGF-1 induces sex-specific oxidative damage and mortality. (manuscript in review)

IGF-1 induces sex-specific oxidative damage and mortality in the Bearded reedling

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Abstract

The insulin-like growth factor-1 (IGF-1) is a pleiotropic hormone that regulates essential life-history traits and is known for its major contribution to determining individual ageing processes. High levels of IGF-1 have been linked to increased mortality and are hypothesised to cause oxidative stress. This effect has been observed in laboratory animals, but whether it pertains in wild vertebrates has not been tested. This is surprising because studying the mechanisms that shape individual differences in lifespan is important to understanding mortality patterns in populations of free-living animals. We tested this hypothesis under semi-natural conditions by simulating elevated IGF-1 levels in captive bearded reedlings, a songbird species with an exceptionally fast pace of life. We subcutaneously injected slow-release biodegradable microspheres loaded with IGF-1 and achieved a systemic 3.7-fold increase of the hormone within the natural range for at least 24 h. Oxidative damage to lipids showed marked sexual differences: it significantly increased the day after the manipulation in treated males and returned to baseline levels four days post-treatment, while no treatment effect was apparent in females. Although there was no overall difference in survival between the treatment groups, high initial (pre-treatment) IGF-1 and low post-treatment plasma malondialdehyde levels were associated with enhanced survival prospects in males. These results suggest that males may be more susceptible to IGF-1-induced oxidative stress than females and quickly restoring oxidative balance may be related to fitness. IGF-1 levels evolve under opposing selection forces, and natural variation in this hormone's level may reflect the outcome of individual optimization.

Introduction

Mortality, a pivotal demographic parameter, profoundly influences fitness, and is central to our understanding of the patterns that contribute to individual differences in longevity. Within the realm of Several genetic and genetic and physiological mechanisms have been identified that affect thecontributing to animal longevity of animals. Among those mechanisms,, the insulin/insulin-like growth factor 1 signalling (IIS) pathway stands out as a key regulator. , because tThis evolutionarily conserved pathway, is present in the wholethroughout the animal kingdom, and has been reported to be demonstrated associated associations with longevity in diverse species, including worms, insects and various vertebrates, including such as humans (Bartke 2017). The primary ligand of this pathway in vertebrates, the peptide hormone insulin-like growth factor 1 (IGF-1) has an antagonistic pleiotropic effect on different fitness components: it stimulates growth and reproduction, but increases mortality (Dantzer and Swanson 2012). Conversely, a repressed activity of the IIS pathway augments self-maintenance or survival functions resulting in extended lifespan (Kenyon 2010; Lind et al. 2019).

Despite the robust patterns observed, the direct mechanisms by which a repressed IIS activity extends lifespan are not fully understood. The prevailing hypothesis is that the positive effects of blunted IIS signalling are in part due to an increased resistance to oxidative stress (Holzenberger et al. 2003); reviewed by (Tatar et al. 2003; Kenyon 2010; Dantzer and Swanson 2012). However, the relationship between IGF-1 and oxidative status is somewhat paradoxical. On the one hand, IGF-1 activates enzymatic antioxidant defences (most notably, glutathione peroxidase) and therefore is considered to have protective roles against oxidative stress, at least in rodents (Sukhanov et al. 2007; Higashi et al. 2010; Aksu et al. 2013; Ayadi et al. 2016; Montivero et al. 2021; Arjunan et al. 2023). On the other hand, IGF-1 is intricately linked with cellular metabolism and growth, and may cause increased productions of reactive oxygen species and thus may induce oxidative stress (Papaconstantinou 2009).

This disparity may, in part, be attributable to the fact that resistance to oxidative stress may not be equally experienced by males and females. In fact, sex-specific effects of IGF-1 regarding its protective or adverse effects have been documented in laboratory model organisms. For instance, in female mice, high serum IGF-1 levels induces bone loss, while in males the effect is the opposite (Elis et al. 2011). Sex-specific effects of IGF-1 may also be observed in the regulation of the immune response (Pinto-Benito et al. 2022), which may contribute to a general sex difference in immunity, with potential implications for divergent longevities (May 2007). Despite its importance, the underlying mechanisms of sexual differences in physiology and their repercussions for mortality patterns remain often unknown and clearly warrant further research.

However, studies investigating the role of IGF-1 in coordinating fitness and oxidative stress have been almost exclusively performed in the laboratory and studies in wild animals are surprisingly scarce (reviewed by Dantzer and Swanson 2012; Lodjak and Verhulst 2020). It is still contentious whether a high IGF-1 titre triggers oxidative damage, and this hypothesis has never been tested in any wild species (Dantzer and Swanson 2012; Lodjak and Verhulst 2020).

We carried out an experiment with juvenile bearded reedlings (Panurus biarmicus), a common Eurasian passerine, previously used in several behavioural (Romero-Pujante et al. 2002; e.g. Hoi and Griggio 2012) and physiological studies, including that of IGF-1 (Tóth et al. 2018, 2022; Mahr et al. 2020; reviewed by Lendvai 2023). Our aim was to achieve an increase in plasma IGF-1 levels over several days. To achieve this goal, we used a novel, minimally invasive manipulation technique (Mahr et al. 2023) by injecting either IGF-1 encapsulated in slow-release biodegradable microspheres (treatment group) or the dispersion medium without IGF-1 (control group). Our primary aim was to assess the short-term effect of IGF-1 treatment by measuring oxidative damage. To this end, we chose malondyaldehide (MDA) as a surrogate measure of oxidative damage, due to its stability as a byproduct of lipid peroxidation, quantifiable nature allowing for comparisons across samples, and

widespread use in scientific research. Furthermore, because the significance of even a temporary upsurge of oxidative damage are not known, we also tested whether the experimental increase in IGF-1 and the potentially associated oxidative stress have long-term fitness consequences by monitoring the mortality of individual reedlings in captivity over 16 months.

Material and methods

(a) Study species, experimental setup, mortality

Forty-one (16 female and 25 male) juvenile bearded reedlings were caught with mist nets at Hortobágy-Halastó (N47.6211, E21.0757) between July 28 and 30, 2017. Upon capture, birds were ringed with an individually numbered metal ring and their body mass was recorded (±0.1g). Only juveniles (hatched in the year of capture) were used in this study: the age and sex were determined based on plumage and bill colouration (Robson 2020). Birds were initially housed in groups of four individuals in cages (100 × 30 × 50 cm) placed in an outdoor aviary (3.65 × 3.35 × 2.75 m). Two cages contained three and two birds, respectively, which was necessary because of the odd number of the individuals and to prevent any bird being housed alone in a cage.ventv Food and water were provided ad libitum throughout the study. The birds were fed a mixture of freshly grated carrots, apples, quark, hard boiled eggs, an insectivorous bird food and ground cat food as a protein supplement, live mealworms daily and occasionally small crickets, grasshoppers and immature Turkestan cockroaches.

After at least 10 days of acclimation, the individuals were randomly assigned to receive either IGF-1 or a control treatment. Treatments were started in a staggered manner over two weeks, meaning that the four-day treatment period was started on different calendar days for different birds (the order within each cage was randomized), to minimize the number of experimental birds and thus handling time on any particular day. Controlling for the experimental order in the analyses had no effect on the results. On the morning of the treatment (day 0), we took a baseline blood sample (mean handling time: 190 ± 100 SD sec time measured from entering the aviary) from each target individual and we recorded their body mass. Total blood volume was approximately 70 µL, average plasma volume collected was 37 µL. Subsequently, we injected subcutaneously 100 µL dispersion containing either slow-release PLGA (poly(lactide-co-glycolide)) microspheres loaded with recombinant human IGF-1 (PeproTech, UK) (treatment; 2.2 mg microspheres containing 272 ng/mg IGF-1) or only the dispersion medium (control). Due to the high structural similarity between human and avian IGF-1, the human peptide has been successfully used in birds (McGuinness and Cogburn 1991; Lodjak et al. 2017; Lendvai et al. 2021). Microspheres were designed to release IGF-1 over several days, as described previously (Meinel et al. 2001; Luginbuehl et al. 2013). In summary, we conducted

microencapsulation of recombinant human IGF-I using a solvent extraction method from a W1/O/W2 dispersion. The internal aqueous phase (W1), comprising IGF-1, 10 mmol/l sodium succinate, 140 mmol/l sodium chloride (pH 6.0), and bovine serum albumin as a stabilizer, was emulsified with a solution of PLGA in dichloromethane (O) through ultrasonication. This W1/O dispersion was then introduced into a 5% (w/v) aqueous PVA solution (W2) to create, under mechanical stirring, a W1/O/W2 dispersion. For solvent extraction, the W1/O/W2 dispersion was diluted with de-ionized water and stirred using a magnetic stirrer. The resulting microspheres were collected on a regenerated cellulose (RC) membrane filter and dried under reduced pressure at room temperature overnight. The microspheres had an IGF-1 loading of 272 ng IGF-I mg/microspheres. Treated birds received a total of 600 ng IGF-1 per injection, with 100 µl of the dispersion administered subcutaneously between the shoulders. The control birds followed the same protocol, except they were injected with 100 µl of the dispersion medium only. The dispersion medium consisted of 1.5% (m/m) carboxymethylcellulose, 5% mannitol and 0.02% polysorbate 80 in sterile saline solution.

Immediately after the treatment, the birds were returned to their cages. Additional blood samples (using the same procedure as above) and body mass measurements were taken after 24 h and 96 h (day 1 and day 4 post-treatment) to assess the short-term physiological effects of the treatment. Once all birds had ondergone day 4 post-treatment sampling, birds from half of all cages (chosen randomly) were released back to the aviary, where the cages were placed, while the other half were released into another outdoor aviary (3.7 × 3.5 × 2.2 m). Both aviaries contained dense bundles of reed and cattail, and a water pool of ~1 m2 surface area to mimic a natural environment. Sufficient branches for perching and small boxes for hiding and resting were provided to enrich the environment. Food (as described above) was provided ad libitum in both aviaries.

At three months post-treatment, between November 20 and 22, 2017, all birds were recaptured to take another blood sample for testing long-term repeatability of circulating IGF-1 levels. Birds were then released back into the aviaries for additional 13 months (i.e., 16 months in total). Bearded reedlings are short-lived passerines with high juvenile mortality (Peiró 2013). Therefore, the entire study period was sufficiently long to detect enough mortality events for statistical analyses.

Mortality events were recorded on a daily basis. While the immediate cause of mortality remains unknown (autopsy and post-mortem pathology were not performed), most of the birds died in apparently good condition, without any visible injuries, suggesting intrinsic physiological causes of juvenile death. Body mass did not differ between treatment and control groups at any time point (all $p > 0.2$). Birds gained significant amount of mass during acclimation (1.3 g ± 0.22 s.e.m., $p < 0.001$) that remained constant during the short-term phase of the study (day 0 – 4) and gained additional mass (0.9 g ± 0.26 s.e.m., $p < 0.001$) by November, three months later,

indicating good conditions and no adverse effects of captivity in the aviaries. The sexes did not differ in the body mass gain at any time point (all $p > 0.1$). After 16 months in captivity, on December 8, 2018, all surviving birds (31%, n = 13; 7 controls and 6 treated individuals) were released at the site of capture.

(b) Physiological measurements

Plasma IGF-1 levels were measured without extraction by an in-house ELISA assay, as described elsewhere (Mahr et al. 2020). Plasma malondialdehyde (MDA) concentration reflects the level of peroxidative damage to cell membrane lipids and is a toxic oxidant itself and thus considered as a reliable measure of systemic oxidative stress (Del Rio et al. 2005; Vágási et al. 2019). MDA was measured by high performance liquid chromatography, as detailed elsewhere (Vágási et al. 2019).

(c) Statistical analyses

All statistical analyses were carried out in R version 'Bird Hippie' (4.1.2.) (R Core Team 2021). We analysed treatment effects on circulating IGF-1 and MDA levels (both log transformed) (n = 41 individuals) by generalised mixed-effects models (GLMMs) with treatment, sex and sampling time (days 0, 1, and 4) and their interactions as fixed factors, and individual identity as random intercept as implemented in package 'lme4' (Bates et al. 2015). To analyse the main effects and interactions, we computed Type III analysis of variance using the Satterthwaite's approximation of degrees of freedom, as implemented in the package 'lmerTest' (Kuznetsova et al. 2017). Based on these models, we compared predicted marginal means of the IGF-1 treatment and control groups within each time point and sex, and report these results. These pairwise comparisons were implemented using the function 'pairs' in the package 'emmeans' (Lenth et al. 2023), and p-values were adjusted using the Tukey HSD method. Repeatability of IGF-1 level was estimated using the package 'rptR' (Stoffel et al. 2017). Survival analyses were carried out by Aalen's regression (function 'aareg' in package 'survival' (Therneau et al. 2023)) that allows for additive effects on the cumulative hazard function. Individuals alive at the end of the study, and one individual that escaped from captivity were right-censored in the models. First, we analysed the effect of treatment and sex as factors on the survivorship. Second, we asked how IGF-1 and MDA measured on day 0, 1 and 4 affected survivorship. We included aviary ID in all of the models either as a fixed factor or as 'strata' (i.e. calculating a different baseline hazard for each aviary), but this effect never approached statistical significance or altered the conclusions, therefore it was removed from the models we report here. To avoid overparameterisation of the models, we modelled survival in several steps. First, we considered treatment, sex and their interaction as factors. Next, we included sex and its two-way interactions with both IGF-1 and MDA levels as covariates for the pre-treatment (day 0) and the post-

treatment (day 4) periods, respectively. Model structure was then simplified to retain only significant interactions. Finally, based on the earlier models, we constructed a model containing sex and its interaction with either IGF-1 and MDA found to be influential at the pre-treatment or post-treatment period.

Results

At the onset of the experiment, neither IGF-1 nor MDA levels were different between the treatment groups (IGF-1: $t = 0.47$, $p = 0.640$; MDA: $t = 0.88$, $p = 0.382$). While pre-treatment MDA levels were higher in males than in females ($t = 2.03$, $p = 0.049$), it was not related to pre-treatment IGF-1 levels ($t = –0.33$, $p = 0.741$).

Hormone treatment increased IGF-1 levels ($F_{2,74} = 27.13$, $p < 0.001$) in both sexes (Fig. 1). Although males had overall higher levels of IGF-1 than females ($F_{1,37} = 11.25$, $p = 0.002$), the magnitude of increase in IGF-1 from day 0 to day 1 was similar in males and females (day × treatment × sex: $F_{2,74} = 0.02$, $p = 0.978$, Fig. 1). IGF-1 levels were similar in the two treatment groups before the manipulation (i.e., day 0) in both sexes (females: $p = 0.639$, males: $p = 0.674$), but it was higher in the treated group than in the control group on day 1 after injection of the IGF-1-loaded microspheres (both sexes: $p < 0.001$, Fig. 1). By day 4, this difference between the two groups disappeared in both sexes (females: $p = 0.835$, males: $p = 0.948$, Fig. 1). Individual identity accounted for 17% of variance in IGF-1 not attributable to fixed effects (conditional $R2 = 0.66$). Inter-individual variation in IGF-1 levels remained consistent throughout the study period, resulting in significant repeatability over three months (controlling for day, sex and treatment: $R = 0.34$ (±0.16 SE), 95% confidence interval: 0.06-0.66, $p = 0.022$, $n = 29$).

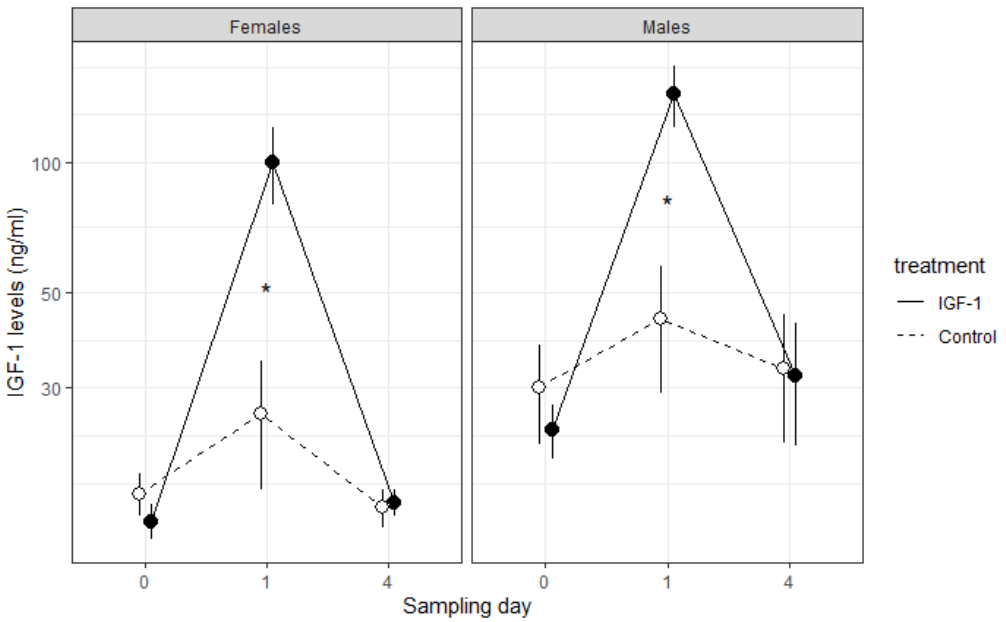

Figure 1. Injection with insulin-like growth factor 1 (IGF-1)-loaded microspheres resulted in a significant increase in circulating IGF-1 levels measured 24 h later (day 1) in captive bearded reedlings, but these effects disappeared by day 4. Mean ± s.e.m. are shown, asterisks denote significant ($p < 0.05$) differences between the treatment and control groups on day 1. The effect of treatment was similar in both sexes, but males had overall higher IGF-1 levels..

The hormone treatment induced a significant difference between the treatment groups in MDA levels, in a sex-specific manner, indicating that the IGF-1 treatment had opposing effects on MDA levels in males and females (day × treatment × sex: $F_{2,72} = 3.42$, $p = 0.038$, Fig. 2). Post-hoc analyses revealed that before the treatment (day 0), there was no difference between experimental groups in their MDA levels in either sex (females: $p = 0.382$, males: $p = 0.804$). However, IGF-1-injected males had higher MDA on day 1 than control males ($p = 0.002$), but this difference disappeared by day 4 ($p = 0.316$, Fig. 2). In contrast, while females appeared to show the opposite pattern, the difference in MDA between the hormone-treated and control groups did not reach statistical significance on either day 1 or day 4 ($p = 0.172$ and 0.493, respectively). Individual variation explained 7.1% of variance in MDA not attributable to fixed effects (conditional $R2 = 0.26$).

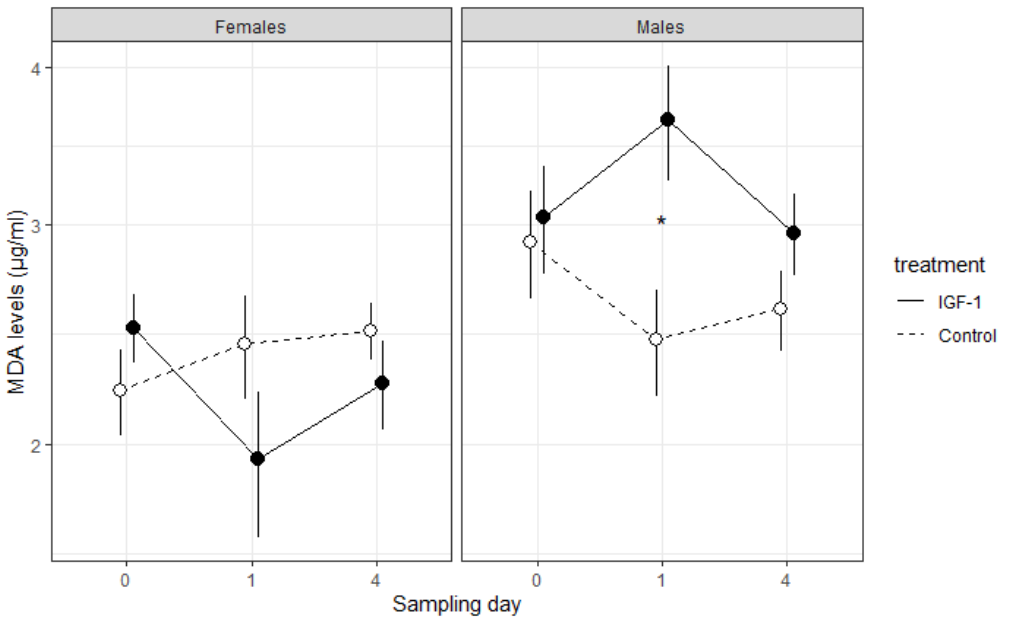

Figure 2. njection with insulin-like growth factor 1 (IGF-1)-loaded microspheres resulted in a significant increase in cellular oxidative damage (malondialdehyde, MDA) measured 24 h later (day 1) in male, but not in female bearded reedlings. Mean ± s.e.m. are shown, asterisk denotes significant ($p < 0.05$) difference between the treatment and control groups in males on day 1. The effect of treatment was sex-dependent and males had overall higher MDA levels.

Survivorship over 16 months did not differ between the IGF-1-treated and control groups ($z = 0.28$, $p = 0.773$, Fig. 3.) or between sexes ($z = 0.15$, $p = 0.883$) and the interaction of these two predictors was also non-significant ($z = 0.50$, $p = 0.620$). Birds with higher pre-treatment (day 0) IGF-1 levels were slightly more likely to survive ($z = –2.65$, $p = 0.008$), while pre-treatment MDA and sex had no significant effect in the model (MDA: $z = 0.26$, $p = 0.799$, sex: $z = 0.42$, $p = 0.675$). Neither peak (day 1) MDA and IGF-1 levels nor sex affected survivorship (all $p > 0.610$). However, MDA on day 4 was associated with survivorship in a sex-specific manner: relatively higher MDA levels on day 4 co-occurred with higher mortality in males ($z = 2.07$, $p = 0.039$), while females showed the opposite pattern ($z = –2.09$, $p = 0.037$). IGF-1 on day 4 showed no such relationship with survival and remained non-significant in both sexes (females: $z = –0.37$, $p = 0.711$, males: $z = 0.34$, $p = 0.737$). Finally, we combined the significant effects of pre-treatment IGF-1 and post-peak MDA levels in a sex-specific model, that corroborated the conclusions for MDA, but the effect of pre-treatment IGF-1 was only significant in males (Table 1).

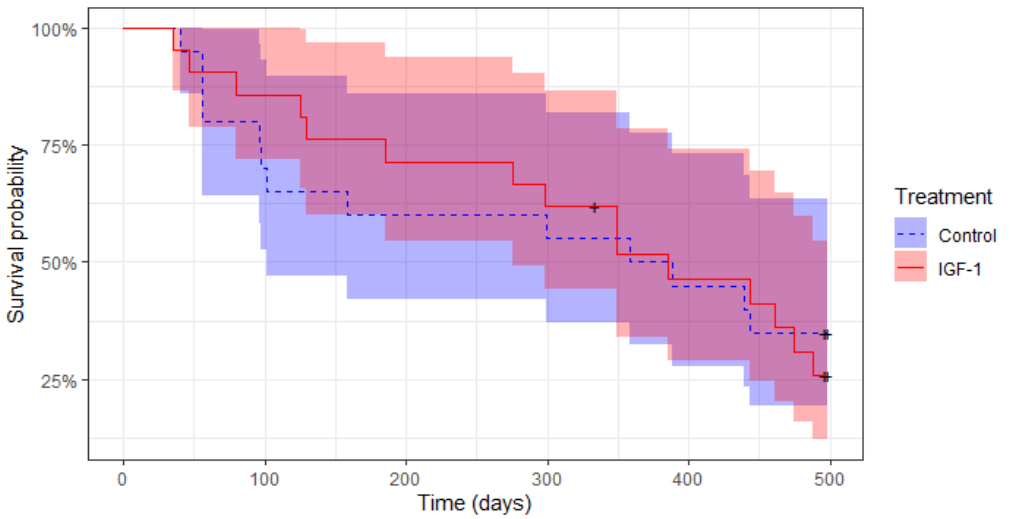

Figure 3. Insulin-like growth factor 1 (IGF-1) treatment did not affect survivorship in bearded reedlings. The solid and the dashed lines represent the Kaplan-Meier survival curves for the IGF-1 treatment and control birds, respectively, and shaded areas denote the corresponding 95% confidence intervals. Cross symbols show censored values. Treatment groups did not differ in survivorship.

Table 1. Survival model predicts that the likelihood of mortality increases over time, but higher pre-treatment (day 0) insulin-like growth factor 1 (IGF-1) levels reduce mortality in males. Post-peak (day 4) levels of malondialdehyde (MDA), a marker oxidative damage to lipids, increase mortality in males, while it has the opposite effect in females.

fixed effects	**estimate ± s.e.m.**	***z***	***p***
baseline hazard	0.187 ± 0.081	2.30	0.022
sex (males)	–0.155 ± 0.085	–1.81	0.070
Pre-treatment IGF-1 (day 0)	0.002 ± 0.001	1.64	0.100
Pre-treatment IGF-1 × sex (males)	–0.004 ± 0.002	–2.46	0.014
Post-peak MDA (day 4)	–0.075 ± 0.032	–2.36	0.019
Post-peak MDA × sex (males)	0.090 ± 0.034	2.64	0.008

Discussion

IGF-1 is a pleiotropic hormone having antagonistic effects on life-history traits (Dantzer and Swanson 2012; Lodjak and Verhulst 2020), but the adaptive value of among-individual variation in its plasma levels remains unknown. Higher IGF-1 titres might be associated with increased mortality in reptiles and mammals (mice, hyenas and humans), though effect sizes differ between studies and according to the sex and

age of individuals (Holzenberger et al. 2003; Andreassen et al. 2009; Sparkman et al. 2009; Milman et al. 2016; Garratt et al. 2017; Lewin et al. 2017). Phylogenetic comparative analyses also report a negative relationship between circulating IGF-1 levels and lifespan in birds and mammals (Swanson and Dantzer 2014; Lodjak et al. 2018). Although the exact mechanism of such increased mortality remains uncertain, several studies suggested oxidative stress as a mediatory agent (Holzenberger et al. 2003); reviewed by (Tatar et al. 2003; Brys et al. 2007; Kenyon 2010; Dantzer and Swanson 2012).

Here, for the first time, we showed experimental support for the hypothesis that an elevation of circulating IGF-1 levels may cause oxidative damage at short-term in individuals originating from a wild population. This result is consistent with a previous correlational study where circulating baseline levels of IGF-1 were found to be positively associated with MDA in adult house sparrows (Passer domesticus) (Vágási et al. 2020). However, the role of IGF-1 in oxidative stress is complex. While higher activity of the IIS pathway has been shown to be associated with oxidative damage, concurrently, it also upregulates antioxidant defences. For example, a study on nestling pied flycatchers (Ficedula hypoleuca) found that daily IGF-1 injections increased the levels of the antioxidant enzyme glutathione peroxidase (Lodjak and Mägi 2017), which might reflect lowered oxidative stress and/or up-regulated antioxidant activity in response to oxidative stress. This upregulation of antioxidant defences may contribute to the protective effects of IGF-1 in certain tissues (especially in the neural system), so much that in clinical settings, the therapeutic use of IGF-1 is also tested to prevent neurodegenerative disorders (Ayadi et al. 2016; Arjunan et al. 2023). Experimentally induced oxidative stress in mice tripled circulating levels of IGF-1 and lead to increased receptor activation (Xu et al. 2014). However, an increased activity of the IGF-1 system is known to generate reactive oxygen species and may lead to lipid peroxidation in rodents (Papaconstantinou 2009; Elis et al. 2011) and systemic augmentation of IGF-1 is associated with an increase of all-cause mortality in humans (Andreassen et al. 2009).

This paradoxical position of IGF-1 in oxidative balance regulation may be partly due to sexual differences in its effect (May 2007; Elis et al. 2011). In this context, we demonstrate that the IGF-1-induced oxidative damage showed marked sexual differences: while the treatment equally increased IGF-1 in males and females, it induced a transient oxidative damage in males only, while in females there was no difference between the treatment groups and they tended to show the opposite pattern. Experimentally-induced IGF-1 levels remained in the natural physiological range of this hormone in this species, as in previous studies we found that some individuals had similarly high or even higher IGF-1 values than the day-1 experimental birds in this study (Mahr et al. 2020, 2023).

As IGF-1 concentration returned to pre-treatment levels at day 4, the difference in oxidative damage also disappeared between the groups. Microspheres were found to

release encapsulated IGF-1 over several days in mice (e.g. Luginbuehl et al. 2013), and in a follow-up study in the bearded reedlings, we also found significant elevation of IGF-1 levels up to 3 days. After temporary regression another wave of release sustained elevated levels up to 7 days following the injection of IGF-1 loaded microspheres (Mahr et al. 2023). Whereas treatment effects disappeared by day 4 in the current study, it is remarkable that a single injection with microspheres achieved sustained increase in IGF-1 for at least 24h (and potentially more), which is considerably longer than the average half-life (32 minutes, regardless of the dose) of simple IGF-1 injections used in previous studies (McGuinness and Cogburn 1991).This finding highlights the usefulness of slow-release, biodegradable microspheres as a promising drug delivery system to manipulate hormones with minimally invasive administration (Mahr et al. 2023).

Although MDA levels also returned to baseline by day 4, males that persistently had relatively higher post-peak oxidative damage levels were more likely to die. Intriguingly, females showed the opposite pattern, where relatively lower MDA levels were associated with higher mortality. Males had overall higher IGF-1 and MDA levels than females and were more susceptible to IGF-1-induced oxidative damage. Studies in mice and humans found the opposite pattern, where females seem to be more sensitive to variation in IGF-1 levels (Holzenberger et al. 2003; Van Heemst et al. 2005; Elis et al. 2011; Xu et al. 2014). It is remarkable, because compared with mammals, birds also show a reversed sex-specific mortality pattern, where males tend to have longer lifespans (Bronikowski et al. 2022). Our study highlights the possibility that the IGF-1 related physiology and oxidative damage may contribute to sex-specific mortality patterns.

Notably, higher baseline IGF-1 (but not MDA) levels measured before the treatment were associated with lower mortality (especially in males). This result was unexpected, since higher IGF-1 activity has been associated with higher mortality in various species (see above). However, it is also noteworthy that individuals possessed large, repeatable natural variation in IGF-1 levels, which may be the result of individual optimization (recently coined as the Optimal Endocrine Phenotype Hypothesis; (Bonier and Cox 2020)). In this context, most of the inter-indidvidual variation of IGF-1 levels may reflect adaptive plastic responses to variation in environmental or internal conditions, where individuals express endocrine phenotypes that are optimal in their current conditions, but which differ among them. Therefore, high-quality individuals may afford to bear the costs of elevated IGF-1 levels (e.g. in terms of oxidative damage, accelerated ageing or increased risk of cancer (Shanmugalingam et al. 2016; Montoya et al. 2022b; Nelson et al. 2023)) while benefiting from its fitness-enhancing effects (e.g. boosting fecundity or anti-inflammatory responses (Higashi et al. 2010)) as expected for wild species exposed to forces of natural selection. Therefore, despite the high levels of circulating IGF-1, the overall balance of its antagonistic effects may still be positive for high-quality

individuals, who, by definition, have better survival prospects. However, it is crucial to recognize that the association between natural variations in pre-treatment IGF-1 or post-treatment MDA levels and observed mortality patterns is correlational, and a direct causal relationship cannot be concluded. Birds with diverse IGF-1 or MDA levels likely exhibit variations in numerous physiological aspects. The intricacies of IGF-1 signalling involve complex processes, including interactions with various binding globulins that can alter hormone signalling (McMurtry et al. 1997; Reindl and Sheridan 2012; Allard and Duan 2018). Moreover, tissue-specific modulation of receptor densities or local IGF-1 production acting in autocrine or paracrine manners adds layers of complexity to regulation. Additionally, given IGF-1's ability to bind to insulin receptors, potential alterations in glucose metabolism may also influence avian longevity (Montoya et al. 2018, 2022a). These intricate mechanisms could contribute to or obscure any relationship between baseline IGF-1 or MDA levels and mortality.

We measured survival in a semi-natural environment under ad libitum diet regime and shelter from predators, but under the exposure to inclement weather, parasites and other pathogens. Fluctuations in environmental conditions and stress stimuli is known to substantially reorganize the physiological network and, therefore, alter the adaptive value of a given endocrine phenotype (Vágási et al. 2020). IGF-1 levels showed high inter-individual variability and significant repeatability over three months indicating that the circulating levels of this hormone may be a consistent individual phenotypic marker, subject to individual optimization. Whether individuals with naturally high IGF-1 levels also realize fitness advantages under more challenging natural conditions remains to be investigated.

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Competing interests. We declare we have no competing interests.

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Study 4

Lendvai, Á.Z., **Tóth, Z.**, Mahr, K., Osváth, G., Vogel-Kindgen, S., Gander, B.A. (2021) Effects of experimental increase in insulin-like growth factor 1 on feather growth rate, moult intensity and feather quality in a passerine bird. *Journal of Experimental Biology* 224(14) jeb242481

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RESEARCH ARTICLE

Effects of experimental increase in insulin-like growth factor 1 on feather growth rate, moult intensity and feather quality in a passerine bird

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ABSTRACT

Moulting is a crucial, yet often overlooked life-history stage in many animals, when they renew their integumental structures. This life-history stage is an energetically demanding somatic growth event that has particular importance in birds because feathers play a crucial role in flight, insulation and communication. Somatic growth processes are regulated by the evolutionarily conserved peptide hormone insulin-like growth factor 1 (IGF-1). However, the role of IGF-1 in feather growth remains unknown. In this study, we captured 41 juvenile free-living bearded reedlings (*Panurus biarmicus*) that had started their first complete moult and brought them into captivity. Then, we manipulated their circulating IGF-1 levels using poly-(lactic-co-glycolid acid) microparticles (microspheres) that provide a sustained release of IGF-1. The treatment increased IGF-1 levels but did not affect the feather growth rate. However, 2 weeks after the treatment, birds in the increased IGF-1 group were moulting more feathers simultaneously than the controls and were at a more advanced stage of moult. Birds with experimentally increased IGF-1 levels had better quality feathers (measured by a lower number of fault bars) than the controls. These results suggest that an increase in IGF-1 does not speed up feather growth, but may alter moult intensity by initiating the renewal of several feathers simultaneously. This may shorten the overall moulting time but may imply costs in terms of IGF-1-induced oxidative stress.

KEY WORDS: IGF-1, Feather growth rate, Feather quality, Moult intensity, Life-history, Hormones, Bearded reedling, *Panurus biarmicus*

INTRODUCTION

Growth is a metabolically expensive process and is associated with severe costs (Werner and Anholt, 1993; Yearsley et al., 2004). These costs can result in important life-history trade-offs, with fast-growing individuals often suffering from higher mortality and a shorter lifespan than conspecifics growing at a more moderate pace (Metcalfe and Monaghan, 2003; Monaghan and Ozanne, 2018; Werner and Anholt, 1993). The significance of growth in shaping life-history trajectories has therefore been widely recognized (Gélin et al., 2016; Huot et al., 2014; Mangel and Stamps, 2001; Stamps, 2007). The term 'growth' often refers to structural and longitudinal growth events during embryonic and postnatal development. However, many organisms maintain growth throughout their lifetime (e.g. fish, amphibians and reptiles) and, even in animals that have reached their final adult body size, somatic growth events frequently occur. One particularly interesting example of such processes is moulting (also referred to as shedding in some taxa).

Moulting is an often overlooked somatic growth period that occurs regularly in adult organisms (Jenni and Winkler, 2020b; Payne, 1972). It serves in renewing the integumental structures (e.g. fur, scaled skin or feathers) covering the entire or most of the body surface, and similar to postnatal structural growth, it invokes serious fitness-related or physiological costs (Dawson, 2015; Jenni and Winkler, 2020b). These costs can involve reduced flight performance (Hedenström and Sunada, 1999), or the temporal loss of defensive armour (e.g. exoskeleton; Harvey, 1993), resulting in increased vulnerability and predation risk (Lucas et al., 2000). Moreover, moulting individuals often undergo major physiological changes that significantly alter metabolic requirements (e.g. higher protein demand) and thermoregulatory costs (Lindström et al., 1998; Murphy and Taruscio, 1995).

Birds provide an excellent system to study the evolutionary, ecological and physiological aspects of moult because the quality of their integuments (i.e. feathers) directly influences their survival and fitness (Dawson et al., 2000; Jenni and Winkler, 2020b; Serra et al., 2007). For example, in flying species, plucking a single flight feather may reduce flight performance and increase individual energetic costs, while some penguins are impaired in foraging and have to fast over weeks during their moult (Groscolas and Cherel, 1992; Hedenström and Sunada, 1999). However, apart from locomotion and insulation, feathers and plumage ornaments also play a crucial role in species recognition and camouflage, they allow adults to be distinguished from immatures and males to be distinguished from females, and they underlie sexual selection (Groscolas and Cherel, 1992; Hill and McGraw, 2006). Therefore, the process of renewing feathers gives rise to strong selection processes and arguably plays an important role in shaping avian life history (Kiat and Sapir, 2018). Given the critical importance of feathers in a bird's life, one may expect that selection enforces plumage maintenance and renewal of the feathers in an impeccable state. To achieve this, all birds drop their worn feathers and regrow them regularly; for some species, even multiple times a year. As

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moulting represents a major life-history stage, its timing is tightly controlled to be optimal for the given species' environment and life history (Barta et al., 2008; De La Hera et al., 2009; Holmgren and Hedenström, 1995; Kiat and Sapir, 2017). Despite the strong evolutionary implications, we know remarkably little about the physiological regulation of moult in birds (Dawson, 2015; Jenni and Winkler, 2020b). Although several hormone families have been recognized to affect moult (e.g. glucocorticoids, prolactin, thyroid hormones), the role of one of the most fundamental growth-regulating hormones, insulin-like growth factor 1 (IGF-1), has been entirely disregarded.

IGF-1 is an evolutionarily conserved peptide hormone that exists in all metazoan animals (Chan and Steiner, 2000). It is the main ligand of the insulin/insulin-like growth factor signalling pathway that regulates metabolism, cell proliferation and survival in the majority of invertebrate and vertebrate taxa (Barbieri et al., 2003; Schwartz and Bronikowski, 2016). In vertebrates, IGF-1 is considered to hold an important role in regulating key life-history transitions and thus has been suggested as a major mediator of life-history decisions (Dantzer and Swanson, 2012). Among the many functions of IGF-1, one of the best documented is its effect on somatic growth. In fact, IGF-1 increases the rate of post-natal growth in fish (Reinecke et al., 2005; Wood et al., 2005), reptiles (Sparkman et al., 2010), mammals (Lewin et al., 2017; Swanson and Dantzer, 2014) and birds (Lodjak et al., 2014, 2017). In mammals, IGF-1 also induces the proliferation of hair follicles and inhibits apoptosis, and therefore helps to keep them longer in the active (so-called anagen) phase and delay their transition to the regressive (catagen) phase (Li et al., 2014; Weger and Schlake, 2005). Transgenic mice over-expressing IGF-1 in the skin show earlier hair follicle development than controls, albeit at the price of developing dermal abnormalities and spontaneous tumour formation (Bol et al., 1997). IGF-1 also increases the rate of hair growth in tissue cultures (Ahn et al., 2012). Consistent with the role of IGF-1 in hair formation, some medical conditions characterized by either an excess or a lack of IGF-1 levels are also associated with abnormal hair growth. For instance, patients with primary IGF-1 deficiency (known as Laron syndrome) show sparse hair growth, while women affected by hirsutism (extreme excess of facial hair), have unusually high IGF-1 levels (Trüeb, 2018).

While these results suggest a role for IGF-1 in the regulation of hair growth, these studies were mostly performed on human tissue cultures or in rodents. Studies of how IGF-1 affects moult in other vertebrates are extremely scarce, but when available, seem consistent with its role in the regulation of moult. For instance, naturally shedding garter snakes were found to have higher circulating IGF-1 levels than non-shedding snakes (Sparkman et al., 2009), while a recently published study indicated a positive relationship between plumage quality, feather vane length and IGF-1 in a passerine species (Mahr et al., 2020). However, to date, there is only one experiment focusing on the relationship between moulting and IGF-1 in birds, revealing that stress-induced moult was associated with an increase in plasma IGF-1 levels in the broiler chicken (Mazzuco et al., 2005). To the best of our knowledge, no study has ever tested experimentally whether systemic IGF-1 affects the growth of feathers during moult.

In this study, we experimentally manipulated circulating IGF-1 levels in a passerine species undergoing a natural moult and investigated whether an elevation of IGF-1 resulted in an increased growth rate of flight feathers, higher moult intensity (increasing the number of feathers replaced at once) and changes in the quality of feathers. We expected that a systemic signal of elevated IGF-1 levels during natural moulting would induce faster growth of the feathers and/or higher moult intensity.

MATERIALS AND METHODS

Study animals and general protocol

We studied bearded reedlings, *Panurus biarmicus* (Linnaeus 1758), a common wetland specialist Eurasian songbird. Between July and October, this species undergoes a complete post-juvenile moult to acquire the first adult plumage. During this time, birds moult intensively, by growing several primary feathers and often some or all of their tail feathers simultaneously, which reduces their flying ability (Pearson, 1975; Spitzer, 1972; Wawrzyniak and Sohns, 1986). Between 28 and 30 July 2017, we captured 41 juvenile bearded reedlings using mist nets at Hortobágy-Halastó (47°38′ 13.7N and 21°04′42.8E, Hungary). We ringed all birds using numbered metal rings and measured their body mass (to the nearest 0.1 g) and tarsus length (to the nearest 0.1 mm). We determined the moulting stage immediately after capture by examining growing feathers in the wing, tail and body plumage. Detailed quantification of the moulting stage was achieved by scoring the moult of the primary wing feathers and tail feathers on a scale of 0–5, using the standard protocol for recording the progress of feather growth as suggested by the British Trust for Ornithology and further described in Jenni and Winkler (2020a).

Individuals that had not yet started the moult were released at the field site, resulting in a total of 41 moulting juveniles that were transferred into the housing facilities at the University of Debrecen. The bearded reedling is a highly social species and individual housing might result in stress; therefore, we kept 4 birds with similar moulting scores in a single cage (measuring 100×30×50 cm L×W×H). The cages were placed in an outdoor aviary, so the birds were protected from rain but experienced natural daylight and temperature fluctuations. Food (a mixture of freshly grated carrots, apples, quark, hard-boiled eggs, cracked dried fish, a commercial soft food mixture for insectivorous birds and ground cat food as a protein supplement, live mealworms daily and occasionally small crickets, grasshoppers and immature Turkestan cockroaches) and water was provided *ad libitum*. Once the experiment was finished, all birds were released in good condition into two spacious outdoor aviaries. We followed all applicable international, national and institutional guidelines for the use of animals. The study was approved by the institutional animal care and use committee and the regional government agency (licence no HBI/01/2708/2015).

IGF-1 manipulation

We manipulated IGF-1 levels using an injection of poly-(lactic-co-glycolid acid) (PLGA) microspheres prepared by S.V.-K. and B.A.G. as described previously (Luginbuehl et al., 2013; Meinel et al., 2001). Briefly, microencapsulation of recombinant human IGF-I was performed by solvent extraction from a $W_1/O/W_2$ dispersion. For this, the internal aqueous phase (W_1), containing IGF-I, 10 mmol l^{-1} sodium succinate and 140 mmol l^{-1} sodium chloride (pH 6.0) with bovine serum albumin as stabilizer, was emulsified in a solution of PLGA in dichloromethane (O) by ultrasonication. This W_1/O dispersion was introduced into a 5% (w/v) aqueous PVA solution (W_2) to form, under mechanical stirring, a $W_1/O/W_2$ dispersion. For solvent extraction, the $W_1/O/W_2$ dispersion was subsequently diluted with de-ionized water and stirred with a magnetic stirrer. The resulting microspheres were collected on a regenerated cellulose (RC) membrane filter and dried under reduced pressure at room temperature overnight. The microspheres had a loading of 272 ng IGF-I mg^{-1} microspheres.

The treated birds received a total of 600 ng IGF-1 per injection. We injected 100 µl of dispersion subcutaneously into the back of the birds, between the shoulders. We followed the same protocol for control birds, with the exception that they were injected with 100 µl of the dispersion medium only.

Immediately before the manipulation, the necessary amount of microsphere particles was measured on an analytical balance (±0.1 mg) and an aqueous dispersion medium (containing 1.5% carboxymethyl cellulose sodium, 5% mannitol, 0.02% polysorbate 80) was added. The dispersion was then vigorously vortexed for 30 s, during which time it became homogeneous. The dispersion was vortexed again before injection.

We used a randomized block design, where half of the birds in a cage were randomly allocated to the treatment, while the remaining two birds were used as controls. The treatments were staggered so that two blocks (i.e. 8 birds) were processed in a day.

Experimental protocol

Experiments started following an acclimation period of, on average, 2 weeks. On the day of the treatment (day 0), two experimenters entered the aviary and captured the birds in the assigned blocks and collected a blood sample (~75 µl) into heparinized capillary tubes by puncturing the brachial vein with a 26G needle. Assistants recorded the time when the experimenters entered the room, and when blood samples were collected for each bird. Variation in sampling time did not affect our conclusions. Samples were kept on ice until transferred to the lab (within 1 h). Samples were centrifuged for 10 min and the plasma was removed with a Hamilton syringe and stored at −20°C until hormone assay.

After blood sampling, we recorded the body mass of each bird, scored their moulting stage as described above and evaluated moult intensity by counting the number of primary and tail feathers growing simultaneously (Fig. 1). Moult index was calculated as the sum of the moulting scores of individual feathers (with left and right side averaged) (Vágási et al., 2010). We also measured the planar length of the growing primary and tail feather vanes. We measured the part of the feather that emerged from the sheath using a digital calliper (to the nearest 0.01 mm) and refer to these measurements as 'feather vane length' throughout the paper. For freshly growing feathers that had only a tubular part (i.e. sheath still closed), this value was 0. After the measurements, birds were injected with the IGF-1 or control dispersion and released back into their cage and left undisturbed for the rest of the day. On day 1 and day 4, blood sampling, body mass and growing feather vane length measurements were repeated as described above, but the moulting scores were not recorded, to minimize handling time and because it was not reasonable to expect changes in the moulting stage during this short time. Day 1 and day 4 blood samples and measurements were always taken at the same time of day as the first samples to avoid diel variation in the hormone levels. On day 15, birds were measured again, and this time the moult stage was also recorded. All measurements were taken by the same person, who was blind to the treatment of the individuals.

After the last measurement, birds were released back into their aviary and were kept on *ad libitum* food and water. Once all birds had completed their moult, we measured their wing and tail length (to the nearest mm) and, for males, the length of their beard with a calliper (to the nearest 0.01 mm). We also plucked the innermost longest tail feather (Ta1). The quality of Ta1 tail feathers was assessed by measuring four parameters: feather length, rachis diameter, feather mass and the number of fault bars (Dawson et al., 2000; Jovani and Rohwer, 2017; Pap et al., 2008). Feather length was measured with a ruler (±0.5 mm) as the distance between the base of the calamus and the tip of the vane. The rachis diameter was measured across the dorsoventral plane with a digital calliper to the nearest 0.01 mm at the base of the vane (superior umbilicus). Tail feather mass was measured with a digital analytical balance (Axis AGN200, accuracy class I, e=0.001 g; d=0.0001 g). Fault bars are narrow malformations in feathers that appear as translucent bands oriented almost perpendicular to the rachis, where the feather vane and even the rachis may break (Jovani and Rohwer, 2017).

Statistical analyses

Statistical analyses were performed in R version 3.6.3 (http://www.R-project.org/). The effectiveness of the treatment was analysed in a linear mixed model, with log-transformed IGF-1 levels as the dependent variable, sex, treatment and days since the onset of treatment as a factor, the treatment×day interaction as fixed factors and individual identity as a random intercept. All these factors were significant, so further model reduction was not possible. Because the treatment×day interaction was significant, we calculated predicted marginal means and compared the IGF-1 and control group within each treatment day, and reported these results. These pairwise comparisons were implemented using the function pairs in the package emmeans, and *P*-values were adjusted using the Tukey HSD method.

Feather growth was analysed as the daily growth rate of feathers. This value was obtained by fitting a linear model to each individual feather with feather vane length as the dependent variable and day (0, 1, 4) as a linear independent variable, and extracting the slope from these models. Different statistical approaches provided very

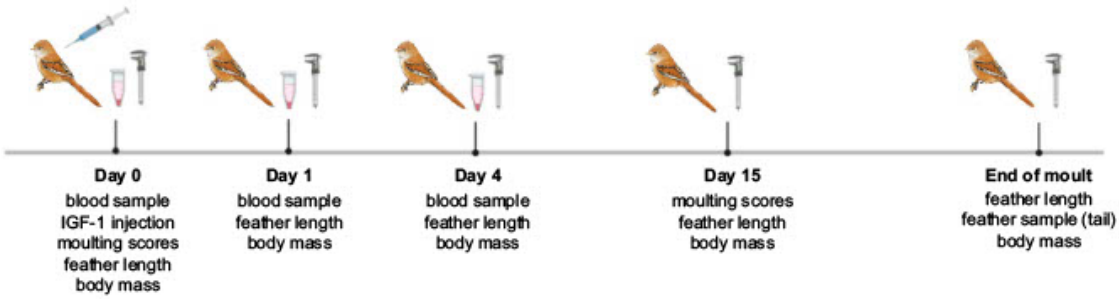

Fig. 1. Schematic representation of the experimental protocol. The syringe indicates the insulin-like growth factor 1 (IGF-1) manipulation; the Eppendorf tube and calliper symbolize the events of blood sampling and measurements, respectively.

similar results. Feathers grew at a steady rate (see Results) so linear models had a good fit to the data (average adjusted R^2=0.93); thus, these estimates had very little uncertainty. When a feather was not yet visible on day 0 and day 1, the 0 value from day 0 was excluded before fitting the linear model. Growth rate was analysed in linear mixed models with Gaussian error distribution and with individual identity as a random effect. Moult intensity (the number of feathers growing simultaneously) and the number of fault bars were analysed with a Poisson distribution. For variables analysed with the Poisson distribution, we report z-values for statistical results, whereas for all other variables, we report t-values.

RESULTS

Before treatment

On day 0 of the experiment, all birds were moulting at least one inner primary feather, and many of them were also moulting their tail feathers (Fig. 2); 49% of the birds were also moulting parts of their flank feathers, 41% were moulting parts of their back feathers and only 5% started moulting the feathers on the head. The number of primary and tail feathers moulting was not different between sexes (z=1.639, P=0.101) and was not related to baseline IGF-1 levels (z=−0.427, P=0.669), but was positively related to body mass at the beginning of the experiment (z=2.260, P=0.023). On day 0, we found no difference between the treatment groups in any measure, including body mass, IGF-1 levels, moulting stage and length of growing feathers (all P>0.5).

Treatment effects

The treatment increased circulating IGF-1 levels the day following microsphere injection (day 1, P<0.001), but this difference disappeared by day 4 (P=0.809; Fig. 3).

Feather growth rate, moult intensity and feather quality

In the short term (over the first 4 days), primary feathers grew faster than tail feathers (t=−2.845, P=0.004), but IGF-1 treatment did not affect the growth rate of either primary or tail feathers (t=0.456, P=0.651, Fig. 4).

In the longer term, IGF-1 treatment altered the intensity of moult: 2 weeks after treatment, control birds were moulting fewer feathers than at the start of the experiment, while IGF-1-treated birds showed the opposite pattern and increased the number of feathers being moulted simultaneously, albeit with substantial individual variation (z=−2.064, P=0.039; Fig. 5A). As a result, 2 weeks after treatment, IGF-1-treated birds were in a more advanced stage of moult than controls (t=2.205, P=0.039; Fig. 5B). Once moult was completed, the final wing length and tail length did not differ between the treatment groups (wing: t=−0.33, P=0.741, tail: t=−0.321, P=0.750). The longest tail feather collected did not differ between the treatment groups in terms of feather mass (t=−0.94, P=0.354) or rachis diameter (t=−0.581, P=0.565). However, IGF-1-treated birds had fewer fault bars than control birds (z=−3.10, P=0.001; Fig. 6), indicating greater feather quality.

DISCUSSION

Our work is the first to explore the potential role of IGF-1 in regulating somatic growth events outside the postnatal period in a wild bird species, by examining its effects on feather moult. We conducted an exogenous hormone manipulation in wild-caught juvenile bearded reedlings and subsequently recorded moulting patterns and feather development in captivity. Our primary goal was to test whether an elevation of systemic IGF-1 affects the growth of feathers at the time when these feathers are growing during natural moult. The results of our experiments indicate that elevated IGF-1 levels were not related to feather growth rate. However, IGF-1 administration altered the intensity of moult: 2 weeks after the onset of the experimental procedure, birds that received IGF-1 treatment displayed a higher number of actively moulting primary and tail feathers than control birds and were at a more advanced stage of moult. Interestingly, IGF-1 did not affect feather length, mass and rachis diameter, but IGF-1 elevation affected feather quality, reflected in a lower number of fault bars on tail feathers of individuals that underwent hormone manipulation.

Based on previous research on the effects of IGF-1 on integument growth, renewal/replacement and development, we expected that

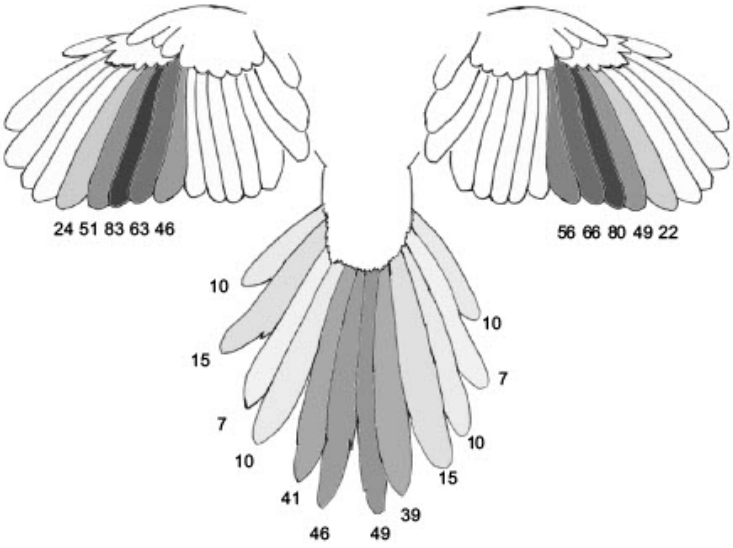

Fig. 2. Moulting status of juvenile bearded reedlings, *Panurus biarmicus*. Data were obtained at the start of the experiment (day 0, n=41 birds), prior to treatment. Shading is proportional to the percentage of birds moulting the given feather, which is also indicated numerically.

Journal of Experimental Biology (2021) 224, jeb242481. doi:10.1242/jeb.242481

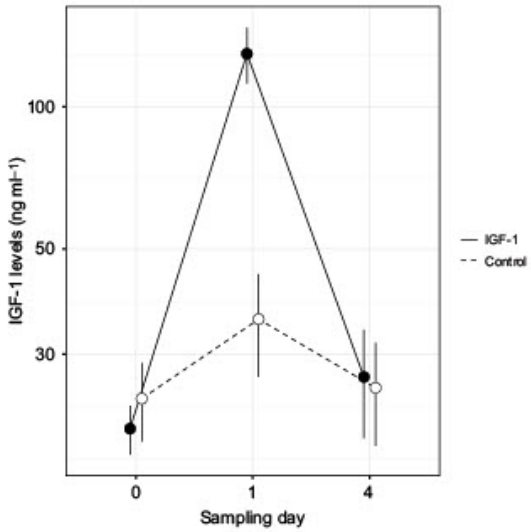

Fig. 3. Microsphere injection of IGF-1. IGF-1-injected birds showed increases in circulating IGF-1 levels relative to controls that lasted at least 24 h, but returned to baseline levels 4 days after the treatment (means±s.e.m.). IGF-1 levels are shown on a logarithmic scale.

IGF-1 supplementation would prolong and accelerate feather growth, resulting in longer and faster growing wing and tail feathers (Li et al., 2014; Weger and Schlake, 2005). We did not find this effect, even though natural variation in tail feather length has previously been shown to be associated with baseline IGF-1 levels in this species (Mahr et al., 2020). We consider the IGF-1 manipulation successful because a single injection of PLGA-microspheres elevated IGF-1 levels significantly above control on day 1 post-treatment (Fig. 3). While the treatment caused a pronounced elevation in IGF-1 levels on day 1, the peak hormone concentrations remained within the physiological range and were close to the highest concentrations found in naturally moulting individuals (Mahr et al., 2020). Our next sampling point was on day 4, by which time IGF-1 levels had returned to the baseline (and control) levels. Although the study design did not allow us to reveal the exact time point of the decline, follow-up investigations on the same species and method showed that significant treatment effects last up to 3 days and after an initial regression a second wave of release may last up to 7 days (K.M., Franz Gabor and Á.Z.L, unpublished results). These patterns indicate an exposure to elevated IGF-1 levels for at least 24 h in the current study and potentially longer, which is considerably longer than the average half-life (32 min, regardless of the dose) of simple IGF-1 injections used in previous studies (McGuinness and Cogburn, 1991), and highlights the usefulness of slow-release, biodegradable microspheres as a promising drug delivery system to manipulate hormones with minimally invasive administration. Despite this clear surge in IGF-1 levels, the growth rate of the primary and tail feathers remained very similar between the control and treatment groups between day 0 and day 1, and after that until day 4 (Fig. 4). Although we are not aware of any comparable study on feather growth, our results contradict some previous experiments in mice, showing that IGF-1 over-expression is related to exaggerated hair growth (Bol et al., 1997). In contrast to hair growth, the growth of primary wing feathers might be more tightly regulated, because they play a crucial role in flight efficiency. From that perspective, an insensitivity towards variation of systemic IGF-1 levels may be adaptive. IGF-1 may exert its effect locally by autocrine or paracrine regulation (Su et al., 1999), and birds may benefit from disentangling the control of feather growth from fluctuation of endocrine source of IGF-1. For example, stressors may cause a decrease in circulating IGF-1 levels (Tóth et al., 2018), and it may not be beneficial if that would affect the growth rate or the size of the developing flight feathers. The fact that the growth of the tail feathers was also unaffected by the manipulation is more surprising as they are sexually selected ornaments in this species (Romero-

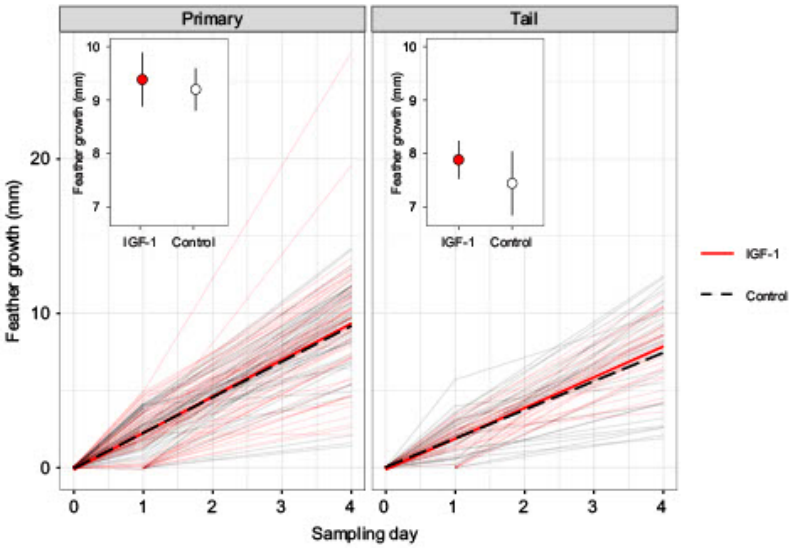

Fig. 4. Effect of IGF-1 on feather growth rate. The growth rate of primary and tail feathers did not differ between IGF-1-treated and control birds, but primary feathers grew faster than tail feathers. Note that the vane of some feathers emerged only on day 1. The thick lines represent the average growth per treatment, while the thin lines show the growth pattern of individual feathers. Values on the *y*-axis show the actual feather vane length minus the feather vane length on day 0, i.e. the amount of feather grown. The insets show feather growth (means±s.e.m.) during the 4 days per treatment.

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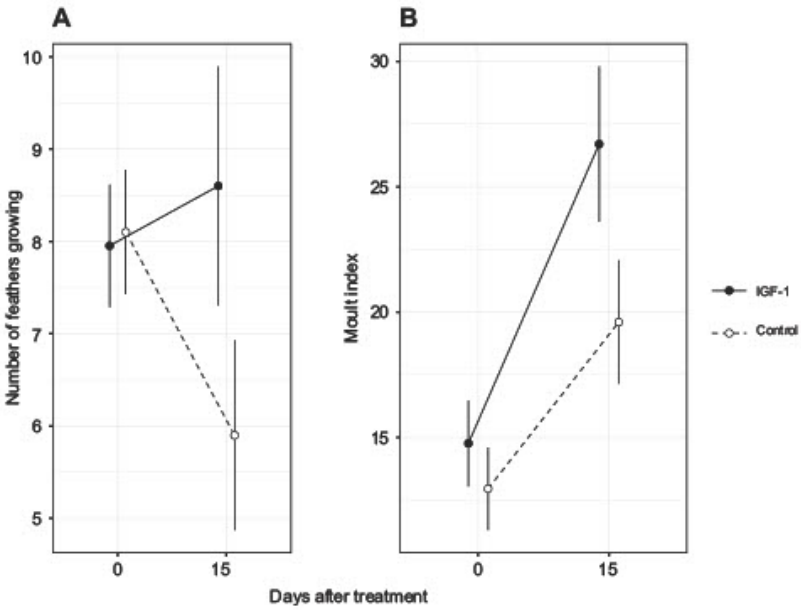

Fig. 5. Effect of IGF-1 on moulting. (A) Moult intensity (means±s.e.m.), measured as the number of simultaneously growing primary and tail feathers, changes in the opposite direction in birds treated with IGF-1 and controls. (B) As a consequence, 2 weeks after treatment, IGF-1-treated birds were at a more advanced stage of moult compared with controls.

Pujante et al., 2002) and natural variation in their length has been shown to be associated with baseline IGF-1 levels (Mahr et al., 2020). Sexually selected signals have been suggested to be related to insulin signalling as an assurance of their honesty (Warren et al., 2013).

Despite the robust lack of effect on feather growth speed, IGF-1 treatment maintained or even increased the number of feathers moulted simultaneously within 2 weeks, while the number of simultaneously growing feathers dropped in controls within the same period (Fig. 5). These results resemble the experiment by Li et al. (2014), who demonstrated that IGF-1 manipulation in wild-type mice stimulated cell growth and led to an increase in the number of hair follicles growing. This is further supported by *in vitro* studies, which have shown that IGF-1 increases the rate of hair growth in tissue cultures and is positively related to early follicle development (Ahn et al., 2012). In contrast to hair, the number of feather follicles that produce wing and tail feathers is small and shows no variation within species (~35 hair follicles mm^{-2} in mice versus the fixed number of 20 primaries and 12 rectrices). Furthermore, the moult of avian species often follows particular strictly controlled species-specific patterns, because gaps in the wing and tail feathers can greatly affect locomotion, with serious effects on individual fitness by increasing energy expenditure and vulnerability towards predators (Swaddle and Witter, 1997). Some species, however, moult several wing and tail feathers simultaneously, which also applies to the bearded reedling (Buker et al., 1975; Massi and Spina, 1996; Pearson, 1975). The question arises how IGF-1 might affect moulting patterns and whether elevated IGF-1 levels can serve as an adaptive function to facilitate faster moulting under specific environmental conditions. In mice, IGF-1 supplementation led to a premature transition of hair follicles to the anagen phase and protected them from turning into the catagen (regressive) phase (Ahn et al., 2012). These effects might also apply to feather growth in birds, facilitating a faster replacement of moulted feathers. While the primary feathers are replaced in a regular sequence in the bearded reedling, other body parts (especially the tail feathers) show a much less regular pattern and the moult may naturally decelerate in more advanced stages (Buker et al., 1975; Massi and Spina, 1996). Whether the decrease in the number of growing feathers in the control group was due to the natural moulting dynamics in this species or was an artefact of the captive conditions remains unknown; the important observation is that the same effect was not observed in the IGF-1-treated

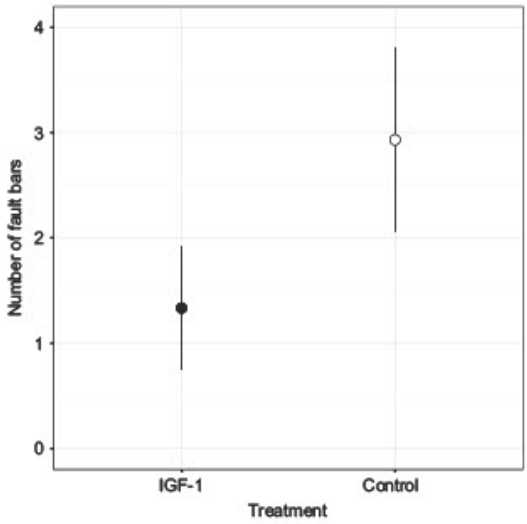

Fig. 6. Effect of IGF-1 on feather quality. At the end of the moult, IGF-1-treated birds had fewer fault bars in their longest tail feather than control individuals (means±s.e.m.).

group. Even though our results indicate that elevated IGF-1 levels do not increase growth rate per se, the moulting pattern may be affected and thus the overall time to moult might decrease. Moulting is an important life-history stage with major metabolic changes and there is a strong indication that different stressors can affect the synchronization and pace of feather growth in some passerine species. These disruptive factors may affect moulting in different ways. In starlings, for example, dietary restriction delayed the replacement of plucked feathers, whereas stress, simulated via corticosterone implants, decreased growth rate (Strochlic and Romero, 2008). IGF-1 is an important regulator of life-history changes and it is highly sensitive to the nutritional status of an individual and to dietary components (e.g. proteins) (Fontana et al., 2008; Miura et al., 1992). These characteristics are tightly linked to its important role in regulating energy allocation into cell proliferation, growth and protein synthesis (Dantzer and Swanson, 2012). Considering the metabolic function of IGF-1, the question arises whether IGF-1 also regulates the allocation of energy resources, required for feather growth (Mazzuco et al., 2005). Protein synthesis in tissues, for example, is partly promoted by and regulated through IGF-1 and this might also be of importance during the growth of feathers, in particular considering the increased demand for proteins during moulting (Murphy and King, 1992). Similar to experimental food restriction or supplementation, IGF-1 supplementation might lead to metabolic changes at the cellular level. These changes might affect moult intensity, i.e. the number of feathers growing simultaneously, rather than growth rate. It has to be considered, though, that we did not control for an effect of the dietary regime on moulting. All birds received an *ad libitum* diet in captivity, and the IGF-1 supplementation might have enhanced the effect of the high nutrient availability on feather growth. Moreover, as our study was designed to measure remiges and rectrices, our conclusions are restricted to these large individual feathers. We cannot exclude the possibility that our hormonal treatment may have affected the development of small feathers covering the entire body, similar to rodents and humans, where IGF-1 increases the number of developing hair follicles (Castro et al., 2012; Li et al., 2014). Rapid regrowth of feathers may be advantageous, because it shortens the time of reduced plumage functionality (e.g. reduced flight capability, weakened insulative capacity and signalling functions), but fast moult does have severe costs, such as a decrease in feather quality (Dawson et al., 2000; Vágási et al., 2010). In our study, moult intensity increased in the IGF-1-treated birds, indicating an accelerated moult rate, but we did not detect any negative effects on parameters of feather quality such as rachis diameter or feather mass. However, our results imply that IGF-1 might affect some aspects of feather quality, because birds receiving IGF-1 treatment had significantly fewer fault bars than birds of the control group (Fig. 6). These findings support a recent study by Mahr et al. (2020), showing that IGF-1 levels were positively correlated with structural plumage colouration in male bearded reedlings. Fault bars (also known as stress bars) are malformations of the feather, resulting from disruptions of feather growth that are often associated with stress or disease (Jovani and Rohwer, 2017). Similarly, the growth and regularity of the nano-sized structures that underlie structural plumage colours also partly depend on parameters of individual physiological condition during moult, with body condition being of particular interest. Hence, the discussed role of IGF-1 in regulating growth, cell proliferation and differentiation in relation to the nutritional status of an individual might also contribute positively to maintain feather structure throughout growth. In mammals, IGF-1 is also known to antagonize the effects of molecules inducing apoptosis (Ahn et al., 2012); this protective effect might also explain the lower number of fault bars observed in the IGF-1 treatment group. This function might be of particular importance during stressful events, when corticosterone levels rise substantially, while IGF-1 levels tend to decrease, but show large individual variation in this response. The interaction of corticosterone and IGF-1 has been suggested to predict fitness in growing songbirds (Lodjak et al., 2016). Corticosterone levels are known to have a seriously detrimental effect on growing feathers (Almasi et al., 2012; DesRochers et al., 2009; Jenni-Eiermann et al., 2015; Vágási et al., 2018), and it would be interesting to investigate how variation in IGF-1 levels during environmental challenges may contribute to escaping the damaging effects of stress-induced corticosterone. It is important to note that our manipulation increased circulating IGF-1 levels for 1–3 days, which is shorter than the time needed for the formation of the full feathers, which were analysed for quality indicators. However, the IGF-1 treatment had consequences well past the 1–3 day time frame (moult intensity and quality of the fully grown tail feathers), indicating that the treatment initiated physiological changes that persisted even after the circulating IGF-1 dropped back to original levels, and/or that the microspheres continued to release IGF-1 after a transient drop around day 4 (K.M., Franz Gabor and Á.Z.L., unpublished results).

If elevated IGF-1 levels accelerate moulting while simultaneously facilitating the maintenance of high feather quality, one might expect strong selection pressure towards high circulating IGF-1 levels. However, a recent study also indicated that the acute increase in IGF-1 levels was also associated with an increase in oxidative damage (measured as the levels of malondialdehyde, a marker of cellular membrane peroxidation and a reactive toxic agent in itself) (Lendvai et al., 2020 preprint). These results were further corroborated by another study in pied flycatchers showing that IGF-1 injection induced a significant increase in antioxidant levels, potentially to fight against increased oxidative stress (Lodjak and Mägi, 2017). Finally, we also found a relationship between circulating IGF-1 levels and oxidative damage in house sparrows, indicating some generality in the association between high IGF-1 levels and oxidative stress (Vágási et al., 2020). These results illustrate IGF-1's antagonistic relationships with several vital processes and therefore underlie its role as a major proximate effector of life-history trade-offs (Dantzer and Swanson, 2012).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.Z.L., Z.T., K.M.; Methodology: A.Z.L., G.O., S.V., B.A.G.; Formal analysis: A.Z.L.; Investigation: A.Z.L., Z.T., K.M., G.O.; Resources: S.V., B.A.G.; Data curation: A.Z.L.; Writing - original draft: A.Z.L.; Writing - review & editing: A.Z.L., Z.T., K.M., G.O., S.V., B.A.G.; Supervision: A.Z.L.; Project administration: A.Z.L., Z.T.; Funding acquisition: A.Z.L., K.M.

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Study 5

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ORIGINAL ARTICLE

Insulin-like growth factor 1 is related to the expression of plumage traits in a passerine species

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Abstract

Avian plumage colors and ornaments are excellent models to study the endocrine mechanisms linking sexually selected traits and individual parameters of quality and condition. Insulin-like growth factor 1 (IGF-1) is an evolutionarily highly conserved peptide hormone. Its regulatory role in cell proliferation and differentiation and its high sensitivity to the nutritional state of individuals suggest it as an interesting candidate, possibly providing a link between body condition and individual capacity to grow elaborated ornamental features. We investigated whether IGF-1 levels during molting correlate with the expression of multiple ornaments in a sexually dichromatic passerine species, the bearded reedling (*Panurus biarmicus*). We collected blood samples of males and females shortly before the molting completed and measured the size and colors of ornamental traits. Our results indicate that in males, structural plumage colors, the size of the melanin-based ornament (beard), and tail length are independent traits. IGF-1 levels are associated with the length of the tail and the expression of male structural plumage components (UV coloration), but not the melanin-based ornament. In females, plumage color and tail length were independent traits, which were not related to IGF-1 levels. To the best of our knowledge, this study provides the first evidence that IGF-1 could play a role in the development of secondary sexual characters in a bird species.

Significance statement

IGF-1 is an evolutionarily highly conserved peptide hormone, which recently entered the center stage of research enquiry in evolutionary biology. It is considered as one of the key factors shaping individual life histories, but little is known about its effects on sexually selected traits. We investigated whether IGF-1 levels during molting predict the elaboration of multiple ornamental plumage traits in male and female bearded reedlings (*Panurus biarmicus*). Our results indicate that higher IGF-1 levels had positive effects on male structural plumage colors and tail feather length. This is the first study, bringing indication for a potential role of IGF-1 in the expression of plumage ornaments in a bird species. Our findings suggest that IGF-1 might serve as an ideal candidate to study the mechanisms linking condition and the capacity to develop sexually selected ornaments.

Keywords Plumage coloration · Sexual selection · Physiology · IGF-1 · Condition

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Introduction

The physiological processes shaping the relationships between showy ornaments and individual parameters of quality and condition have been investigated intensively over the past decades. In that respect, hormones and their pleiotropic effects present one of the key mechanisms, linking individual physiology and the capacity to develop elaborated sexually selected traits (Nolan et al. 1992; Peters et al. 2006; Roberts et al. 2009; Flatt and Heyland 2011; Laucht and Dale 2012; Hudson and Wilcoxen 2018). The best studied examples are androgens, which orchestrate the investment into behaviors related to reproduction and stimulate the growth of secondary sexual characters (Andrew 1969; Alonso-Alvarez 2001; Peters et al. 2006; Roberts et al. 2009; Flatt and Heyland 2011). Their effects, however, come along with costs, which result in the suppression of the immune system and an increase in oxidative stress, which force the bearer of an ornament into a trade-off between the differential allocation of resources into ornamental features or self-maintenance (e.g., growth, immunecompetence) (Nolan et al. 1992; Ketterson and Nolan 1999; Hau 2007; Flatt and Heyland 2011). The capacity of individuals to cope with these trade-offs might constitute one of the key factors linking condition and ornament expression in many vertebrate species.

There is a phylogenetically ancient hormonal pathway, the insulin/insulin-like growth factor 1 (IGF-1), which has been less investigated than sex steroids but plays a crucial role in the mediation of life-history trade-offs (Zera and Harshman 2001; Barbieri et al. 2003; Harshman and Zera 2007; Sparkman et al. 2009; Dantzer and Swanson 2012; Shit et al. 2014). IGF-1 is an evolutionarily conserved polypeptide metabolic hormone, which serves as the main mediator of the growth hormone (GH). Its expression is regulated along the hypothalamic–pituitary–somatotropic axis (HPS axis). After stimulation (through GH) of the production in the liver, IGF-1 is released to the bloodstream. It is an organism-wide integrator regulating development, growth, and reproduction and moreover life span in vertebrates by stimulating cell proliferation, migration, and differentiation and protein synthesis in almost every cell of the body (Liu et al. 1993; Doublier et al. 2000; Dantzer and Swanson 2012). Stress, infection, and nutritional status (e.g., malnutrition) affect IGF-1 secretion and its effects on individual physiology (Dantzer and Swanson 2012; Emlen et al. 2012; Lodjak et al. 2016; Tóth et al. 2018). There is a compelling body of evidence that environmental factors and external stimuli (Ciucci et al. 2007), as for example resource limitation or ambient temperature (Gabillard et al. 2003), have effects of IGF-1 expression and secretion in vertebrates (Dantzer and Swanson 2012). The high responsiveness of the IGF-1 system towards external stimuli and its regulatory functions give it an important role in shaping life history traits, which also involve the development of sexually selected characters (Suttie et al. 1985; Ditchkoff et al. 2001; Emlen et al. 2012; Lewin et al. 2017).

We propose that the capacity of IGF-1 in mediating the effects of environmental factors on individual life-history traits makes it an interesting candidate to link parameters of body condition and the expression of avian plumage ornaments. Ornamental plumage traits appear in different forms like, for example, elongated feather structures, striking colors, or ornamental patterns (Andersson 1994b; Andersson et al. 2002; Hill and McGraw 2006b; Hill and McGraw 2006a; LaFountain et al. 2015; Roulin 2016). Despite their remarkable diversity, they share the same characteristics: they serve as "quality indicators" (phenotypic and genetic) and hence correlate with individual access to resources and future reproductive success (Andersson 1994a; McGraw et al. 2002; Delhey and Kempenaers 2006; Hill and McGraw 2006a, b; Jacot and Kempenaers 2006; McGlothlin et al. 2007; Murphy and Pham 2012; Musgrove and Wiebe 2016; Hudson and Wilcoxen 2018). Intensity, size, and elaboration of plumage colors and ornaments are determined during molt, and certain types of ornamentation are regarded as costly to produce. The development of plumage colors (e.g., carotenoid-based colors) and feather structures (e.g., tail length), for example, was shown to be negatively affected by malnutrition or physiological stress, which makes these ornamental traits highly sensitive towards parameters of condition during molting (Svensson and Merilä 1996; McGraw et al. 2002; Loyau et al. 2005; Griggio et al. 2009; Hudson and Wilcoxen 2018). The renewal and growth of feathers is a long and energy-demanding process, which requires major changes in the metabolic rate, a vast increase in cell proliferation rate, cell differentiation, and body protein synthesis (Kuenzel 2003). Considering that IGF-1 (i) regulates all of the aforementioned processes, which are tightly linked to the physiological requirements of molting, (ii) is highly sensitive towards the nutritional status (Gunnell et al. 2003; Mazzuco et al. 2005; Clemmons 2012; Dantzer and Swanson 2012), and (iii) signals the availability of resources (Bartke et al. 2003; Mattson et al. 2004), it might also affect the individual capacity to grow condition-dependent ornamental features. Whereas the role of IGF-1 in growth and development of avian species has been investigated (McMurtry et al. 1997; Beccavin et al. 2001; Lodjak et al. 2014, 2016, 2017), to the best of our knowledge, the relationship between plumage ornaments, body condition, and IGF-1 concentrations during the molt has yet not been studied.

Conspecifics often evaluate individuals based on several cues and signals, rather than one single trait, and in many species, males and females display multiple ornaments (Andersson 1994a; Andersson et al. 2002; Candolin 2003; Alonso et al. 2005; Mahr et al. 2016). These different traits can provide redundant information and/or amplify a signal, provide different information, or have no further informational content ("unreliable" signal) (Andersson et al. 2002; Candolin 2003; Ornelas et al. 2009; Griggio et al. 2016). Therefore, in

order to gain insight into the role of IGF-1 as a possible link between parameters of condition and ornament expressions, we investigated multiple ornamental plumage traits. Our model species is a free-living European passerine, the bearded reedling (*Panurus biarmicus*). While displaying a strong sexual dichromatism, males and females carry multiple ornamental features (Surmacki et al. 2015), with different characteristics, which might affect the information these traits convey to receivers (Hill and McGraw 2006a) (Fig. 1). Male bearded reedlings display a distinct melanin-based ornament, the black beard, which is an honest signal and underlies inter- and intrasexual selection. It was shown, for example, that males with larger beards are more dominant and, further, females display a preference for males with a more elaborated beard (Hoi and Griggio 2008). Previous studies on this species also demonstrated that the length of the tail is a sexually selected trait in males and females (Romero-Pujante et al. 2002; Peiró et al. 2006; Hoi and Griggio 2008; Griggio et al. 2016). Moreover, males are characterized by a conspicuous blue head and a rose/pink flank region and both males and females possess an achromatic bright chin (Fig. 1). These plumage regions

Fig. 1 Female and male bearded reedlings display a strong sexual dichromatism. The conspicuous blue head and rose flank region of the males as well as the achromatic bright chin reflect in the UV range (original artwork provided by G. Rédai, Department of Evolutionary Zoology and Human Biology, University of Debrecen, Debrecen, Hungary)

are characterized by reflection in the UV range (Fig. 2), which indicates the presence of structural plumage components (Osorio and Ham 2002; Mays et al. 2004; Shawkey and Hill 2005). Structural plumage colors are the result of keratin structures and air spaces embedded in the spongy medullary layer of the feather (Hill and McGraw 2006a, b). Their regularity, which is highly sensitive towards parameters of individual condition during the molt, predicts the degree of UV reflectance in the feathers (Keyser and Hill 1999; Griggio et al. 2009, 2010b). We therefore examined structural plumage components, by measuring UV chroma in addition to the melanin-based plumage colors of the body feathers in both sexes.

In order to explore whether IGF-1 serves as possible link between body condition and plumage traits, we captured free-living bearded reedlings at the final stage of their molt and measured their baseline IGF-1 levels. The timing of the study was based on findings in other species, showing that IGF-1 secretion increases strongly towards the end of the molt (Mazzuco et al. 2005) and circulating levels are significantly higher in comparison to non-molted birds. We brought the birds into a spacious aviary under semi-natural conditions, and after completion of the molt, we measured plumage traits in both sexes. We then tested for a relationship between the multiple ornamental traits and parameters of body size and condition (Hoi and Griggio 2008; Griggio et al. 2016) and investigated whether baseline IGF-1 levels collected during late molting stages predicted the expression of plumage characteristics. We expected that ornamental features, which are sensitive towards body condition during molt, would be related to condition parameters. If IGF-1 increases individual capacity to invest into the development of sexually selected characters, we expected to observe a positive relationship between IGF-1 plasma concentrations and parameters of plumage quality.

Methods

General methods

Free-living bearded reedlings were captured at Lake Neusiedl (47° 46′ 10.5″ N, 16° 45′ 20.1″ E, Burgenland, Austria) in September and October 2016. During this time of the year, bearded reedlings have almost finished their molt and have moved through the area in large flocks (HH, OV, and KM personal observations).

Mist netting was conducted between 0700 and 1600. Immediately after the capture, we collected blood samples (70–140 μl) by puncturing the brachial vein. The blood samples were drawn within 3 min (mean ± SD, 138.65 ± 59.67 s) from the time when the bird hit the net, and therefore, these samples are considered as baseline measurements (Wingfield and Romero 2001; Romero and Reed 2005; Tóth et al. 2018). Immediately after sampling, we transferred the blood into 0.5-

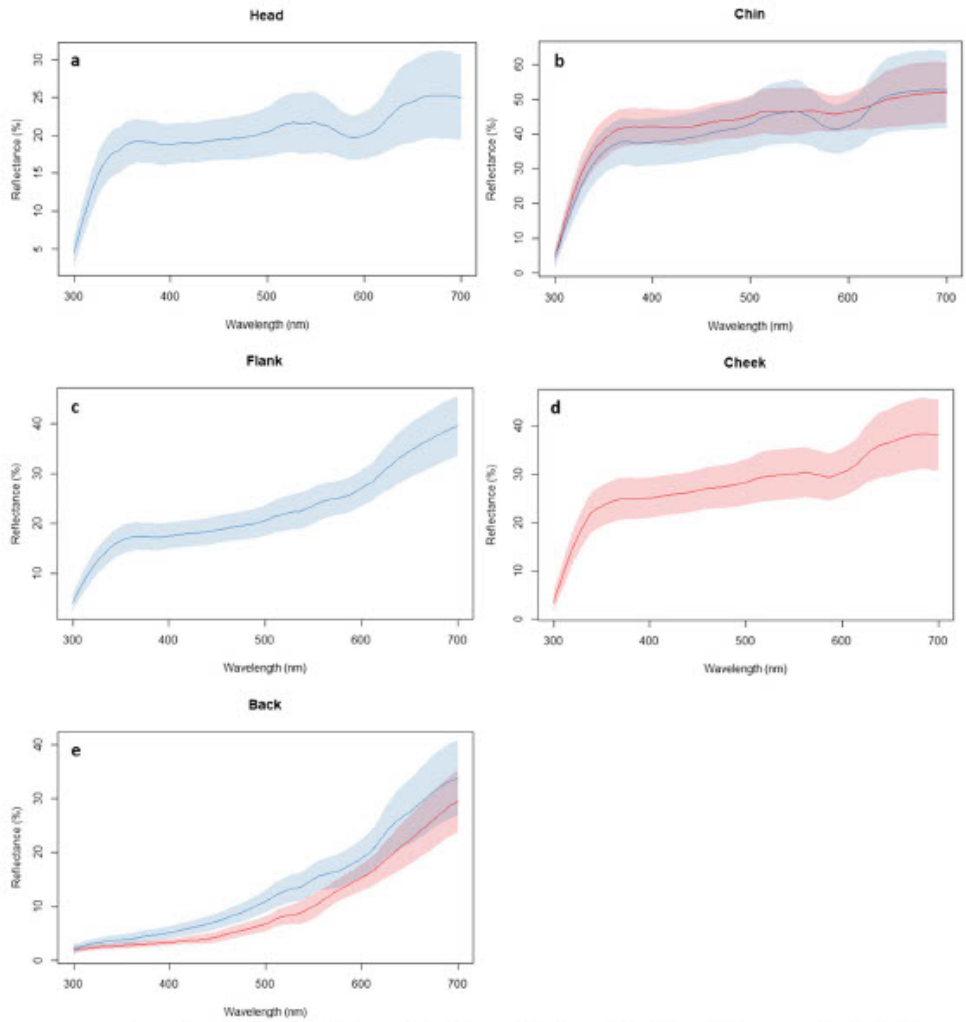

Fig. 2 The average spectral curves based on 23 male (blue) and 20 female (red) bearded reedlings. Note that the head, flank, chin, and cheek (panels a–d) indicate reflectance in the UV range (320–400 nm), while the brown coloration of the back (panel e) does not reflect in the UV range. The lines show the average reflectance, and the shaded areas show the corresponding 95% confidence intervals

ml microcentrifuge tubes, which were stored in a cooling box until further processing in the laboratory. The plasma was separated from red blood cells, by centrifuging the sample for 5 min at 2000*g* and removing the plasma with a Hamilton syringe (50 μl). After each sample, the Hamilton syringe was flushed 5 times by drawing up 50 μl of distilled water. We stored the samples in a −20 °C freezer until they were assayed for IGF-1 by enzyme-linked immunosorbent assay (ELISA; see details below).

All birds were banded with a unique combination of darvic color rings. Only individuals in adult plumage, which have molted more than two thirds of their plumage, entered the study ($n = 42$ birds; 11 birds were excluded at this point); the stage of the molting was estimated by looking at the number of feather quills in proportion to newly grown feathers at the given time (following the protocol provided by the Vogelwarte Radolfzell). The sex was determined by plumage coloration (Svensson 1992). Subsequently, we measured body mass (to the nearest 0.01 g), tarsus length (to the nearest 0.01 mm), and tail length (to the nearest 0.5 mm). Immediately after the measurements, we transferred the birds to individual cages. Bearded reedlings usually adapt to captive conditions very fast, but some individuals may be unusually stressed by the captive conditions. This was tested by providing each bird with 5 mealworms, and after 2 h, we counted the remaining mealworms. Only birds that resumed feeding

within 2 h after capture were regarded as capable to cope with the captive situation (80% of all birds tested), and were transferred to the housing facilities of the Konrad Lorenz Institute of Ethology, University of Veterinary Medicine, Vienna, Austria. The birds were kept in a mixed-sex flock in a large outdoor aviary (10 × 5 × 4 m), under natural light regime and conditions simulating their natural environment. The aviary contained dense vegetation of naturally growing *Sambucus nigra*; we also provided reed bundles and papyrus plants (*Cyperus papyrus*). The birds received water, a mixed diet of commercial insectivorous food (protein mash, apples, quark, egg, carrots), seeds (canary seeds, millet, hemp seeds), and mealworms ad libitum. Taking the birds into captivity was necessary because recapturing all the sampled birds in the field, once they completed molting, would not have been possible. Furthermore, all individuals finished molting under the same standardized conditions, although effects of captivity on wild-caught birds cannot be excluded.

Characterization of plumage traits

We characterized plumage traits in male and female bearded reedlings after all individuals had finished molting. To determine the end of the molt, we controlled the progress of the molt weekly; after 6 weeks, no growing feather quills were visible. The freshly molted plumage coloration was measured using a USB-2000 spectrometer and a DHS-2000-FHS deuterium halogen lamp, connected through a bifurcated fiber-optic probe (Ocean Optics, Eerbeek, Netherlands). By fitting a black rubber cylinder on the top of the probe, we minimized disturbance by outer light sources and ensured a standardized distance and angle (90°). Prior to each measurement, the spectrophotometer was recalibrated. For the calibration of white, we used a white standard (Avantes, Eerbeek, Netherlands); for black, we removed the probe from the light source and closed the cap of the plug (Griggio et al. 2009; Mahr et al. 2012, 2016).

We obtained 3 measurements from distinct regions of the plumage in males (head, flank, back, chin) and females (chin, cheek, back) (Fig. 1). Based on the obtained spectral curves (Fig. 2) and the literature (McGraw et al. 2005; Hill and McGraw 2006a, b), we decided to focus on two standard descriptors of reflectance variables, which were generated from the raw reflectance data: brightness and UV chroma. We used brightness to estimate the degree of melanization of the brown back of males and females (Fig. 2), which we calculated as the average percent reflectance in the 320–700-nm range. Brightness was previously shown to reflect the melanin content of plumage features, with low brightness indicating higher pigmentation (McGraw et al. 2005). The spectral curves of the head, flank, and chin of males and the chin and cheek of females, respectively, revealed the presence of plumage components facilitating the reflectance in the UV range (here defined between 320 and 400 nm) (Fig. 2). In order to quantify UV reflectance of the feathers, we calculated UV chroma, which is the proportion of reflectance in the UV range compared to the total reflectance (320–700 nm) (Hill and McGraw 2006b; Griggio et al. 2010a; Mahr et al. 2012). Spectral measurements were restricted to a range between 320 and 700 nm, which reflects the avian color vision spectrum (Hill and McGraw 2006b).

In addition to the colorimetric variables, we measured the tail length for both sexes and quantified the length of the black male beard, which were previously shown to be sexually selected traits in this species (Romero-Pujante et al. 2002; Hoi and Griggio 2008; Griggio et al. 2016). Tail length was measured using a ruler (to the nearest 0.5 mm). To measure the beard length (to the nearest 0.01 mm), three photographs (left/right side and front) of each male individual were taken by placing the birds in front of a millimeter paper at a standardized distance (25 cm) from the camera (Nikon D 60, Nikon Corporation, Tokyo, Japan) mounted on a tripod (Manfrotto, Vitec Imaging Solutions Spa, Cassola, Italy). All pictures were taken without a flash; to standardize conditions, the ambient light was only provided by artificial light under laboratory conditions. We used the software ImageJ (Rueden et al. 2017) to determine the beard length, by measuring a known distance on the millimeter paper as a scale (10 mm on each picture) and calculating the distance between the lowest and highest point of the beard in millimeters. For the analyses, we calculated the average beard length (in mm). All measurements were conducted by the same person to minimize variation through measurement errors. To minimize observer bias, blinded methods were applied during all measurement procedures in the field and the laboratory and the subsequent analyses of the data.

IGF-1 plasma concentrations

Plasma IGF-1 levels were measured in duplicates by a competitive ELISA developed in our laboratory at University of Debrecen (AZL et al. unpublished data). Ninety-six-well NUNC microplates were coated overnight at 4 °C with 100 μl of an antibody raised against IGF-1 in rabbits. The capture antibody was incubated for 2 h at room temperature (24 °C) with 20 μl known concentrations (in serial dilutions starting at 500 ng/ml) of synthetic chicken IGF-1 or 20 μl of sample and 100 μl biotinylated IGF-1. After incubation, the microplate was washed three times with 250 μl of PBS buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 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 min during which the enzyme conjugate bind to the biotinylated IGF-1. The incubation was followed by another washing cycle (3 times), and then, 100 μl of tetra-methylbenzidine was added to the wells and incubated at room

temperature for 30 min. The enzymatic reaction was stopped by adding 100 μl of 1 M H_2SO_4, 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). In this assay, we did not use any extraction protocol on our samples. This assay was validated for bearded reedlings by showing that serial dilutions of plasma (pooled from 5 individuals) ran parallel to the standard curve (Fig. S1). Samples were only analyzed on a single plate, so we could not calculate inter-assay CV specifically for the reedling samples (repeatability of samples was 94.1%). However, including the plate number as a random factor in the analyses did not change the conclusions.

Statistical analysis

In total, $n = 23$ males and $n = 19$ females were sampled. However, we excluded one female from the analyses of the plumage coloration, because we did not succeed in gathering reliable spectral measurements from more than one sample point, which would not be sufficient to provide reliable data for further analyses. We further excluded one male from the analyses of the beard length because the quality of the picture did not allow precise measurements of the beard length.

Statistical analyses were conducted using Statistica 7.1 (Statsoft Inc., Tulsa). All data were tested for normal distribution and no data transformations were required. To test the relationship between the different ornamental features in males and females, we conducted principal component analyses (PCAs) for each sex separately. Therefore, we combined male tail length, beard length, and the spectral variables (brightness and UV chroma), describing the coloration of the head, chin, flank, and back into a PCA. We applied the same procedure to females using tail length and colorimetric variables describing the coloration of the female cheek, chin, and back. We applied "varimax" rotations and normalized the data. The factor scores of the consecutive PCs explaining the highest variation within the chosen variables were extracted using the Kaiser criterion (eigenvalues higher than 1). Based on the findings of the PCA (see "Results" section), we conducted a second PCA on the UV chroma variables of different body parts to produce a single variable representing the structural plumage coloration (males: eigenvalue = 2.62, variance explained = 87.46%; females: eigenvalue = 1.51, variance explained = 75.28%) for further analyses. We refer to this variable as "overall UV chroma."

We used GLMs to explore the relationship between baseline IGF-1 levels, structural components of the plumage coloration (overall UV chroma), brightness, beard length, and the morphological plumage trait (tail length). Each of these variables was tested separately, with each plumage trait entering the initial model as dependent variable and IGF-1 being the explanatory variable. Tarsus length and body mass were incorporated as covariates. Since IGF-1 might be linked to the nutritional state of individuals, we also included an interaction between body mass and IGF-1 levels.

Model selection was conducted using stepwise elimination and re-introduction of terms. Starting with the interaction, the non-significant terms were eliminated step by step from the model. Only significant variables were retained in the final model, and each removed variable was re-entered separately into the final model to test their effects (Grafen and Hails 2002; Engqvist 2005; Mahr et al. 2016) (for the list of all models, see Supplementary Material Tables S1–S7). Model assumptions were verified by graphically checking the distribution of residuals. The variance inflation factor was calculated to assess possible multicollinearity, but none was detected. We provide parameter estimates ± SE and two-tailed tests throughout.

Results

Integration of multiple plumage ornaments

PC1 and PC2 capture the most variation in plumage coloration of the colorimetric variables (PC1: eigenvalue = 3.39, total variance = 56.57%; PC2: eigenvalue = 1.13, total variance = 18.86%). Brightness of the back loads negatively, whereas UV chroma of the same areas loads strongly positively on PC1, suggesting that high reflectance of structurally based components of the plumage indicates low brightness (Table 1). The three UV chroma variables show very similar loadings. PC2 explains the most variation in the beard length (Table 1), whereas tail length loadings differ from both colorimetric variables and beard length.

Female coloration showed a similar pattern to male coloration: PC1 (eigenvalue = 1.57, total variance = 39.3%) and PC2 (eigenvalue = 1.18, total variance = 29.45%). Similar to males, the two UV chroma variables show very similar loadings, which is perpendicular to the brightness of the back and opposite from tail length, indicating that brightness of the back and UV chroma of the structural plumage components might

Table 1 Loadings of PC1 and PC2 and in male bearded reedlings

	PC1	PC2
Tail length	0.34	0.59
Beard length	−0.24	0.79
UV chroma head	0.89	0.17
UV chroma flank	0.89	0.16
UV chroma chin	0.94	−0.04
Back brightness	−0.84	0.31

be independent traits, whereas tail length is negatively related to brightness in females (Table 2).

Do IGF-1 levels predict plumage colors, tail length, and ornamental patterns?

Male tail length was positively related to IGF-1 ($F_{1,21} = 6.65$, $\beta = 0.49 \pm 0.19$, $p = 0.02$, Fig. 3a). In contrast, female tail length was not related to IGF-1 or tarsus length (tarsus length: $F_{1,17} = 1.65$, $p = 0.22$; IGF-1 levels: $F_{1,17} = 0.23$, $p = 0.64$). However, after stepwise elimination of non-significant terms and the re-introduction of single terms, a trend, indicating a positive relationship between tail length and body mass, became apparent ($F_{1,17} = 3.87$, $p = 0.07$).

Beard length was not related to any of the variables measured during the molt (tarsus length: $F_{1,20} = 0.51$, $p = 0.48$; body mass: $F_{1,20} = 1.36$, $p = 0.26$; IGF-1 levels: $F_{1,20} = 0.26$, $p = 0.62$). The brightness of the male back plumage was also unrelated to tarsus length ($F_{1,21} = 0.003$, $p = 0.95$), body mass ($F_{1,21} = 0.79$, $p = 0.39$), or IGF-1 levels ($F_{1,21} = 1.53$, $p = 0.23$). However, we found a positive relationship between IGF-1 levels and overall UV chroma in males ($F_{1,21} = 5.09$, $\beta = 0.44 \pm 0.19$, $p = 0.03$; Fig. 3b).

In females, the brightness of the back showed a complex relationship with body mass and IGF-1 levels. While increasing levels of IGF-1 were associated with darker coloration in birds with low body mass, heavy birds showed the opposite pattern (body mass: $F_{1,14} = 8.90$, $\beta = -2.89 \pm 0.97$, $p < 0.01$; IGF-1: $F_{1,14} = 9.28$, $\beta = -10.67 \pm 3.5$, $p < 0.01$; body mass * IGF-1 interaction: $F_{1,14} = 8.84$, $\beta = 10.06 \pm 3.39$, $p = 0.01$; Fig. S2).

Overall female UV chroma was not related to either IGF-1 ($F_{1,16} = 0.532$, $p = 0.48$), body mass ($F_{1,16} = 2.84$, $p = 0.11$), or tarsus length ($F_{1,16} = 1.78$, $p = 0.20$).

Discussion

This study provides the first evidence that the IGF-1 signaling pathway may facilitate the development of sexually selected plumage traits in birds. Our results show that in male, but not female, bearded reedlings, IGF-1 levels during molting predict tail length and UV reflectance of the plumage (Fig. 3). Our findings are corroborated by previous studies on bearded reedlings and bring indication that different ornamental features, such as plumage colors and tail length, do not correlate.

Table 2 Loadings of PC1 and PC2 in female bearded reedlings

	PC1	PC2
Tail length	0.15	−0.76
UV chroma cheek	−0.87	0.07
UV chroma chin	−0.85	−0.07
Brightness back	0.07	0.80

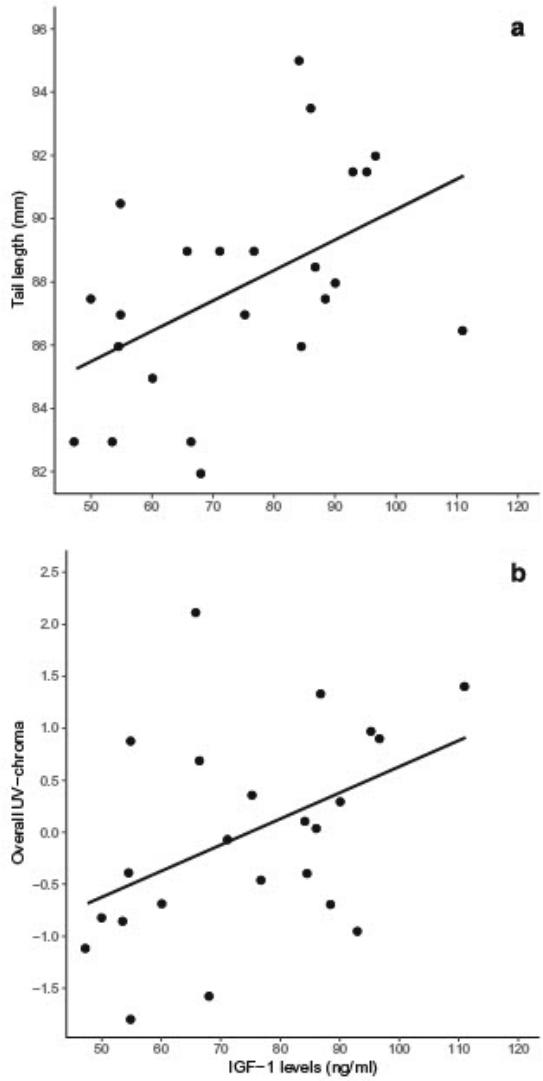

Fig. 3 Baseline IGF-1 levels during molt are related to sexually selected ornaments in male bearded reedlings. Males with higher IGF-1 levels a have longer tails and b develop plumage with higher reflectance in the UV range

This raises the idea that they either reveal a different set of information (rather than amplifying another signal) or carry no further informational content (Hoi and Griggio 2008; Griggio et al. 2016). Within this context, the question arises how IGF-1 can affect the development of ornaments and whether it provides a causal link between body condition and parameters of plumage quality.

Molting is an energy-demanding process, with concomitant major metabolic changes and an increased demand for cell growth, proliferation, and differentiation (Kuenzel

2003). The corresponding physiological requirements might be an important factor, linking feather development to parameters of individual condition before and during the molt (Murphy et al. 1988; Jovani and Blas 2004; Pap et al. 2008). In white crowned sparrows (*Zonotrichia leucophrys gambelii*) and house sparrows (*Passer domesticus*), for example, malnutrition had severe effects on the quality of feathers and the length of flight feathers (Murphy et al. 1988; Pap et al. 2008). In addition, late hatching, late arrival from overwintering sites, and poor body condition often force individuals to increase molt speed to keep pace with conspecifics and to be able to compete for mating partners or resources (Bojarinova et al. 1999). This compensatory measure has negative effects on feather length and plumage quality (Dawson et al. 2000; Vágási et al. 2012). IGF-1 is highly responsive towards the availability of environmental resources, due to its sensitivity to the nutritional status of an individual and to dietary components (e.g., proteins) (Miura et al. 1992; Fontana et al. 2008). It can affect whether energy is allocated into cell proliferation, growth, and protein syntheses (Blumenthal et al. 2011; Dantzer and Swanson 2012; Tighe et al. 2016), which might constitute an important adaptive adjustment to the environmental conditions with long-lasting effects on individual life history (Holzenberger et al. 2003; Dantzer and Swanson 2012; Lewin et al. 2017). The same properties might not only affect growth and life span, as was shown previously (Dantzer and Swanson 2012), but might also have the potential to regulate the allocation of energy into feather growth (Mazzuco et al. 2005). In molting birds, higher individual levels of IGF-1 might stimulate feather development and facilitate the display of more elaborated plumage features. IGF-1 levels were shown to be negatively affected by dietary restrictions (e.g., reduction of the diet and/or protein content) in several vertebrate species, and circulating levels are suggested to play a role in reflecting the nutritional status of cells and regulating energy metabolism (Breier 1999; Regan et al. 2019). Furthermore, IGF-1 promotes protein synthesis in tissues, which might play an important role in the development and growth of plumage, in particular considering the high demand for proteins during feather growth (Murphy and King 1992). Indeed, we show that in bearded reedlings, IGF-1 correlates with male, but not female, tail feather length and UV reflectance. The development of structural plumage components, which are suggested to be responsible for the coloration in the UV range, is complex and requires the regulation of protein synthesis and breakdown of keratin (Hill and McGraw 2006a; Hudson and Wilcoxen 2018). There is strong indication that, similarly to longitudinal growth of feathers, the growth of the regularity of the nanoscaled structures, which cause the reflection in the UV range, is linked to parameters of individual physiological condition during the molt. This was shown in different species; for instance, in blue tits (*Cyanistes caeruleus*), accelerated molt causes a decrease in the saturation of the UV-blue crown feathers (Griggio et al. 2009). Also, in dark-eyed juncos (*Junco hyemalis*), the availability of resources (dietary restrictions) during molting negatively affected the growth of structural plumage components (McGlothlin et al. 2007).

In brown-headed cowbirds (*Molothrus ater*), structural plumage traits, but not melanin-based plumage coloration, were shown to be affected by nutritional stress (McGraw et al. 2002). These findings support our results, which did not reveal a relationship between the measured melanin-based ornamental features (beard length and brightness of the back), IGF-1 levels, body mass, or tarsus length. This is particularly interesting because the beard of male bearded reedlings is a melanin-based plumage ornament, which underlies strong sexual selection processes. Males with longer beards are more dominant, and females clearly display a preference for them as potential mates (Hoi and Griggio 2008, 2012; Griggio et al. 2016). These characteristics raise the idea that the beard might be costly to produce and serves as an honest indicator of quality (Andersson 1994b; Hoi and Griggio 2008; Griggio et al. 2016). Indeed, the degree of melanization and the size of some types of melanin-based ornaments can be sensitive towards environmental factors and dietary components and hence serve as potential indicators of condition during the molt (Jawor and Breitwisch 2003; Roulin 2004; Musgrove and Wiebe 2016; Roulin 2016), but this idea remains controversial as the costs of melanin-based plumage ornaments are yet not well understood (McGraw et al. 2002; McGraw 2008; Guindre-Parker and Love 2014). One example, which resembles the beard length of the male bearded reedling, is the black throat badge of the male house sparrow (*Passer domesticus*). It is a melanin-based ornament which is considered to serve as an honest signal and indicates dominance to conspecifics, but its size was not affected during a dietary restriction experiment by McGraw et al. (2002) and McGraw (2008). Similar findings in great tits (*Parus major*) also suggest that the black breast band was not affected by nutritional condition (Senar et al. 2003). Whereas the growth and the development of feather structures require high amounts of proteins, the degree of melanization is not necessarily dependent on the availability of these energy resources. The modulation of melanin pigmentation is most likely connected to genetic prerequisites and other physiological constraints, such as, for example, the availability of certain elements and amino acids, and further might underlie the regulation of androgen levels (Senar et al. 2003; McGraw 2008; Roulin 2016). However, we did not measure the exact type and ratio of the specific pigment types in our study. Melanin-based plumage colors result from two specific types of melanin pigments, namely eu- and phaeomelanin, which differ substantially in their regulation and costs (Hill and McGraw 2006a, b; Roulin 2016). We suggest that future investigations should consider these differences.

In female bearded reedlings, neither of the ornamental features were significantly correlated with IGF-1 levels. This is particularly surprising, because tail length underlies mutual mate choice in bearded reedlings and we found a weak correlation between tail length and body mass, which corresponds with the idea that it serves as a potential indicator of quality (Romero-Pujante et al. 2002). Interestingly, our study indicates that dependent on the body mass, IGF-1 might positively affect the melanin pigmentation of the brown back in females, whereas no such relationship became apparent in males. Heavier females with lower IGF-1 and smaller females with high IGF-1 displayed darker back plumage than heavy birds with high IGF-1 or light birds with low IGF-1. One possible explanation is that this effect is due to different selection pressures acting on males and females (Romero-Pujante et al. 2002). We cannot rule out that IGF-1 might have effects, which facilitate the growth of more pigmented feathers in females, but it should be considered that overall, our study did not reveal a significant relationship between body mass or size during the molt and any of the plumage ornaments in both sexes. However, we obtained only one measure of body condition and molting birds undergo an energetic demanding period, which was previously demonstrated to significantly affect parameters of body condition. Muscle score, body fat, and mass in molting birds are often low (Dolnik and Valery 1979; Swaddle and Witter 1997; Minias et al. 2010) which may explain why we did not find a relationship between body mass and the ornamental features.

Overall, our study is the first to report a relationship between IGF-1 levels during molting and the elaboration of ornamental feather traits and colors in adult birds. The distinct properties of IGF-1, namely its sensitivity towards nutrition and its important role in the mechanisms stimulating growth and development, make it a potential candidate to further investigate the mechanisms linking parameters of individual condition and the capacity to display elaborated ornaments.

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Author contribution AZL, HH, OV, and KM designed the study; OV, KM, and ZT conducted the experiment and took samples and measurements; AZL and ZT conducted the lab analysis; KM and AZL conducted statistical analyses; and KM and AZL wrote the manuscript with significant contributions from OV, ZT, and HH. All of the authors read and approved the final manuscript.

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Data availability Data is available as supplementary material.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval We followed all applicable international, national, and institutional guidelines for the use of animals. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution and approved by the Austrian Federal Ministry of Education, Science and Research (§26 of the Law for Animal Experiments, Tierversuchsgesetz 2012—TG 2012, permit number: GZBMWF-68.205/0012-WF/V/3b/2017).

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