Charles University, Faculty of Science
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Lizard growth and the ontogeny of sexual size dimorphism

Růst a ontogeneze pohlavní dvojtvárnosti ve velikosti těla u ještěrů

Doctoral Thesis

Brandon Meter, M.Sc.

Supervisor: Zuzana Starostová, Ph.D.
Advisors: Lukáš Kubička, Ph.D. and Prof. Lukáš Kratochvíl, Ph.D.

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Author contribution statement

I declare that this thesis is my original work and has not been submitted for the purpose of obtaining a different or similar type of university qualification. My exact involvement in the research presented in this thesis is expressed below. All literature sources used when writing this thesis have been properly cited.

Meter, B., Starostová, Z., Kubička, L., and Kratochvíl, L. (2020). The limits of the energetical perspective: life-history decisions in lizard growth. *Evolutionary Ecology,* 34, 469-481. doi.org/10.1007/s10682-020-10054-0

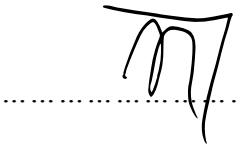
This review was written by Brandon. All three coauthors (Starostová, Z., Kubička, L., and Kratochvíl, L.) helped in reviewing the final version of this paper.

Meter, B., Kratochvíl, L., Kubička, L., and Starostová, Z. (2022). Development of male-larger sexual size dimorphism in a lizard: IGF1 peak long after sexual maturity overlaps with pronounced growth in males. *Frontiers in Physiology,* 13, 917460. doi.org/10.3389/fphys.2022.917460

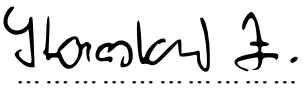
Brandon designed the RT-qPCR assay based on the study established by other coauthors then collected and analyzed the RT-qPCR data. The first draft of the manuscript was written by Brandon and coauthors before subsequent discussion and revision.

Meter, B., Kratochvíl, L., Starostová, Z., Kučera, T., and Kubička, L. (2024). Complex ontogeny of sexual size dimorphism in a female-larger gecko with determinate growth. Submitted in *Evolution & Development*

Brandon was the main person responsible for animal care for the Paroedura vazimba *individuals used in this experiment. He participated in sample collection for hormone and histological analysis and performed analysis of the growth data. He designed the RT-qPCR assay for IGF1 analysis as well as collected and analyzed its data. Alongside coauthors he participated in writing the manuscript for this project.*



Brandon Meter



Zuzana Starostová, Supervisor

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List of publications

I. Meter, B., Starostová, Z., Kubička, L., and Kratochvíl, L. (2020). The limits of the energetical perspective: life-history decisions in lizard growth. *Evolutionary Ecology.* 34, 469-481. doi.org/10.1007/s10682-020-10054-0.

II. Meter, B., Kratochvíl, L., Kubička, L., and Starostová, Z. (2022). Development of male-larger sexual size dimorphism in a lizard: IGF1 peak long after sexual maturity overlaps with pronounced growth in males. *Frontiers in Physiology.* 13, 917460. doi.org/10.3389/fphys.2022.917460.

III. Meter, B., Kratochvíl, L., Starostová, Z., Kučera, T., and Kubička, L. (2024). Complex ontogeny of sexual size dimorphism in a female-larger gecko with determinate growth. Submitted to *Evolution & Development*

Abstract

Growth has historically been considered indeterminate in reptiles. This common assumption has been challenged in recent years with accumulating evidence of determinate growth in lizards. These disputes being spearheaded by the analysis of the bone growth plates showing their closure and therefore indicating a final body size. In parallel to this growth's expected indeterminate nature, long lasting assumptions of the functions of the energetical budget in growth have also been challenged. This shift in perspective not only impacts our understanding of growth but also extends to the study of the related trait - sexual size dimorphism (SSD) - the difference in structural size between the sexes in a species. There have been three leading theories on the proximate causes of SSD: the cost of reproduction, the control by male androgens and the control by ovarian hormones. This thesis presents further evidence of the determinate nature of growth in lizards and discusses its consequences for SSD evolution. The review on growth focusing on male-larger gecko *Paroedura picta* highlights the canalized nature of growth while giving a foundation in the past literature surrounding the proximate causes of SSD. In this thesis, I present evidence of the likely role of ovarian hormones alongside the role of insulin-like growth factor 1 (IGF1) in the development of SSD through experiments with species in the genus *Paroedura*. To this end, we targeted male-larger species *P. picta* and female-larger species *P. vazimba* by studying their sex hormone levels as well as the hepatic gene expression of *IGF1* throughout growth. We discovered a spike of hepatic *IGF1* gene expression independent of sexual maturity in *P. picta* that coincides with elevated plasma levels of estrogen in the females. Distinctly, in *P. vazimba* no such spike was found but, in this species, sexual maturity and termination of growth coincided with elevated levels of progesterone in females. Importantly, the respective growth curves of each of these species are also significantly different. *P. picta* show a more classical growth pattern, i.e. similar growth patterns in early growth in both sexes before a more pronounced growth in males. In contrast we found a more complex pattern in the female-larger species with early dimorphism in growth curves for *P. vazimba* with females growing faster early on and closing their growth plates prior to males. Overall, this thesis aims to clarify the nature of growth in being determinate and canalized in lizards and elucidate the proximate causes of SSD.

Abstrakt

Růst byl u plazů historicky považovaný za neukončený. Tento obecně sdílený předpoklad byl v posledních letech zpochybněn a narůstaly důkazy o ukončeném růstu u ještěrů. Tento obrat způsobila především analýza růstových plotének kostí, která ukázala jejich uzavření a tím nemožnost dalšího prodlužování kostí, což způsobuje dosažení konečné velikosti těla. Paralelně s touto změnou v nahlížení na ukončený růst byly zpochybněny i dlouhodobé předpoklady o zásadní přímé roli energetického rozpočtu a s ním spojených *trade-offs* v ovlivňování růstu. Tento posun v perspektivě ovlivňuje nejen naše chápání individuálního růstu, ale také se odráží ve studiu souvisejícího znaku - pohlavního dimorfismu ve velikosti (SSD) - rozdílu ve velikosti mezi pohlavími v rámci druhu. Existují tři hlavní hypotézy o příčinách SSD u plazů: pohlavně-specifické náklady na reprodukci, ovlivnění růstu samčími gonadálními androgeny a ovariálními hormony. Tato práce přináší další důkazy o ukončeném růstu u ještěrů a diskutuje jeho důsledky pro evoluci SSD. Review o růstu zaměřené na gekona *Paroedura picta*, kde jsou větší samci než samice, zdůrazňuje značnou kanalizovanost růstu a současně poskytuje základ o úvahách týkajících se proximátních příčin SSD. V této práci dále předkládám podporu pro pravděpodobnou roli ovariálních hormonů a inzulinu podobného růstového faktoru 1 (IGF1) při ontogenezi SSD prostřednictvím experimentů s druhy rodu *Paroedura*. Studoval jsem hladiny pohlavních hormonů a expresi IGF1 v játrech během růstu u obou pohlaví druhů *P. picta* a *P. vazimba*, kde jsou větší naopak samice. Zaznamenal jsem významný nárůst exprese genu *IGF1* v játrech nezávislý na pohlavní dospělosti u samců *P. picta*, který je patrně potlačen zvýšenými hladinami estrogenů u samic. U *P. vazimba* nebyl takový nárůst zjištěn, ale u tohoto druhu souvisela pohlavní dospělost a ukončení růstu se zvýšenými hladinami progesteronu u samic. Pohlavně-specifické růstové křivky se mezi těmito dvěma druhy významně liší. Druh *P. picta* vykazuje klasický růstový vzor, tj. zpočátku podobný růst u obou pohlaví před výraznějším růstem samců. Naopak u *P. vazimba* samice zpočátku rostly rychleji, ale uzavřely své růstové ploténky dříve a ve větší velikosti než samci. Práce přinesla další evidenci pro ukončený a kanalizovaný růst plazů a navrhla proximátní model pro evoluci SSD.

Introduction

General introduction

The study of evolution seeks to understand how organisms have adapted and changed over time. A prevalent objective in the study of evolution is the discovery of ultimate causes - the causes and pressures defining 'why' a specific trait has evolved in a given species. As the crux of my PhD thesis, I have come to appreciate that understanding the proximate causes - the immediate mechanisms and processes defining 'how' a specific trait evolves - is equally significant. This is especially of interest for life history traits - that define organisms throughout their life. A key principle in evolutionary ecology is the concept of trade-offs and energetical budget or the compromises organisms make to optimize their survival and reproduction in a world of limited resources. However, as highlighted by Flatt and Heyland (2011) in '*Mechanisms of Life History Evolution: The Genetics and Physiology of Life History Traits and Trade-Offs'* the interplay between these proximate mechanisms and these energetical constraints provides a nuanced perspective on evolutionary dynamics. The gears and cogs creating the opportunity for the evolution of a trait are reflected by hormonal control and fluctuation, which may be sex-specific in a given species. Evolutionary biology therefore lies in the interaction between genetic and hormonal factors that orchestrate life history traits and the available resources permitting traits to evolve.

A major trait that is a subject of study in evolutionary biology is body size. Body size can vary significantly between closely related species or even within species. This difference within species is transcribed by the difference in structural size between the sexes or sexual size dimorphism (SSD) (Badyaev, 2002). In recent years, there has been accumulating research, in reptiles, on the proximate causes of SSD and the study of the trait that governs body size – growth. As I will present throughout this thesis some expectations of the nature of growth in lizards are questioned resulting in shifts in the study of body size plasticity and SSD. Within the scope of this thesis, through a series of meticulously designed experiments, I aim to illustrate the canalized and determinate nature of growth/body size in lizards and contribute to the understanding of the functional architecture underpinning both structural growth patterns and SSD.

Sexual dimorphism

Sexual dimorphism is prevalent across vertebrates (Owens and Hartley, 1998; Cox et al., 2007; Mcpherson and Chenoweth, 2012). Dimorphism can take many forms affecting anatomical and physiological features, secondary sexual characteristics that show clear differences between the sexes in coloring or ornaments. A common form of sexual dimorphism consists of differences in size (Owens and Hartley, 1998; Cox et al., 2007; Mcpherson and Chenoweth, 2012). In most cases, the assumption of the evolution of sexual dimorphism in vertebrates comes from sexual selection and sex-specific reproductive roles (Darwin, 1871). Although, differences due to sex specific niche divergence can also occur (Mcpherson and Chenoweth, 2012).

Sexual dimorphism takes many forms in reptiles. Dimorphism in color (Stuart-Fox et al., 2004; Chen et al., 2012; Olsson et al., 2013; Pérez I de Lanuza et al., 2013; Rossi et al., 2019) or in the presence of ornaments such as casques in males (Taylor et al., 2017; Bauerová et al., 2020) are amongst prevalent forms in lizards. More common forms of dimorphisms are related to differences in size. Notably, differences in structural size or SSD. There can be a relation between the different forms of sexual dimorphism. Dichromatism is positively correlated with SSD in some taxa such as Lacertidae and Agamidae (Rossi et al., 2019) while presence of male casques is correlated to bigger body size (Taylor et al., 2017; Bauerová et al., 2020).

The most common form of SSD in lizards is male-larger SSD but female-larger species are also found in nearly every family. Male biased sexual dimorphism is more common in some families (Iguanidae, Varanidae; Cox et al., 2007) while the direction of SSD is non-directional in others (e.g. Gekkonidae, Scincidae; Cox et al., 2007). The difference between male biased and female biased SSD also reflects in the more extreme pronunciation in male-larger species. In some species of anoles, tropidurids and marine iguanas males can be up to 50% longer than females. By contrast, females exceed males by as much as 20% in some polychrotids, skinks and pygopodids (Cox et al., 2007). The Rensch's rule states that larger species tend to be male-larger while female-larger species tend to be smaller (Butler et al., 2000; Reyes-puig et al., 2023). This rule was supported in some lizards and challenged in others (Starostová et al., 2010; Liang et al., 2021; Liang et al., 2022) demonstrating variance in this trait. The variation of SSD in lizards is also exemplified below in figure 1 by the relative percentage in the order Gekkota with noticeable variation in the

different families and confirmed patterns of non-dimorphic, male and female-larger species in our genus of interest, *Paroedur*a (Cox et al., 2007; Starostová et al., 2010).

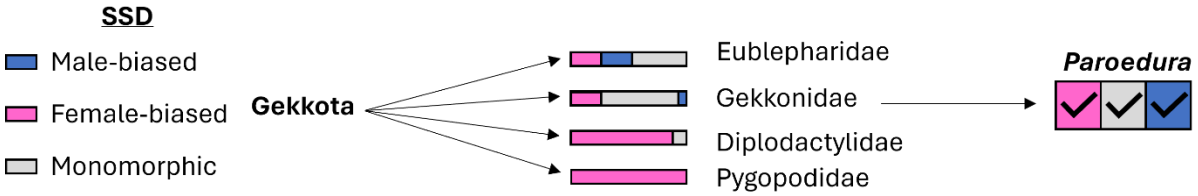

Figure 1: Example distribution of SSD in order Gekkota (after Cox et al., 2009 and Starostová et al., 2010).

Sexual selection is expected to be the major driver of SSD in lizards. The extensiveness of male biased SSD can be explained by the frequent male-male aggressive behavior found in a number of lizard species (Kratochvíl and Frynta 2002; Cox et al., 2003; Taylor et al., 2017; Bauerová et al., 2020). This combative nature in males creates a selection for larger size as males will use parts of their body especially their head as weapons (Kratochvíl and Frynta 2002). Furthermore, male aggressivity does increase for a number of male-larger species (Cox et al., 2009). In contrast, female-larger SSD can be explained in the lens of reproduction with larger females having bigger clutches (Cox et al., 2007; Yu et al., 2014). Habitat could also contribute to explaining patterns of SSD as represented by the studies targeting ecomorphs of *Anolis* showing patterns of SSD divergence depending on the specific ecological niche of each ecomorphs (Butler et al., 2000; Butler, 2007). As for the proximate causes of SSD, there have been three leading theories in lizards. First, through the lens of the trade-offs and relationships of growth and reproduction - the cost of reproduction hypothesis (e.g. Cox et al., 2006). Then in the lens of the role of sex steroids with both ovarian hormones and male androgens having been tested (e.g. Cox et al., 2009; Starostová et al. 2013; Kubička et al., 2017) as we discuss in more detail in **paper I**. However, we believe that to fully understand the ontogeny of SSD a fundamental knowledge of the nature and mechanisms of growth are first needed as we will explore below.

Growth

Growth, just as reproduction, is expected to be influenced by energetical budgets (Warner et al., 2008; Werner et al., 2018) and the idea of the impact on life history traits of energetical allocation through nutrition is a focal point in evolutionary biology (Ng'oma et al., 2017). In contrast, as we discussed in our review using our model species the Madagascar ground gecko *Paroedura picta* (Peters 1984) (**paper I**), growth can be canalized - less affected by energy trade-offs and the environment than expected by classical templates and leading to a defined final body size. There is still evidence in other lizards of possible influence of energy on growth. A trade-off with growth was found in cases of combined tail autotomy and food restriction in *Eublepharis macularius* and *Podarcis muralis*, respectively (Lynn et al., 2013; Fernández-Rodríguez et al., 2022). We do not believe there is no influence of energy trade-offs on growth but simply that this should not be the automatically assumed naively applied framework when studying life history traits. The energetical budget also determined the modeling of growth. Classical models used this assumption of a trade-off with energetical traits - namely reproduction - in their design (von Bertalanffy, 1957; West et al., 2001; Martin et al., 2019; Sibly and Brown, 2020). For instance, according to West et al. (2001), energy allocated for growth is split into three components: to the maintenance of existing tissue, the replacement of cells and the formation of new tissue. Then, a large part of the available energy is later distributed to reproduction and, in term, reduces growth. Updated models have focused on the trade-offs due to maintenance to explain the reduction of growth (Sibly and Brown, 2020; White et al., 2022). Still, these classical and revised models function under a mechanistic modeling of growth which restricts to a given pattern. In lizards, a commonly used model is the von Bertalanffy model (von Bertalanffy, 1957) which uses a mechanistic but also asymptomatic approach in describing lizard growth. Another key aspect of growth in lizards that has been disputed is its indeterminate nature.

Growth can be considered either determinate or indeterminate. Determinate growth being characterized by its cessation during the lifespan of an individual while indeterminate growth is distinguished by continuation throughout the lifespan (Frýdlová et al., 2020). This also creates a possible difference in the expected plasticity of the trait with indeterminate growth being able to change due to environmental conditions throughout life, while determinate growers have a more genetically guided growth trajectory that can be only somewhat influenced by the environment (Sebens, 1987). It is also presumed that determinate growers tend to stop growth before sexual maturity (Frýdlová et al., 2020). Historically,

endothermic tetrapods have been considered determinate growers while ectothermic tetrapods are expected to be indeterminate growers with some documented exceptions (Frýdlová et al., 2020). This view of growth has however been shaking in reptiles through accumulated evidence (Frýdlová et al., 2020). Notably, through evidence of skeletal growth arrest by microcomputed tomography of bone growth plates in several lizards (Frýdlová et al., 2019). This is also represented throughout this thesis. In **paper I**, the determinate nature of growth in lizards was not discussed as it was less established in literature, and we wanted to focus on the role of the energetical budget. In **papers II and III**, we support this determinate nature, but the models used for depicting the growth curves diverge. In **paper II**, the growth curves presented were based on the data from Kubička et al., (2022) which used the asymptomatic von Bertalanffy model. In contrast, we used General Additive Models (GAM, Pedersen, et al., 2019) in **paper III,** as we explained, this type of modeling has the advantage of not being restricted to a particular pattern as in the asymptomatic von Bertalanffy model. As mentioned above, more mechanistic models of growth expect a direct influence of energy budgets (von Bertalanffy, 1957; West et al., 2001; Martin et al., 2019; Sibly and Brown, 2020; White et al., 2022). We stated in **paper III** that the use of GAM is advantageous as it does not have the constraint of the assumptions as in classical mechanistic models. The statistical approach of GAM allows fitting to any type of curve which can elucidate the patterns of growth especially in cases like lizards where the nature of growth is revisited. We believe the shortcomings of GAM modeling namely over or under fitting a curve pattern are outweighed by its flexible nature. In figure 2, below, the proposed shift in the growth patterns from indeterminate to determinate growth is schematically represented. The shift in modeling of growth as we propose is driven by two factors. First, the evidence of determinate growth, we encourage determining the cessation of growth through techniques such as growth plate analysis. Secondly, in the tools used to depict growth curves, we believe that switching to GAM allows for better representation of growth curves.

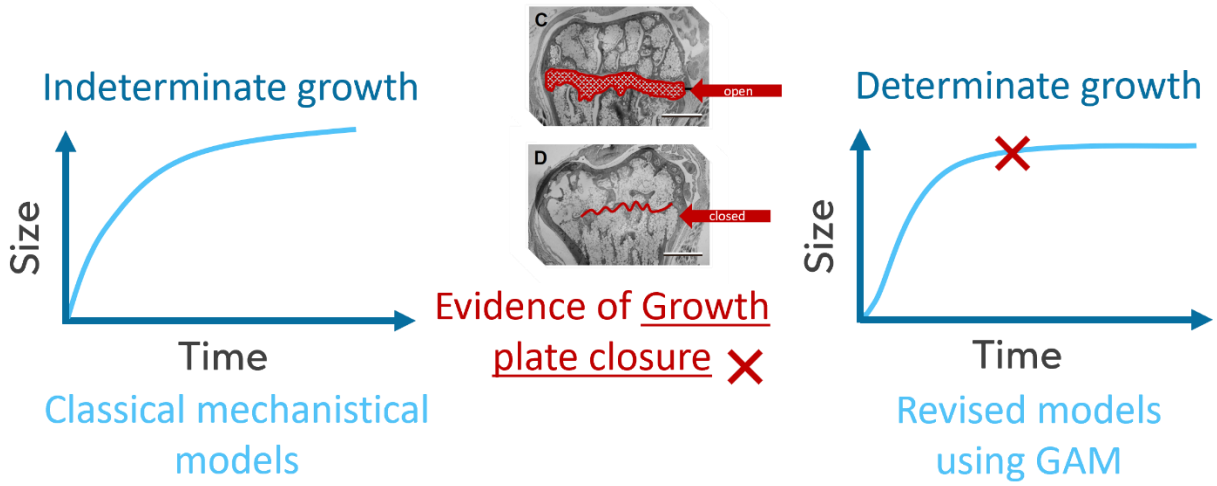

Figure 2: Schematic representation of the shift in the modeling of growth in lizards (growth plate pictures adapted and modified from Kubička et al., 2022).

These two novel views of determinate and canalized growth are seemingly more compatible. As featured more recently, this constrained nature reviewed in **paper I**, can be explained by a canalization to a sex specific - determinate - final body size while still having some plasticity in growth rates (Starostová et al., 2024). Overall, there are some supported assumptions of the role of trade-offs and constraints on life history traits but as we argued in our review (**paper I**) a simple vision of energy allocation may not explain the nature of all traits. In the context of growth, trade-offs in the maintenance of tissues could answer why growth does not continue throughout the lifetime and the existence of mechanisms such as the bone growth plate closure guiding determinate growth. However, to fully understand the impact of trade-offs and constraints on traits a foundational knowledge of the genetic and hormonal mechanisms is also needed (Braendle et al., 2011). With this framework in mind, to also recognize why traits can be less flexible to energetical budgets there is a need for exploring the mechanisms guiding this canalization. As presented in **papers II and III,** the determinate nature of growth in lizards demonstrates the utility in long term growth studies to fully grasp the complexity of growth trajectories and the mechanisms that guide growth and SSD. In this lens, alongside long-term growth studies, we focused on the genes and hormones that are likely the proximate mechanisms of this elaborate life history trait by studying the role of sex steroids and the insulin-like growth factor network.

Molecular pathways controlling growth

The insulin-like growth network (figure 3) has a principal role in the ontogeny of growth. The growth hormone (GH) is secreted into the bloodstream from the anterior pituitary gland to bind to the GH receptor (GHR) with the liver being the main target. This leads to the stimulation of the insulin-like growth factors I and II (IGF1 and IGF2) and a negative feedback loop of IGF1 on GH. In time, both enter the bloodstream and bind to their respective receptors. IGF1 onto the insulin receptor (IR) and the insulin-like growth factor I receptor (IGF1R) while IGF2 binds to insulin-like growth factor II receptor (IGF2R) and IGF1R (Ohlsson et al., 2009; Schwartz et al., 2016; Yakar et al., 2018). In addition, IGFs are also regulated by insulin-like growth factor binding proteins 1-6 (IGFBPs) (Schwartz et al., 2016).

When it comes to structural growth the role of IGF1 has been well documented while the role of IGF2 is still understudied (Ohlsson et al., 2009; Yakar et al., 2018). The role of IGFs is known in longitudinal and radial bone growth as well as in the differentiation and maturation of chondrocytes of the growth plate (Ohlsson et al., 2009; Yakar et al., 2018; Racine and Serrat, 2020). Both hepatic IGF1 and local bone IGF1 have their role in longitudinal growth (Ohlsson et al., 2009). The two sources of IGF1 may have overlapping effects on bone growth (creating redundancy) with IGF1 from the liver not being able to be replaced (Ohlsson et al., 2009). Furthermore, manipulations of endocrine hepatic IGF1 in mice shows that radial growth remains dependent on hepatic IGF1 (Yakar et al., 2018).

Sex steroids have also been found to modulate the insulin-like growth network contributing to sex specific liver function and gene expression patterns (Ohlsson et al., 2009; Zhongbo et al., 2016; Yakar et al., 2018). Testosterone stimulates bone size and IGF1 levels through GH mediation (Callewaert et al., 2010; Zhongbo et al., 2016; Yakar et al., 2018). The role of estrogens in bone growth is well documented, mainly by its inhibitory effect on GH (Leung et al., 2004; Ohlsson et al., 2009; Zhongbo et al., 2016). Estrogens also influence IGF1 levels independently of GH (Callewaert et al., 2010; Zhongbo et al., 2016; Yakar et al., 2018) and may also affect bone growth plate senescence directly (Nilsson et al., 2005; Nilsson et al., 2014). The role of progesterone on bone growth is less studied and the present evidence suggests a less significant role than estrogens (Rickard et al., 2008; Yao et al., 2010; Mills et al., 2021). Evidence suggests a limited role of progesterone in bone mass during adolescence/rapid growth period in mice (Rickard et al., 2008; Yao et al., 2010). However, knocking out progesterone receptors may also interfere with other hormones then

progesterone (Mills et al., 2021). The role of sex steroids is also represented by their receptors on bone tissues. Androgen receptor (AR) was found to affect bone growth in male mice (Zhongbo et al., 2016). Estrogen receptors alpha and beta (ESR1 and ESR2) were found to have their respective roles in skeletal growth, ESR1 being the predominant sex receptor affecting bone morphology and IGF1 expression (Zhongbo et al., 2016). Still, most evidence about the role of sex steroids and the insulin-like growth network in structural growth is brought by studies in humans and rodents.

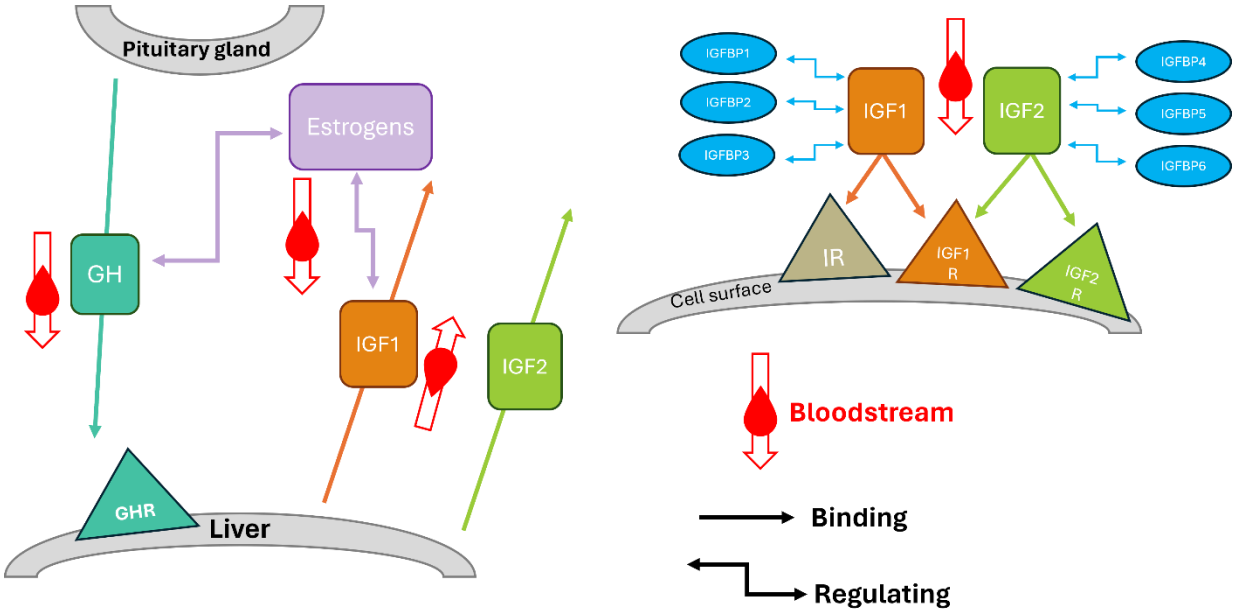

Figure 3: Insulin-like growth network and the probable role of estrogens (after Ohlsson et al., 2009, Schwartz et al., 2016, Zhongbo et al., 2016 and Yakar et al., 2018).

As for specific studies in reptiles concerning the insulin-like growth network there has been increasing literature in recent years. The role of IGF1 and IGF2 in growth was highlighted in several reptiles (Reding et al., 2016; Beatty & Schwartz, 2020; Cox et al., 2022; Xie et al., 2023) with most of these papers focusing on the role of hepatic IGF1. *IGF2* mRNA levels were found to be much higher than *IGF1* in garter snakes *Thamnophis elegans* (Reding et al. 2016), in lizard *Anolis carolinensis* (Marks et al. 2021) and throughout development in lizard *Anolis sagrei* (Beatty & Schwartz; 2020). Components of the insulin-like growth network, particularly *IGF1*, were found to be expressed in a sex biased fashion through RNAseq analysis in male-larger lizard *Anolis sagrei* (Cox et al. 2017; Hale et al., 2022). *ESR2* was also found to have a likely inhibitory role in bone growth of cranial skeleton of *Anolis carolinensis* by evidence of its sexually dimorphic mRNA expression

(Sanger et al., 2014). The functional architecture of growth is therefore complex and still understudied in reptiles.

The proximate mechanism of SSD in lizards has been a subject of debate for a number of years. The evidence of the determinate nature of growth and the limited influence of simple energy allocation opens up to the possibilities of studying these causes in a lens of a mechanical function instead of a trade-off framework.

Aims of the thesis

This thesis aims to uncover the proximate causes that dictate growth and SSD while better understanding the nature of growth in lizards using the *Paroedura* genus as a model. We wanted to provide further evidence on the determinate nature of growth in lizards and challenge relying on energy trade-offs as a framework to study growth as a life history trait. Understanding and implementing an updated view on the nature of growth necessitates a full picture of the mechanisms that guide its development. We therefore also needed to better understand the key genes and hormones guiding the endogenous control of SSD and growth. To fully encapsulate the endogenous control of SSD we targeted male-larger species *Paroedura picta* and female-larger species *Paroedura vazimba*. Long term growth studies were performed for both species, *P. picta* as illustrated in **paper II** from the study by Kubička et al. (2022) and for *P. vazimba* as performed in **paper III**.

For *P. picta* we looked at the gene expression of genes of the insulin-like growth factor network and receptors of sex steroids to study their expression throughout growth. In addition, we studied the evolution of plasma sex steroids levels during development in both *Paroedura* species. Finally, the gene expression study for *P. vazimba* was focused on the gene *IGF1* following the results found in *P. picta*. With all this gathered information we aimed to give a comprehensive review of the endogenous control of SSD and its influence in the development and nature of growth.

Results and discussion

Main results

As stated in the introduction, the review on *P. picta* (**paper I**) highlighted the canalized nature of growth in this species but also established the very likely role of ovarian hormones in the ontogeny of SSD. **Paper I** reviewed several manipulative experiments in *P. picta* to draw these conclusions. First, the experiment focusing on food restriction showed little to no effect on growth while both reproduction and fat storage were constrained (Kubička and Kratochvíl, 2009). The experiment on tail autotomy rejected the hypothesis that growth would be hindered by the energetical cost of tail regeneration (Starostová et al., 2017). Finally, the experiments regarding SSD, namely through ovariectomy and castration did not indicate a trade-off between growth and reproduction as traditionally expected (Starostová et al., 2013; Kubička et al., 2015, 2017). The evidence from each of these experiments alone was not enough to presume on the effect of simple energy trade-offs and growth. However, combining the data and conclusions from all these experiments allows for a broader and more decisive picture of the nature of growth. Still, challenging the supposedly evident role of energy in molding a major life history trait needed not only accumulated evidence but also a synthesis revisiting some commonly held views in ecology. As we stated in the paper, we admit there are some limits to the approach of this review but we believe it gives a strong exemplary perspective to approaching life history traits and their relation to energy expenditure.

Table 1 below summarized the three leading theories of the ontogeny of SSD in squamate reptiles. These were tackled in the different experiments with *P. picta* as outlined in **paper I**: the cost of reproductive hypothesis, the role of male androgens and the role of ovarian hormones (Starostová et al., 2013; Kubička et al., 2015, 2017). The cost of reproduction hypothesis stating that the cost of producing gametes and in the case of females eggs/embryos could explain the sex difference in structural growth as one sex allocated more energy to reproduction at the expense of growth. This hypothesis was mainly defended by full ovariectomy experiments which prohibited female reproduction (Cox et al., 2006, 2010, 2014). As reviewed in **paper I**, manipulative experiments in *P. picta* consisting in unilateral ovariectomy reducing reproductive output to half and social isolation of females preventing mating and hence reproduction showed that the change in growth in fully ovariectomized females is not explained by the cost of reproduction (e.g., frequent egg laying) but by the

hormones produced by the gonads. The role of androgens was not supported in a number of squamates (Kubička et al., 2013; Bauerová et al., 2020), including *P. picta* (Starostová et al. 2013; Kubička et al. 2015), while its evidence in other squamates focused mainly on the application of exogenous testosterone or castration and did not control for its aromatization with dihydrotestosterone (DHT) (Cox et al., 2005,2009; Duncan et al., 2020), an androgen which cannot be further aromatized and therefore can only act through androgen receptors. In contrast, change in growth of females with exogenous estradiol combined with the other manipulative experiments (Starostová et al. 2013; Kubička et al., 3017) called attention to the theory of ovarian hormones as the mechanical originators of SSD.

Table 1: Summary of the theories of the ontogeny of SSD.

	Cost of reproduction hypothesis	Testosterone hypothesis	Ovarian hormones hypothesis
Evidence	✓ Full ovariectomy	✓ Castration ✓ Exogenous testosterone (T)	✓ Partial vs full ovariectomy ✓ Exogenous estradiol ✓ Estrogens (E) levels and SSD onset
Counter-evidence	✓ Female isolation ✓ Partial ovariectomy	✓ No T influence in some species ✓ T aromatization into estradiol → test with dihydrotestosterone	✓ Coincidence of growth and E levels ✓ Independent role of IGF1
Conclusion	➢ Ovariectomy is a better evidence for the role of ovarian hormones than the cost of reproduction hypothesis	➢ Castration and exogenous T do not support an independent role of T in SSD	➢ The different experiments and the known role of E on growth plates and IGF1 provides strong evidence for E being a mediator of SSD

As stated in the introduction, **papers II** and **III** estrogens have a known role in structural growth by mediation of IGF1 and influence on the bone growth plates. We believe that the accumulated evidence presented in the review (**paper I**) and the evidence in **papers II** and **III** show the role of ovarian hormones and most likely estrogens in SSD. The role of IGF1 as a mechanism of SSD was established in the male-larger *P. picta* (**paper II**). More precisely we found a spike of *IGF1* expression in males just after the beginning of the onset of SSD and after reaching sexual maturity. This is in contrast with humans, where a spike of IGF1 during puberty is known to affect growth of both sexes (Cole et al., 2015). On the other hand, the onset of SSD also corresponds to an increase of plasma level of estradiol in *P. picta* females. Given the known role of estrogens on growth through bone growth plates this suggests a plausible inhibition of growth in females coupled with the role of IGF1 in males creating the SSD in this species. In contrast, for female-larger species *P. vazimba* no such spike was found (**paper III**). Plasma levels of estrogens were below the detectable level in

both sexes while progesterone levels increased at time of female sexual maturity. A similar trend was found in *P. picta* with progesterone being at its highest level in reproductively active females (Kubička et al., 2017). Therefore, this hormone may also play a role in the ontogeny of SSD in both species or at least serves as a marker of female sexual maturity connected with increase of other ovarian hormones including estrogens.

We suggested a role of ovarian hormones - namely estrogens and possibly progesterone - in the SSD of both *P. vazimba* and *P. picta.* In *P. picta,* a noticeable male spike of *IGF1* can be suppressed by increased estradiol in females. Overall, the growth strategies in both species are very different with early divergence between the growth of both sexes in *P. vazimba* with females attaining a larger final body size earlier as shown by bone growth plate closure. In contrast, the growth curves overlap in both sexes early in *P. picta* then males grow larger, and the final body size is attained at similar age in both sexes which corresponds to the expected growth curves of a sexually dimorphic species (Badyaev, 2002).
In both studies we monitored their plasma levels in both sexes throughout growth and targeted hepatic *IGF1* gene expression for specific age/sex groups. One major constraint in both these data sets was the difficulty in targeting younger individuals. This was especially a problem in **paper III**, because the total body mass of freshly hatched *P. vazimba* is around 0.2 grams. We suggested a possible spike of *IGF1* in younger individuals that we could not pick up with our analysis. Still the spike of *IGF1* in *P. picta* is clear evidence of its role in SSD while the convergence between the uptake of estrogens in females with the onset of SSD does strongly suggests its role in SSD as well. The evidence in *P. vazimba* is less direct. There is an increase of progesterone in females that corresponds to reproductively active ovaries which would also indicate an increase in estrogens levels. This increase of progesterones coincides with fused vertebrae growth zones indicating the role of ovarian hormones in a sex specific final body size also in this species.

As a counterpoint, SSD may be guided by a spike of *IGF1* expression which can be regulated by estrogens but an independent role is not impossible. Furthermore, one caveat in studying the natural circulating levels of sex steroids is in drawing definitive answers. An increase in estrogens corresponding with the cessation of growth implies its role but may be coincidental. Still we believe the accumulated evidence in both species for a role of ovarian hormones outweigh these unlikely scenarios.

We therefore propose a model in which estrogen has a bipotential effect in reptiles. At lower levels, estrogens stimulate growth while at higher levels it has an inhibitory effect as found in growing female mammals (Cutler et al., 1997; Weise et al., 2001) with their sexual

maturity leading to gradual maturation of follicles resulting in an increase of estrogens production. In *P. picta*, a surge of estrogen levels found in females co-occurs with their onset of sexual maturity and likely prevents the spike of *IGF1* that is found later in males. A direct role of estrogens on the bone growth plates is possible (Nilsson et al., 2005; Nilsson et al., 2014) but its influence on the insulin-like growth factor network - especially through GH mediation - is better documented (Callewaert et al., 2010; Zhongbo et al., 2016; Yakar et al., 2018). In *P. vazimba*, the recorded levels of estrogens are below the detectable levels – indicating its relative low levels throughout lifetime in this species. However, an increase of progesterone at time of sexual maturity indicates an increase of other ovarian hormones including estrogens, possibly resulting in the earlier closure of growth plates in females compared to males of *P. vazimba*. The difference in this bipotential effect of estrogens on male and female-larger species could be explained by a difference in sensitivity of the growth axis to estrogens or in the relative timing of the onset of follicular cycles in female mature ovaries. In *P. picta*, the growth curves only diverge around the time of increase of estrogens in females and the spike of *IGF1* disconnected from sexual maturity in males. While in *P. vazimba*, growth is already divergent on the onset with females growing faster than males but this growth in females stops at sexual maturity connected with an increase in ovarian hormones including estrogens. The two different systems of growth presented here are reflected by the circulation of ovarian hormones while the most prominent of which are apparently estrogens.

Conclusions

We provided evidence of the impact of IGF1 and ovarian hormones in the endogenous control of growth and SSD. We demonstrated that a more likely candidate in the control of growth are estrogens but cannot ignore a possible role of progesterones. We found that growth is complex and variable between species of this genus with respect to the ontogeny of SSD and the mechanisms that guide growth.

From our review on growth in *P. picta* (**paper I**) we demonstrated that growth can be quite canalized with the only major acting factors being temperature alongside hormones (e.g. exogenous estradiol). The aforementioned recent evidence suggests this canalization is in the final body size while the growth rates can be somewhat plastic (Starostová et al., 2024). This further highlights the rigidity of final body size and SSD in lizards. Growth trajectories have a distinct, directed, and final path that is guided by a complex network of hormones with possibly limited environmental guidance (with the exception of temperature e.g. Starostová et

al., 2010). This less flexible nature leading to a canalized body size indicates a form of selection and that this final body size is an adaptive trait. In addition to sexual selection on SSD, final body size may have a form of natural selection. As stated in our review (**paper I**) we hypothesized that species trend towards an optimal body size to fit an ecological niche and a preferred prey size. We believe that the determinate and canalized final body size presented here works well within this hypothesis. Selective pressures guide to a canalized final body size through endogenous hormonal control (by ovarian hormones and IGF1) instead of simple energetical tradeoffs.

Throughout this thesis I aimed to show that to fully encase the proximate causes of growth we need the combination of long-term growth studies with the study of the molecular pathways that guide this trait. This allowed us to provide further evidence of the determinate nature of growth with bone growth plate analysis while the depiction of growth curves between sexes were proven to be complex and diverse across species of the same genus. Studying the molecular pathways that guide growth showed that ovarian hormones have a prominent role in SSD, more precisely we believe that estrogens mediate IGF1 - a key hormone in structural growth and the closure of growth plates. We believe this thesis gave a strong analysis on lizard growth and the ontogeny of SSD but cannot deny there is still much to be studied in this subject.

Prospects

This thesis, just as many other research projects about SSD and growth did mainly focus on the insulin-like growth factor network. This network still needs to be studied in more depth. From 1990 to 2020 only 30% of publications regarding IGFs were about IGF2 (Beatty et al., 2022). It has also recently been shown that postnatal IGF2 is the norm across amniote vertebrates indicating a shortcoming in the study of growth (Beatty et al., 2022). IGFBPs are understudied in reptiles with, to our knowledge, one paper examining their function through gene expression analysis in a reptile species. IGFBP 1-5 were found to be expressed in embryonic stages across tissues in adults of *A. sagrei* (Beatty et al., 2020). IGFBP6 is also believed to have lost its functionality in binding IGF hormones in reptiles after bioinformatic prediction (McGaugh et al., 2015). Furthermore, this network may not be the only one essential in the study of growth. As discovered by Wu et al., (2022), in an evolutionary rate study, targeting 101 body-size-related genes in 28 reptilian genomes. The insulin-like growth factor network is not the only network of importance in growth and body size. Notably, genes

related to the bone morphogenetic protein (BMP) which has its role in bone and cartilage formation (Wu et al., 2022). There are known interactions between BMP-6 and IGF1 (Rico-Llanos et al., 2017) but *BMP-6* was not positively selected in the study in both lizards and snakes (Wu et al., 2022). Alternatively, some *SOX* genes with a known role in embryonic development were also targeted as reptilian body-size-related genes (Wu et al., 2022). The choice of focusing on the role of the insulin-like growth factor network and sexual hormones for our projects was decisive but we cannot ignore that growth and SSD are life history traits that require an intricate and intertwined network of proteins and its endogenous control is complex. Even considering the insulin-like growth factor network alone there are multiple elements guiding this network and fully deciphering is difficult.

Future studies should prioritize long term growth data and we suggest alternatives in the statistical models used to analyze this data. Using general additive models instead of more classically used mechanistic growth models may yield a better representation of growth. Another avenue of research could incorporate a better analysis of the complex growth network by studying gene expression in other tissues then the liver alongside other genes of interest. Targeting the growth hormone receptor (*GHR*) in the liver in *P. picta* around the time of the *IGF1* spike could paint a better picture of the interaction of these hormones and their resulting influence on growth. Furthermore, our results (**paper II**) did not yield any noticeable difference in hepatic expression of estrogen receptor alpha (*ESR1*) but targeting this receptor, and possibly, the other estrogen receptor (*ESR2*) in bone of growing *P. picta* and *P. vazimba* could unclog the specific role of estrogens in SSD. More precisely, its role in bone growth by determining if its effect is direct on the bone growth plate or by mediation of the insulin-like growth factor axis in the liver. The possible role of progesterone in bone growth is less studied than estrogens but a possibility could be a study of its receptor's gene expression in both hepatic and bone tissue. The suggestions above did focus on our genus of interest *Paroedura* but these alternative paths of research in other sexually dimorphic lizard species are more than welcome. Through this thesis I hope I transcribed that challenging long term assumptions about the nature of fundamental traits -such as growth and body size- requires not only evidence from manipulative studies but also by direct study of their proximate mechanisms.

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The limits of the energetical perspective: life-history decisions in lizard growth.

Meter Brandon, Starostová Zuzana, Kubička Lukáš and Kratochvíl Lukáš

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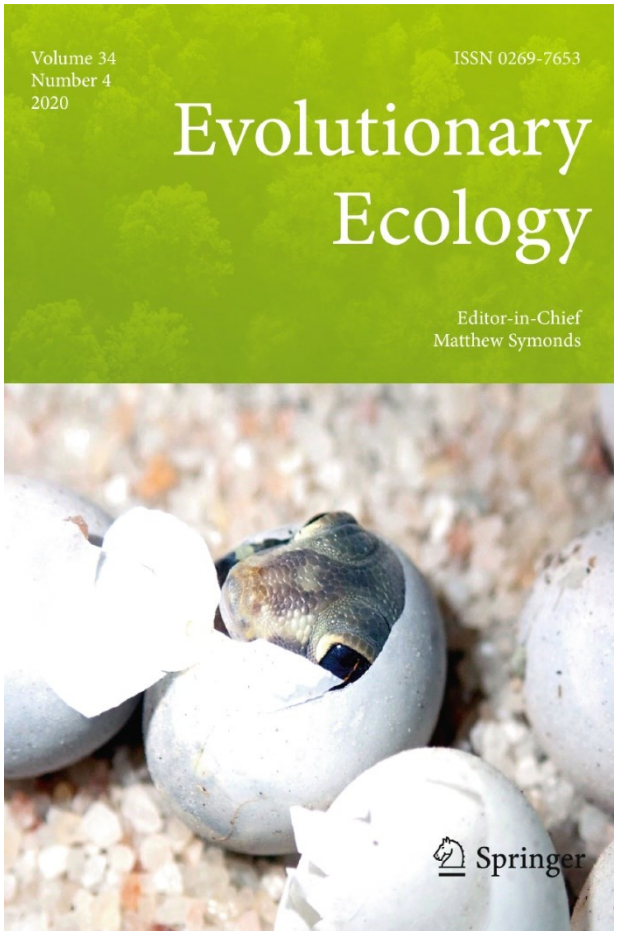

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IDEAS & PERSPECTIVES

The limits of the energetical perspective: life-history decisions in lizard growth

Brandon Meter[1] · Zuzana Starostová[1] · Lukáš Kubička[2] · Lukáš Kratochvíl[2]

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Abstract

The study of energy allocation is essential in understanding the regulation of major life history traits. It is often assumed automatically that the limitation of an energy budget or higher allocation to a single trait affect all life history traits. This assumption was inherently included in influential models of ontogenetic growth. We aim to challenge this perspective by focusing on growth in lizards. Summarizing the results of a series of long-term manipulative experiments in the Madagascar ground gecko (*Paroedura picta*), we show that although growth is generally assumed to be highly plastic in reptiles and other ectothermic vertebrates, it is at least in this species largely canalized and does not seem to be affected by energy limitations under several experimental conditions. Diet restriction, resulting in lower allocation to fat storage and reproduction, and the allocation to energetically demanding traits such as reproduction in both sexes and tail regeneration had little if any effect on structural growth. We document that sexual size dimorphism does not emerge in the ontogeny of the studied species directly due to differential allocation to structural growth in males and females. Instead, sex-specific growth trajectories are driven by a signaling of ovarian hormones as the key proximate mechanism shaping sex-specific allocation decisions during ontogeny. We suggest that the large degree of canalization of the structural growth can reflect hierarchy in energy allocation with the structural growth being prioritized to investment in other traits. The prioritized allocation to structural growth can reflect selective advantage of reaching a final, optimal size for a given sex as fast as possible.

Keywords Growth · Energy · Trade-offs · Gonadal hormones · Sexual size dimorphism · Reptiles

Zuzana Starostová
zuzana.starostova@natur.cuni.cz

[1] Department of Zoology, Faculty of Science, Charles University, Viničná 7, 12844 Prague, Czech Republic

[2] Department of Ecology, Faculty of Science, Charles University, Viničná 7, 12844 Prague, Czech Republic

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Direct split of energy: an untested assumption of growth models

The study of energy in living organisms is essential for understanding the regulation of major life history traits such as growth and reproduction. All living organisms have a limited amount of allocable energy, which force them to optimize allocation to energetically demanding life-history traits such as growth, maintenance and reproduction (Stearns 2000; West et al. 2001; Taborsky 2017). The concept of such energy allocation trade-offs is widespread in evolutionary ecology (Angilletta et al. 2003) and we can hardly imagine an alternative to it. Nevertheless, it is often assumed automatically that the limitation of an energy budget or higher allocation to a single trait affects all other life history traits. Several models that tried to describe the ontogenetic growth trajectories from the energetical perspective are based on this rarely tested assumption (von Bertalanffy 1957; West et al. 2001; Martin et al. 2019; Sibly and Brown 2020). In other words, these growth models assumed that ontogenetic growth is very phenotypically plastic and that changes in growth are directly affected by allocation to other traits, mainly to reproduction. For example, the general model by West et al. (2001) suggests that acquired energy related to metabolic rate is split into three components: to the maintenance of existing tissue, the replacement of cells and the formation of new tissue. According to this model, a substantial portion of energy is later in ontogeny allocated to reproduction, which is accompanied directly due to energy limitations with a reduction in growth (West et al. 2001). Similar logic was also applied to explain the ontogeny of sexual size dimorphism (SSD), i.e. differences in size between the sexes. The so called "reproductive cost" hypothesis states that allocation to growth should be smaller in the sex with higher reproductive cost (Cox 2006), i.e. that the amount of energy allocated to reproduction is directly traded-off with the allocation to growth.

The idea that ontogenetic growth is plastic with respect to the total energy budget or to the amount of energy allocated to reproduction and other energetically demanding traits is so appealing that it is in fact rarely tested. It has straightforward predictions: growth and hence final body size should correlate with the total amount of available energy and with the allocation to other energetically demanding processes such as reproduction and tissue regeneration. Nevertheless, we realized in the series of growth experiments in the model lizard species Madagascar ground gecko, *Paroedura picta* (Peters, 1854), that these intuitive predictions are not followed. Here, we summarized the observed effect of manipulations with diet, allocation to reproduction and tissue regeneration on structural growth, i.e. increase in snout-vent length (SVL) reflecting skeleton dimensions, in this species. As typical reptile with "indeterminate growth", *P. picta* mature at a size representing a small fraction of its final body size (males can mature at the body mass of c. 3 g and continue to final body mass of 35–40 g, females mature at about 4 g and their final mass is around 15–19 g; own data). An enormous fraction of postembryonic growth in this species thus proceeds after the start of reproduction, which is convenient for the manipulative growth experiments focused on energy allocation. We provide insights into the proximate control of sex-specific growth trajectories and suggests an explanation at the ultimate level, why the predictions based on direct differential allocation were not followed.

Sexual dimorphism in growth is not directly related to allocation to reproduction

One field where an energetical perspective on the control of growth became prevalent is the ontogeny of SSD. SSD in body size is widespread in animals including reptiles and male- or female-biased SSD can be found across taxa, sometimes even closely related species being at the opposite side of the spectrum (Cox et al. 2007). For instance, our model species *P. picta* experiences male-biased SSD while the closely related species *P. vazimba* and *P. androyensis* are female larger (Starostová et al. 2010). The "reproductive cost" hypothesis based on an energetical perspective states that a trade-off between reproduction and growth due to the high energetical cost of reproduction is at the core of SSD development. This hypothesis was tested mostly in reptiles with male-biased SSD (Cox 2006), but it can also be relevant for species with female-biased dimorphism since the cost of reproduction has been found for both sexes across taxa (Hayward and Gillooly 2011).

In male-larger species, the "reproductive cost" hypothesis was traditionally tested by removing female allocation to reproduction by ovariectomy, which should remove the energetical cost of reproduction and therefore lead to higher allocation to growth. Higher growth rate and/or larger structural body in females comparable to male-typical pattern was indeed observed in ovariectomized females in the Yarrow's spiny lizard, *Sceloporus jarrovii* (Cox 2006), brown anole, *Anolis sagrei* (Cox and Calsbeek 2010; Cox et al. 2014) and the Madagascar ground gecko *P. picta* (Starostová et al. 2013; Kubička et al. 2017; schematically depicted in Fig. 1), which could be taken (and often was) as support for the "reproductive cost" hypothesis (Cox 2006; Cox and Calsbeek 2010). As characteristic for geckos, *P. picta* lay maximally two eggs per clutch, but the clutches are very frequent in this species leading to enormous reproductive effort (Kubička and Kratochvíl 2009; Kubička et al. 2012; Starostová et al. 2012). The idea that non-reproducing females allocate saved energy to growth is thus seemingly supported.

However, Starostová et al. (2013) also used social isolation as another way of blocking energy allocation to egg production next to ovariectomy in *P. picta* as females of this species do not produce eggs if they do not have access to sperm. Surprisingly, females in reproductive isolation did not differ in growth trajectory in SVL and in final SVL from regularly reproducing females (Fig. 1), which suggests that the female reproductive cost is not responsible for the ontogeny of SSD in this species.

These authors suggested that ovariectomy removed not only reproductive cost but also production of ovarian hormones, which can drive females to female-typical growth trajectory leading to decreased final SVL directly, not via allocation to reproduction. A follow up study by Kubička et al. (2017) extended the test of the "reproductive cost" and "ovarian hormone" hypotheses. They found that contrary to the predictions of the "reproductive cost" hypothesis, unilaterally ovariectomized females that produced around half of eggs in comparison to sham operated females while maintaining normal hormonal cycling, reached a comparable final size in terms of SVL via the same growth trajectory as control sham operated females (Fig. 1). Moreover, ovariectomized females of *P. picta* receiving exogenous estradiol reached a smaller size, which suggests that female growth can be suppressed by gonadal estrogens (Kubička et al. 2017).

Manipulations in males also found little support for the "reproductive cost" hypothesis. Sperm production is energetically demanding and can be restricted by metabolic rate and total available energy (Hayward and Gillooly 2011). Removal of allocation to gonads in growing males should thus lead to higher allocation to structural growth.

However, growth rate and final SVL in males of *P. picta* was not affected by castration both under constant temperature (Starostová et al. 2013) and in a thermal gradient (Kubička et al. 2015) (Fig. 1). These results also suggest that male gonadal androgens are not responsible for the increased growth in males in comparison to females and by extension for the ontogeny of SSD in *P. picta*. Control of male growth by male gonadal androgens was suggested as a major mechanism of evolutionary changes in SSD in squamate reptiles (Cox et al. 2005, 2009). The evidence for masculinized growth by the application of exogenous androgens in females was initially taken as a support for the control of SSD ontogeny by male gonadal androgens (Cox et al. 2009). However, this assumption has been challenged as it is not consistent with the lack of the effect of castration on growth in males *P. picta* (Kubička et al. 2015) and other lizard species (Kubička et al. 2013; Bauerová et al. 2020). Equally as ovariectomy, application of exogenous androgens likely causes defeminization, i.e. the suppression of the development of female-typical morphology via interference with normal ovarian hormonal production, not masculinization of female growth trajectory (Starostová et al. 2013). The agreement of various, mutually complementary experimental data (schematically presented in Figs. 1 and 2) implies that SSD in *P. picta* is caused by suppressed growth in females, which cannot be attributed to their high allocation to reproduction but is likely driven by ovarian hormones as the key proximate mechanism switching between sex-specific growth trajectories.

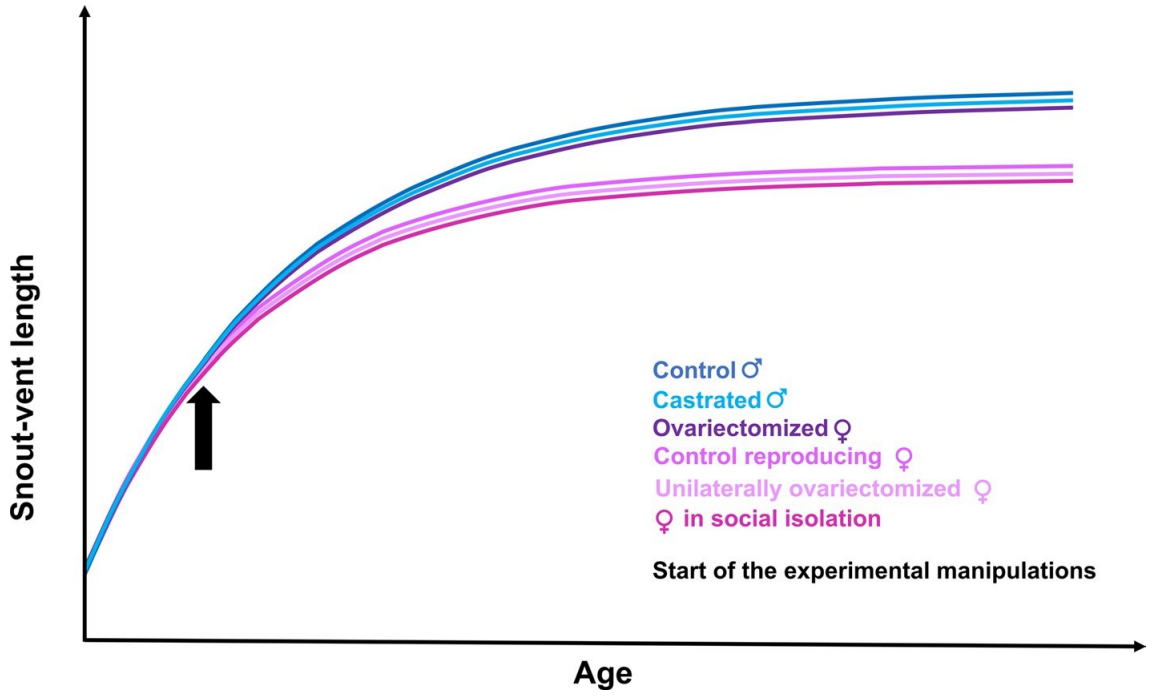

Fig. 1 Schematic depiction of postembryonic structural growth trajectories in accordance to sex and treatment in *Paroedura picta.* Only ovariectomy affects female growth and final snout-vent length likely causing defeminization in absence of hormones produced by ovaries. All other experimental treatments depicted fail to affect the growth of experimental animals. Arrow indicates the start of experimental manipulations. Schematic growth depiction for experimental groups (from above): control males, castrated males, ovariectomized females, control reproducing females, unilaterally ovariectomized reproducing females, females in social isolation

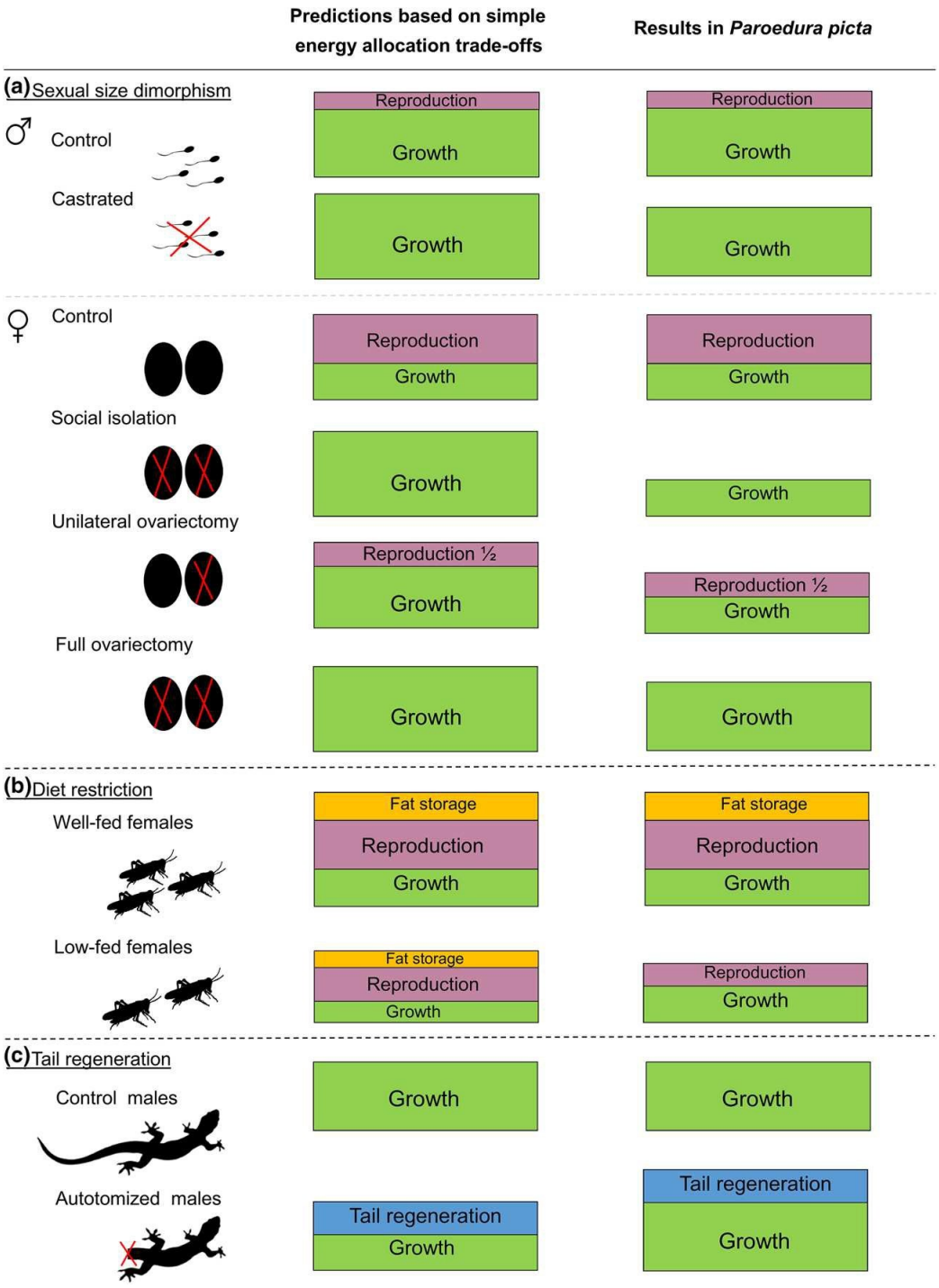

Fig. 2 Summary of the results from case studies in *Paroedura picta* that show that growth does not seem to be influenced by variations in allocable energy. **a** The "reproductive cost" hypothesis predicts that removal of the costs of reproduction should lead to higher allocation to growth in both sexes. However, castrated males attained the same size as non-castrated control males (Starostová et al. 2013). Socially isolated non-reproducing females and females with highly decreased allocation to reproduction due to unilateral ovariectomy maintained similar body size (SVL) and growth rate as control regularly egg-laying females (Kubička et al. 2017). Only full ovariectomy led to higher allocation to structural growth in females, which indicates that ovarian hormones, not directly allocable energy, controls ontogeny of sexual size dimorphism via negative effect on growth in females (Kubička et al. 2017). **b** In the case of food restriction, we expected that restricted diet would lead to reduced allocation to reproduction, growth and fat storage. However, structural growth was not affected by food limitations, which reduced only allocation to reproduction and fat storage (Kubička and Kratochvíl 2009). **c** The simple energy allocation trade-off predicts that growth should be decreased in the lizards during tail regeneration. Nevertheless, geckos with and without growth regeneration had similar growth rates and reached similar structural body size (Starostová et al. 2017). Silhouette images were taken and modified from: https://pixabay.com/

Food restriction: limited reproduction and fat storage, but not structural growth

Energy allocation to growth and reproduction was studied in females of *P. picta* also through restriction of allocable energy via manipulation with food quantity (Kubička and Kratochvíl 2009). Two balanced groups of young, still growing females kept at different food levels were followed for six months until cessation of growth. The expectation based on direct differential allocation was that the limited energy intake would impair growth rate and possibly final structural body size represented by final SVL (Fig. 2). Nevertheless, females on a restricted diet maintained growth rate and attained the same final SVL as females with higher food intake (Kubička and Kratochvíl 2009). They did however compromise on their reproduction. Females on the restricted diet laid smaller eggs in longer intervals. Diet restriction also led to the lower body mass and thus fat reserves when compared to females with higher food intake. A trade-off between growth and reproduction does not seem to occur in its simplest form here with an expected allocation compromise between these processes. In this case study, the allocation to structural growth was clearly more canalized than allocation to reproduction.

Little effect of energy limitation through tail autotomy on structural growth

Another factor that should affect the allocable energy to life history traits is tail autotomy, a widespread defence strategy of a vast number of lizard species, which is commonly followed by tail regeneration (Arnold 1988; Bateman and Fleming 2009). By shedding a tail lizards can lose a substantial proportion of body mass (Jagnandan and Higham 2018) and possibly also an energy reserve since tails are an important organ for fat storage (Pond 1978; Paz et al. 2019). Tails also need to be regenerated since they are important for locomotion and balance (Gillis et al. 2009; Gillis and Higham 2016; Jagnandan and Higham 2018) as well as social interaction (Fox et al. 1990; Martín and Salvador 1993). The energetical cost of tail regeneration can come to the expense of growth (Ballinger and Tinkle 1979; Niewiarowski et al. 1997; Lynn et al. 2013) or reproduction (Dial and Fitzpatrick 1981; Wilson and Booth 1998; Chapple et al. 2002), but the support is ambiguous (e.g., Fox and McCoy 2000; Goodman 2006; Webb 2006). In a study on *P. picta*, the cost of tail regeneration in growing juvenile males was evaluated (Starostová et al. 2017). Tail autotomy was induced in juvenile, approximately four months old males still in the phase of rapid growth and their growth was followed and compared to intact control group for more than five months. The prediction based on a simple direct differential energy allocation was that the growth rate and final SVL of the juveniles that suffered tail autotomy would be hindered compared to intact juveniles (Fig. 2). However, tail autotomy and its subsequent regeneration did not affect structural growth and resulted in a similar SVL. Furthermore, mass-corrected metabolic rate was not significantly affected by tail loss and allocation to regeneration. It seems that fast growing juveniles can compensate tail autotomy at least under unrestricted food conditions without a notable change in mass-specific metabolic rate. Future studies should test whether the same pattern would be observed also under food limitation.

Structural growth is still plastic with respect to temperature

The above discussed findings suggested that structural growth represented by change in SVL during the postembryonic ontogeny in *P. picta* is less phenotypically plastic than generally assumed under manipulation with energetically demanding processes. But, is it plastic with respect to other factors than energy allocation? Considering ectotherms, i.e. animals that rely on external sources for body heat, a clearly essential factor influencing structural growth is environmental temperature. The Temperature size rule states that ectotherms develop faster but mature at smaller body sizes at higher temperatures whereas ectotherms maintained at low temperatures grow more slowly, but attain a larger final body size (Atkinson 1994; Zuo et al. 2012). Temperature has indeed a strong influence on growth in *P. picta* (Starostová et al. 2010). Animals incubated and reared until cessation of growth under three different environmental temperatures did not follow the Temperature size rule, but body size in terms of SVL was higher especially in males at the intermediate temperature (Starostová et al. 2010). The effect of temperature at least in *P. picta* does not operate via changes in number of trunk vertebrae (Kratochvíl et al. 2018), but partially via influence on cell size at least in some tissues (Czarnoleski et al. 2017). The influence of temperature on growth and final body size in animals was considered as an energy allocation problem (Zuo et al. 2012); however, this hypothesis deserves further attention and seems less likely taking into account that growth responses of *P. picta* to manipulation with energetically demanding processes are rather limited.

Why is growth highly canalized when facing energy limitations?

Being indoctrinated by the simple energy allocation perspective before we conducted these growth studies introducing both decreased and increased demands of other energetically demanding processes, we did not expect that growth would be so canalized with respect to manipulations affecting energy and were ever surprised by the results. The growth experiments challenged the idea of simple direct allocation of growth versus other energetically demanding processes (summarized in Fig. 2). Growth was affected neither by reduced energy uptake through food restriction (Kubička and Kratochvíl 2009) nor by hindering the energy balance during growth through tail autotomy and regeneration (Starostová et al. 2017). By extension, simple trade-offs in energy allocation between growth and reproduction was not supported by studies in *P. picta* (Kubička and Kratochvíl 2009; Starostová et al. 2013; Kubička et al. 2017).

The inherent insignificant role of simple energy allocation in the case of growth in *P. picta* is even more evident when comparing the role of energy in another life history trait, reproduction. Reproduction was found to be more sensitive to allocable energy in the study on food restriction (Kubička and Kratochvíl 2009). This difference in variation between growth and reproduction is also highlighted through a substantial difference in environmental plasticity. Reproduction in *P. picta* is also heavily influenced by temperature (Starostová et al. 2012). Overall, females at higher temperatures produced smaller eggs which is consistent with the pattern found in ectotherms (e.g., Blanckenhorn 2000; Oufiero et al. 2007), and rate of reproduction (amount of energy allocated to reproduction per unit of time) was smaller for females at the lowest of the tested

temperatures (Starostová et al. 2012). The plasticity of growth regarding temperature is clearly less significant than in reproduction indicating the more plastic nature of reproduction compared to growth.

Why do the predictions from differential energy allocations fail so much (Fig. 2)? The structural growth rate and final SVL has some capability for phenotypic plasticity as exemplified by manipulations with rearing temperature described above. Also, structural growth trajectories can be shaped in *P. picta* by hormonal manipulations bringing further evidence that growth trajectories are not totally fixed. So, why is structural growth to a high extent canalized with respect to changes in an energy budget, even more so when compared to reproductive traits? One possible explanation is that selection in geckos and possibly other lizards is preferring the allocation rules prioritizing structural growth to other traits such as reproduction, fat storage and regeneration. It is possible that the allocation to particular traits is not totally mutually plastic as commonly assumed under the intuitive logic of "higher allocation to reproduction means less allocation to growth", but that the allocation of energy followed a hierarchical rule with the priority given to structural growth. This hypothesis was suggested by Kubička and Kratochvíl (2009) interpreting the results of their food limitation experiment. They concluded that energy is allocated to reproduction only after demands of structural growth are fulfilled, and to fat storage only when the maximal possible allocation to reproduction was achieved.

The degree of phenotypic plasticity, more specifically canalization (e.g., Walzer and Schausberger 2014), should reflect selective pressures. Canalization—no matter whether against environmental or genetic perturbations—should evolve when there is a stabilizing selection on a trait value (Stearns and Kawecki 1994). At the current stage of knowledge, we can only speculate why structural growth should be prioritized in *P. picta* over other traits. Body size is a crucial fitness-related trait and as such it should be optimized. Body size is connected with food intake—in a gape limited predators like geckos body size determines maximal and minimal prey size (Daza et al. 2009), antipredator strategies (Roth and Johnson 2004), dealing with competitors (Pafilis et al. 2009) and optimal reproductive performance, e.g. due to positive egg size-body size relationship demonstrated in geckos (Kratochvíl and Frynta 2006) and other reptiles (e.g. Escalona et al. 2018). Reaching an optimal size as fast as possible for a given ecological niche and keeping it as long as possible throughout life span might be important. Of course, reptiles including *P. picta* start reproduction well before reaching the final/close to asymptotic structural body size. This hypothesis trying to explain the canalization of allocation to structural growth expects that the performance, including reproductive performance, should be suboptimal before the period of the cessation of growth. We welcome tests of this hypothesis in the future.

The optimal size could be sex-specific, e.g. due to sexual selection or other sex-specific roles (Darwin 1871; Cox et al. 2003; Fairbairn et al. 2007). Males of *P. picta* are highly combative (Golinski et al. 2014; Schořálková et al. 2018) and intrasexual selection can thus be a selective force responsible for male-larger SSD in this species. At the proximate level, it seems that males in this species are not larger because females do not have enough energy for growth as they allocate more to reproduction, but because each sex has their own optimal trajectory of ontogenetic structural growth. Gonadal hormones, particularly ovarian hormones seem to be the signal to cells in the body which of these trajectories should be followed during the ontogeny (Starostová et al. 2013; Kubička et al. 2017).

Limits of our approach

We acknowledge the limits for generalizations brought by focusing on one species. However, in this review we chose to focus on a well-studied—and hopefully not too special and exceptional—gecko since we considered it would help to tell a complex story as best as possible and at the same time to control several potentially confounding issues. While we have attempted to add arguments from other species, we found such parallels between species to be harder to establish, as we doubt that there is another species where similar manipulative long-term growth experiments were performed under so many treatments under so similar conditions, and partial studies (e.g. only a test of the effect of tail autotomy on growth in one species, but of the removal of the allocation to female reproduction in the other) might be confounded by differences in life-history decisions and other aspects of species biology. Although we tried to analyze as many parameters as possible in our growth experiments, we are aware that these studies are not complete. For example, activity has been found to influence distribution of energy and the expression of life history traits such as reproduction or immunity (Lailvaux and Husak 2014; Husak and Lailvaux 2017; Husak et al. 2017), but it was mostly not considered in our experiments. We only found that castrated and control males did not differ in the activity in the open field test performed in the neutral arena (Kubička et al. 2015), but we lack data for other experiments. As all treatment groups in each of our former experiments were held in the same environment (the same thermal environment, social isolation, the same size and equipment of cages) and hence likely possessed similar activity patterns, it is not very likely that the difference in the activity pattern would explain the notable lack in the response in the structural growth. In the case of different activity pattern among treatment group, groups would have to precisely counterbalance the allocation to growth with differences in activity, which seems unlikely. However, the energetical cost of activity and its influence on other life history traits such as growth and reproduction should be more explored in the future. As natural conditions certainly are more demanding for energy intake than conditions in the laboratory, it will be important to do more energetically focused research on growth also in the field, which will be especially important to elucidate the evolutionary context of growth canalization observed by us in the laboratory experiments.

Conclusions and future perspectives

Overall, we documented that although structural growth has some potential to be plastic and it is sexuall dimorphic in *P. picta*, it is at the same time to a large degree canalized with respect to an energy budget. This pattern is consistent with the idea that structural growth is carefully regulated and that allocation to it is prioritized to other life history traits, most importantly to reproduction. Importantly, this observation challenges the general growth models based on dynamic energy budgets as they assume that the limitation of an energy budget or higher allocation to a single trait—mostly to reproduction—affects all other life history traits (von Bertalanffy 1957; West et al. 2001) and neglect that there might exist a strict hierarchical rules shaped by selection for priority allocation of metabolized energy to structural growth (or other traits). The simplified energetical perspective became also influential in macroecology being claimed responsible for major ecological rules in the so called “metabolic theory of ecology” aiming to quantify the processes of acquisition

and use of resources, to explain different biological patterns of life history traits such as growth (Brown et al. 2004; Martin et al. 2019). However, the recent large-scale comparison across eukaryotes suggests that metabolism and thus energy income is not necessarily at the core control of biomass increase (Hatton et al. 2019). Hatton et al. (2019) propose that instead of a fundamental metabolic control and limitation of growth, metabolism adjusts to growth (understood there as a maximum population growth rate, i.e. intrinsic growth rate, multiplied by individual adult body mass) within major groups, which agrees with here advocated perspective that a simple and intuitive energy allocation rule is not operating at the individual level and that growth is carefully regulated endogenously. We argue that this endogenous control reflects past selective pressures shaping the structure of allocation rules. Future growth models should incorporate these findings and be based on carefully tested, not only intuitively appealing assumptions on energy allocations.

Next to growth, analogous situation challenging classical views based on simple trade-offs in energy allocation recently emerged in another key life-history trait, ageing (Lind et al. 2019). The classical disposable soma theory of ageing states that the limited amount of energy can be either used for maintenance and repair or growth and reproduction resulting in trade-offs, with energy limitations for repair leading to the accumulation of unrepaired cellular damage with age (Kirkwood et al. 1979; Lind et al. 2019; Maklakov and Chapman 2019). However, recent evidence suggests that simple energy allocation between life history traits is not at the heart of variability in ageing. Similar to structural growth, delayed ageing is highly endogenously controlled, in the case of aging by a conserved insulin/IGF-1 nutrient-sensing signaling pathway (Lind et al. 2019). In the case of ontogenetic growth, a prominent and well conserved pathway responsible for growth regulation and its variability and plasticity can be the insulin and insulin-like signalling network (Shingleton 2011; Stearns 2011). The ovarian hormones can be an important sex-specific modifier of the structural growth pathways. Evidence brought up for structural growth and aging demonstrates that trade-offs can be mediated at the proximate level by switches in signalling pathways independently from direct simple energy allocations (Flatt et al. 2011; Stearns 2011). As evolutionary ecologists, we should stop thinking in the framework of simple direct differential energy allocation unless based on solid empirical evidence and we should focus on the question how selective forces shape complex, likely hierarchical structure of allocation rules and how it is reflected in proximate mechanisms controlling life-history decisions.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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II

Development of male-larger sexual size dimorphism in a lizard: IGF1 peak long after sexual maturity overlaps with pronounced growth in males.

Meter Brandon, Kratochvíl Lukáš, Kubička Lukáš and Starostová Zuzan

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EDITED BY
Turk Rhen,
University of North Dakota,
United States

REVIEWED BY
Richard M. K. Yu,
The University of Newcastle, Australia
Gabriele Andreatta,
Max F. Perutz Laboratories GmbH,
Austria

*CORRESPONDENCE
Lukáš Kratochvíl,
lukas.kratochvil@natur.cuni.cz

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Development of male-larger sexual size dimorphism in a lizard: *IGF1* peak long after sexual maturity overlaps with pronounced growth in males

Brandon Meter[1], Lukáš Kratochvíl[2]*, Lukáš Kubička[2] and Zuzana Starostová[1]

[1]Department of Zoology, Faculty of Science, Charles University in Prague, Prague, Czechia, [2]Department of Ecology, Faculty of Science, Charles University in Prague, Prague, Czechia

Squamate reptiles have been considered to be indeterminate growers for a long time. However, recent studies demonstrate that bone prolongation is stopped in many lizards by the closure of bone growth plates. This shift in the paradigm of lizard growth has important consequences for questions concerning the proximate causes of sexual size dimorphism. The traditional model of highly plastic and indeterminate growth would correspond more to a long-term action of a sex-specific growth regulator. On the other hand, determinate growth would be more consistent with a regulator acting in a sex-specific manner on the activity of bone growth plates operating during the phase when a dimorphism in size develops. We followed the growth of males and females of the male-larger Madagascar ground gecko (*Paroedura picta*) and monitored the activity of bone growth plates, gonad size, levels of steroids, expression of their receptors (*AR, ESR1*), and expression of genes from the insulin-like growth factor network (*IGF1*, *IGF2*, *IGF1R,* and *IGF2R*) in livers. Specifically, we measured gene expression before the onset of dimorphic growth, at the time when males have more active bone growth plates and sexual size dimorphism was clearly visible, and after a period of pronounced growth in both sexes. We found a significant spike in the expression of *IGF1* in males around the time when dimorphism develops. This overexpression in males comes long after an increase in circulating testosterone levels and sexual maturation in males, and it might be suppressed by ovarian hormones in females. The results suggest that sexual size dimorphism in male-larger lizards can be caused by a positive effect of high levels of *IGF1* on bone growth. The peak in *IGF1* resembles the situation during the pubertal growth spurt in humans, but in lizards, it seems to be sex-specific and disconnected from sexual maturation.

KEYWORDS

body size, bone, growth, hormones, IGF1, reptiles, sexual size dimorphism, testosterone

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Introduction

Animals differ in growth patterns. Some animals stop growing before sexual maturation, e.g. most insects which are not able to change their structural size as imagoes due to rigid exoskeleton, while others used to be assigned as indeterminate growers. Among vertebrates, endotherms (mammals and birds) are traditionally viewed as determinate growers, while non-avian reptiles, amphibians, and fish as indeterminate growers (Kozłowski, 1996; Charnov et al., 2001; Geiger et al., 2014; Hariharan et al., 2015). However, this dichotomy is shaking now. Many reptiles, particularly lizards, do in fact stop growing and attain a final body size. However, they typically do so long after sexual maturation (Frýdlová et al., 2019; Frýdlová et al., 2020). The reasons for this are still unclear, in a previous review we speculated that due to a much lower metabolic rate, growth in ectotherms is slow and that they attain final body size much later, which forces them to start reproduction much before reaching the final, possibly optimal body size. Endotherms with more rapid growth can postpone reproduction after they attain an optimal body size (Meter et al., 2020). Alternatively, it could be explained by the energetically demanding nature of endothermy and a trade-off between reproduction, growth, and body temperature maintenance (Werner et al., 2018). The new perspective on lizards as determinate growers has important consequences for the search for the proximate mechanisms of sexual size dimorphism (SSD), i.e., the size difference between sexes of a species, and its measurement. Under determinate growth, SSD should be best expressed as a size difference between fully grown males and females, and it should reflect the processes largely influencing the cessation of growth in a sex-specific manner.

SSD is widespread in reptiles and can differ in direction even among closely related species (Cox et al., 2007; Starostová et al., 2010). Finding a proximate mechanism of SSD development in vertebrates is a complex task. Several hypotheses on the proximate cause of SSD have been proposed in squamate reptiles. In most squamates as well as in many other vertebrates, there is little if any SSD at hatching, and the early growth is monomorphic (Badyaev, 2002). SSD develops only later in ontogeny through dissociation of male and female growth trajectories. But which sex-specific growth modifiers do in fact drive this dissociation? Sexes in some vertebrates do not differ in genomes, e.g. in sequential hermaphrodites and species with environmental sex determination (Janzen and Phillips, 2006; Johnson Pokorná and Kratochvíl, 2016; Todd et al., 2016; Katona et al., 2021), but still often substantially differ in body size. In them, sex-specific growth modifiers cannot be attributed to genetic differences between sexes. But also, in species with genotypic sex determination, an overwhelming majority of tetrapods (Kostmann et al., 2021), sex-specific modification of growth is mostly triggered by differential expression during development, not directly by sex-linked genes. It can be exemplified by how easily growth can be altered by gonad manipulations, e.g. ovariectomy leads to male-typical growth in several squamate species (Cox, 2006; Starostová et al., 2013; Cox et al., 2016; Kubička et al., 2017; Cox, 2019).

The cost of reproduction hypothesis postulates that SSD is plastic with respect to energy allocation. SSD should reflect energetic costs of reproduction and the sex allocating less to reproduction should grow larger (Cox, 2006; Cox et al., 2009; Hayward and Gillooly, 2011; Cox et al., 2016). This hypothesis was supported by manipulative experiments in male-larger species, where ovariectomized females were masculinized in growth (Cox, 2006; Cox and Calsbeek, 2010; Starostová et al., 2013; Cox et al., 2016). Nevertheless, unilateral ovariectomy, dramatically decreasing the allocation to reproduction, but at the same time preserving the cycling of ovarian hormones, did not lead to enhanced structural growth in male-larger gecko *Paroedura picta*. It suggests that ovariectomy does not remove only the energetic costs of reproduction, but also endocrinologically active organs producing hormones affecting growth (Kubička et al., 2017).

The ontogeny of SSD was also suggested to be controlled by circulating levels of male gonadal androgens, which should trigger enhanced growth in male-larger species and inhibit growth in female-larger species (Cox et al., 2005; Cox et al., 2009; Duncan et al., 2020). However, most of the support came from relatively short-term studies done under the view that lizards are indeterminate growers, and there was thus no control in these studies on whether the animals already stopped growth or not. The long-term growth experiments comparing growth in castrated and control males do not support the role of male gonadal androgen neither in male-larger species, namely the gecko *P. picta* (Starostová et al., 2013; Kubička et al., 2015) and the chameleon *Chamaeleo calyptratus* (Bauerová et al., 2020), nor in the female-larger gecko *Aeluroscalabotes felinus* (Kubička et al., 2013). It was also noted that the increase of testosterone levels in males does not coincide with the period of sexually dimorphic growth in a rattlesnake and the gecko *P. picta* (Taylor and DeNardo, 2005; Kubička et al., 2022).

An alternative proximate mechanism of SSD development is the control by ovarian hormones (Schmidt et al., 2000; Starostová et al., 2013). This hypothesis was supported by a coincidence in the start of female reproductive cycles with the feminization of growth (Starostová et al., 2013); by a pattern of female-typical growth (Kubička et al., 2022); and by the observation of defeminization of growth after ovariectomy and by the strong negative effect of exogenous estradiol on growth in male-larger gecko *P. picta* (Kubička et al., 2017).

The presented pattern of growth in squamates suggests that the sex-specific growth modifier should not be expressed at the early growth when growth is not sexually dimorphic. It should be detectable at the time of dimorphic growth before the cessation of bone prolongation. It should keep temporarily the higher activity

of bone growth plate in the larger sex, and switch again to a non-dimorphic level (or at least it should lose its positive effect on growth in the larger sex) after senescence of growth plates.

To uncover the candidate sex-specific growth modifier, we monitored the growth, the activity of growth plates, size of reproductive organs, and levels of testosterone and estradiol in males and females of *P. picta* from hatching till the age when growth is already stopped, or at least when it is negligible (Kubička et al., 2022). Based on their ontogenetic stage, we selected individual age groups from this experiment, and we measured in them the expression of candidate genes, potential sex-specific regulators in livers, the major metabolic organ. Namely, we measured the expression of receptors of steroid hormones and members of the insulin/insulin-like growth signaling pathway: estrogen receptor alpha (*ESR1*), androgen receptor (*AR*) as well as insulin-like growth factors one and two (*IGF1*/*IGF2*) and their receptors (*IGF1R*/*IGF2R*).

The insulin and insulin-like signaling molecular networks are composed of many receptors but mainly the insulin-like growth factor network has a strong relation to growth. IGF1 and its counterpart IGF2 have roles in cell growth and proliferation as they are mediators of the growth hormone action (Schwartz and Bronikowski, 2016). The role of IGF1 and IGF2 in the growth of reptiles has been well documented (Reding et al., 2016; Beatty and Schwartz, 2020; Duncan et al., 2020; Marks et al., 2021). IGF1 has been assumed to be a postnatal growth factor and IGF2 to be a prenatal growth factor, but this assumption has been challenged in reptiles (Beatty and Schwartz, 2020). IGF1 and IGF2 compete to bind to insulin receptors and IGF1R which have a role in cell proliferation and growth (Denley et al., 2005; Schwartz and Bronikowski, 2016). IGF1 plasma levels have also been associated with reproduction (Guillette et al., 1996; Sparkman et al., 2010), and its gene expression is affected by nutrition in reptiles (Duncan et al., 2015; Marks et al., 2021). The role of IGF1 in growth in humans and mice is best transcribed in its role in postnatal bone elongation. Hepatic levels of IGF1 have a role in longitudinal bone growth as well as local IGF1 levels in growth plates while affecting chondrocyte differentiation and maturation and therefore their closing (Yakar et al., 2018; Racine and Serrat, 2020). Plasma levels of IGF1 were found to be major determinants of sexually biased skeletal dimorphism in mice and tied with puberty (Callewaert et al., 2010). Radial bone growth has also been found to be affected by IGF1 levels (Yakar et al., 2018).

Previous papers on reptiles using absolute quantification gene expression indicated that IGF2 has a role in postnatal growth in reptiles with levels being higher than in IGF1 (Reding et al., 2016; Beatty and Schwartz, 2020). This is similar to the plasma levels trends in other vertebrates (de Beer et al., 2008; Fowke et al., 2010) but not rodents (Brown et al., 1986). IGF2 binds to IGF2R which acts negatively toward IGF2 since it degrades IGF2 to maintain non-excessive levels and therefore negatively regulates the insulin/insulin-like signaling network (Ghosh et al., 2003; Denley et al., 2005). This function is still unclear in reptiles (Schwartz and Bronikowski, 2016). However, we do know that this binding is potentially significant in lizards (Sivaramakrishna et al., 2009). *IGF2* has also been found to have a highly conserved sequence in squamates (Schwartz and Bronikowski, 2016) while in contrast, *IGF1* is quite variable amongst reptiles (Schwartz and Bronikowski, 2016; Yamagishi et al., 2016).

The androgen pathway and namely the hormone testosterone has been linked to sexual dimorphism in the past (Zauner et al., 2003; Cox et al., 2005, 2007). Testosterone and dihydrotestosterone have been found to trigger their biological activities through binding to the AR (Heinlein and Chang, 2002). Estrogens bind to two different receptors, the ESR1, and estrogen receptor beta (ESR2) which are expressed differently in different tissues and can have differential roles (Sanger et al., 2014; Bondesson et al., 2015).

Our study assesses the gene expression of target genes by quantitative reverse transcription PCR (RT-qPCR) to determine relative gene expression between selected stages of growth to uncover genes responsible for sexually dimorphic growth in a male-larger species. We expect that a member of the crucial pathways modifying growth in a sex-specific manner should be differentially expressed during the time when sexual dimorphism emerges in the ontogeny.

Materials and methods

Model species

The gecko *P. picta* has become a model species for studies of reptile growth and ontogeny (reviewed in Meter et al., 2020). The species was used for this experiment specifically due to its male-larger SSD and availability of genetic information with both whole-genome and transcriptome data being available (Hara et al., 2015; Hara et al., 2018), which enabled the design of primers for candidate genes. Our working hypothesis of the male-larger SSD in our model species *P. picta* is the combative and territorial nature of males driving intersexual selection. Such male-typical territorial behavior was confirmed in a series of experiments (e.g., Golinski et al., 2014; Schořálková et al., 2018).

Experimental animals

For the present study, we used individuals of *P. picta* from the specifically selected age groups that were reared in the experiment conducted by Kubička et al. (2022). Briefly, Kubička et al. (2022) followed growth in 256 individuals of *P. picta*, that were the progeny of the wild-caught animals or of the first generation in captivity. The experimental individuals

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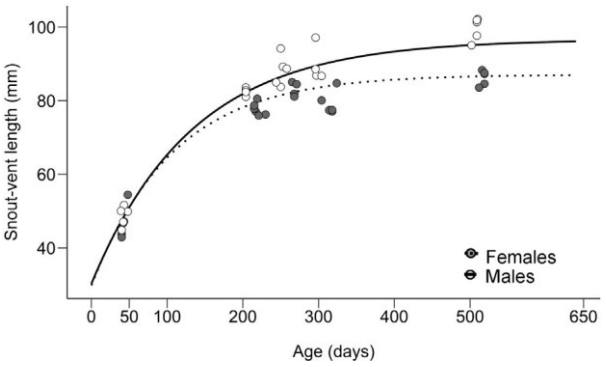

FIGURE 1
Sex-specific growth curves estimated from 256 individuals (data from Kubička et al., 2022.) and snout-vent lengths in males (solid line, white circles) and females (dotted line, grey circles) of *Paroedura picta* used for gene expression measurements.

were incubated and held individually after hatching at the constant temperature of 30°C (±0.2) and 12:12 h light–dark cycle. Animals were kept in a standardized box with a sandy substrate, water dish, and shelter and fed *ad libitum* twice a week with crickets powdered with vitamins and minerals. The growth of experimental animals was followed from hatching to up to the age of *c.* 22 months and divided into 14 age groups with approximately 6-week intervals. Animals were regularly weighed, and their snout-vent length (SVL) was measured. Maintaining typical physiological processes such as hormonal cycles in females was ensured by allowing animals to mate and reproduce. Once a female reached 4 months of age or a body mass of 7 g, it was mated with an unrelated experimental male of the same age. At the time the animals reached the age required for a particular cohort (ranging between 0 and 620 days), they were sacrificed by rapid decapitation, and samples were collected for future analyses. To maintain similar food availability across all age cohorts animals were not starved prior to the decapitation. Based on growth data for all individuals, the onset of sexually dimorphic growth, revealed by the breakpoint analysis, occurred at *c.* 180 days of age (Kubička et al., 2022). For our analyses, we selected animals at the beginning of the growth curve before the onset of dimorphic growth (≈42 days), at the time when males have more active bone growth plates and SSD was clearly visible (≈255 days; with two age groups added for target gene *IGF1* ≈ 211 days; ≈ 307 days) and the end of the growth curve, i.e. after the period of pronounced growth in both sexes (≈512 days) (see Figure 1). Final body mass and SVL were taken immediately before euthanasia. The sex of each individual was confirmed by gonadal inspection and the mass of testicles and ovaries was recorded (except for the ovaries of 42 days old females as they were too small). Plasma for all 256 animals was used for measurements of levels of estradiol and testosterone, as described in Kubička et al. (2022) and showed in Supplementary Figures S1, S2, which gave us information about hormonal profiles across the ontogeny. Due to the necessity of pooling some plasma samples in small individuals, we did not use hormone levels directly in our statistical analyses. All raw data used in Kubička et al. (2022) are available in the Mendeley database (doi:10.17632/fxcdd6j4sh.1).

Sample collection, RNA isolation and cDNA synthesis

Liver samples were collected from individuals of both sexes and different age groups. The liver was chosen as the target tissue because estrogen and testosterone metabolize in the organ while gonads were too small. Furthermore, the liver was also chosen as it had a higher expression of *IGF1* and *IGF2* than other tissues in several reptiles (Reding et al., 2016; Duncan et al., 2020; Marks et al., 2021), and differences in the expression of *IGF1R/IGF2R* in the liver were found between fast versus slow-growing sexually dimorphic snake (Reding et al., 2016). After dissection, the liver samples were quickly cut into small pieces and stored in RNA*later* (Sigma-Aldrich Inc., Missouri, United States) at −20°C until used for RNA extraction. RNA was extracted according to the manufacturer's protocol using the HighPure RNA tissue kit (Roche, Germany) and treated with DNase with a standardized amount of about 10 mg of liver tissue for each extraction. Each RNA sample had its concentration measured using the Quantus fluorometer (Promega Corporation, Madison, United States), and the Agilent 2,100 Bioanalyzer and Agilent RNA 6000 Nano Kit (Agilent Technologies, California, United States), were used to assess RNA integrity. Usually, samples with RNA integrity number (RIN) under 3 are considered heavily degraded, and samples with RIN between 3 and 5 are partially degraded (Farrell 2017). Our samples had RIN ranging from 4.6 to 9.8 and only a single sample had a RIN below 5 (4.6). The purity of RNA measured as *A*260/*A*280 ratios was determined with the NanoDrop One (Thermo Fisher Scientific Inc., Massachusetts, United States) and only samples with acceptable ratios (between 1.9 and 2.15) were kept in this experiment, as ratios around 2 are considered pure RNA (Farrell, 2017). Once extracted RNA samples were divided into aliquots and diluted to 50 ng/μl for the cDNA synthesis. All RNA samples were stored at −80°C. The RNA samples were reverse transcribed using the TATAA GrandScript cDNA synthesis kit (TATAA Biocenter, Sweden) with a standard amount of 500 ng of total RNA in each sample.

Quantitative reverse transcription PCR

Primer pairs for six target genes and three reference genes were designed (Table 1) using the BioEdit software (Hall, 1999)

TABLE 1 Primer pairs of target and reference genes.

Gene	Forward 59-39	Reverse 59-39	Amplicon size (bp)	Efficiency (%)
Target				
IGF1	*GTGGAGCTGAGCTGGTTGAT*	*ACAGCTCTGGAAGCAGCATT*	140	95
IGF1R	*GTGCTGGACTTCCAACCACT*	*GGTGGCAGCACTCATTTTG*	86	88.4
IGF2	*TGTGGTTGATTCTGCCTCTG*	*TTGAGCCGGCCTCTGTTT*	138	85.8
IGF2R	*GCGACCTCGTTTGGTAACAT*	*GGGAAGACACAAAGCTCATCC*	131	94.2
ESR1	*TGCTGACAGAGAGCTGGTACA*	*TCTAGCCAAGCACATTCCAG*	108	94.9
AR	*TGGAAGCTGCAAGGTCTTTT*	*GCATCTTCAGATTGCCCAGT*	190	90.5
Reference				
GAPDH	*AGCTGAACGGCAAACTGA*	*GTCATACTTGGCTGGCTTGG*	101	84.4
18S	*GAGGTGAAATTCTTGGACCGG*	*CGAACCTCCGACTTTCGTTCT*	92	93.4
YWHAZ	*TGATGTGCTGTCTCTTTTGG*	*TTGTCATCACCAGCAGCAA*	129	81.6
HRPT1	*TGACAAGTCCATTCCCATGA*	*TTCCCAGTTAGGGTCGAGAG*	117	91

All primers, except the primer pair for 18S gene (Plot et al., 2012; Rollings et al., 2017) were designed directly for *P. picta*.

and published transcriptome of our target species (Hara et al., 2015; available at https://transcriptome.cdb.riken.jp/reptiliomix). In addition to the *P. picta* transcriptome, mRNA sequences for each gene of interest of different vertebrate species from the GenBank database were used in primer design. Reference gene *18S* primers were the ones previously used in reptiles (Plot et al., 2012; Rollings et al., 2017). Products of each primer pair were sequenced and aligned with other mRNA sequences to verify their authenticity. The efficiency of each primer pair (Table 1) was assessed by creating a standard curve and using the software of the Lightcycler 480 PCR machine (Roche, Germany) for calculation. A pooled cDNA of samples from the different age and sex groups was used to create standard curves in dilution series 100,000; 20,000; 4,000; 800; and 160 copies to assess primer efficiency. The thermal cycle used for all the assays was as follows: 95°C for 30 s of activation, then 40 cycles of 5 s at 95°C followed by 58°C for 15 s and 72°C for 10 s of amplification cycles. Finally, a melting curve of 60−95°C was done after the final cycle to assess for possible secondary products.

For the gene expression measurement, Cq values from 5 samples of each age/sex group were measured in triplicates in each assay (or primer pair). For each assay, triplicates of no-template controls (wells with nuclease-free water instead of cDNA—NTC) were used to assess for contaminations in reagents. In each well was used 5 µl of the TATAA® SYBR® green master mix (TATAA Biocenter, Sweden), 2.6 µl of nuclease-free water, 0.4 µl of forward and reverse primer (10 µM) respectively of the assay and finally 2 µl of the sample cDNA for a total of 10 µl. To ensure that comparisons could be drawn with all the reference and target assays, an interpolate calibrator (TATAA IPC kit, TATAA Biocenter, Sweden) was used on each plate in replicates on the same wells.

The relative gene expression of each individual for each target gene was calculated from the raw Cq values using the program R and the interface R studio (RStudio Team, 2019; Core Team, 2020). We first calculated mean Cq from technical replicates for each individual and target and reference gene. The R script used from our design followed a combination of the Vandesompele and Pfaffl method which considers the efficiency (Pfaffl, 2001) of all targets and the multiple reference genes (Vandesompele et al., 2002). The script also allowed for interplate calibration. This script for data analysis and the figures presented were done using R and several packages including the package "outliers" for statistical analysis, and the "ggplot2" package for a graphical representation (Komsta, 2011; Wickham, 2016). A number of packages were also used for data processing: "Tydiverse", "dplyr", "psych", "stringr", and "writexl" (Wickham, 2019; Wickham et al., 2019; Sievert, 2020; Ooms, 2021; Revelle, 2021; Wickham et al., 2021). Biological outliers of the groups for each target gene were signaled using statistical tests while triplicates were selected in accordance with their coefficient of variation. Triplicate outliers were verified from the raw Cq values while biological outliers were identified using the final relative expression.

For all reference genes and target genes, the efficiency of the primers (Table 1) was considered before normalization and calibration. Considering the efficiency of each gene allows for a more accurate calculation of the relative expression as described by Pfaffl (2001). For normalization the use of several reference genes opted for a more accurate normalization as described by Vandesompele et al. (2002), especially considering the species of interest is not commonly used for gene expression studies and finding a single adequate reference gene would be difficult. We selected four reference genes based on previous experimental work. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) has been used in mammals and reptiles (Coulson et al., 2008; Sanger et al., 2014) with limits under caloric restrictions (Gong et al., 2016; Marks et al., 2021). The 18S ribosomal RNA genes (*18S*) had a primer pair already validated previously in reptiles (Plot et al., 2012; Rollings et al., 2017). Gene tyrosine 3-monooxygenase/tryptophan 5-monooxygenase

activation protein zeta (*YWHAZ*) had previously been used as a reference gene in several RT-qPCR studies in different tissue types (Coulson et al., 2008; Al-Sabah et al., 2016). Finally, the gene hypoxanthine-guanine phosphoribosyl transferase (*HRPT1*) was also found to be a suitable candidate in different species for different tissues (Stassen et al., 2015; Wang et al., 2018). A geometric mean of values of all four reference genes was taken as a reference for the measurement of target gene expression (Vandesompele et al., 2002).

Subsequent to the normalization step a group was chosen as a calibrator (females of age group 42 days) to calculate each sample's relative expression to the mean of that group as described in the established methods (Livak and Schmittgen, 2001; Pfaffl, 2001). Finally, the resulting calculation was log-transformed (natural logarithm transformation) to give the final relative gene expression of each individual for each target gene as described in our Supplementary Material (Supplementary Table S1).

Statistical analysis

All statistical tests were done with the R program and the interface R studio (RStudio Team, 2019; Core Team, 2020). Biological outliers for each age and sex group were assessed using both Dixon and Grubbs' tests using the package "outliers' (Komsta, 2011). For triplicates coefficients of variation above 30% were deemed unacceptable and the replicates were either redone or two of the three Cq-values in a triplicate were kept if one was a clear outlier. Shapiro-Wilk test was used to check for normal distribution in the relative gene expression. Statistically significant differences between groups were assessed using ANOVA tests with sex/age groups as a factor followed by *post hoc* Tukey tests. The relationship between the natural logarithm (ln) transformed mean mass of testicles and ln-transformed *IGF1* expression or ln-transformed body mass was analyzed using Spearman's correlation coefficient. For all statistical tests, the significance threshold was set at $p = 0.05$.

Results

The growth curves of *P. picta* individuals show rapid, non-dimorphic growth at the beginning, then a more pronounced cessation of growth in females, followed by a period of the stagnation of body size in both sexes, with males being in the last two segments considerably larger in body size (Figure 1; Kubička et al., 2022).

Measurements of gene expression identified as biological outliers with both Grubbs' and Dixon tests were taken out of the gene expression analysis. Altogether, there were only few such excluded measurements: the expression value was excluded for the gene *IGF1* in a 211-day-old male (Grubbs' test: $p = 0.041$; Dixon test: $p = 0.036$), for *ESR1* in a 255-day-old female (Grubbs' test: $p = 0.045$; Dixon test: $p = 0.036$) and for *IGF1R* in two 255-day-old females on two separate tests (Grubbs' test: $p = 0.041$; Dixon test: $p = 0.031$/Grubbs' test: $p = 0.024$; Dixon test: $p = 0.025$). Finally, *AR* had two outliers, a 42-day-old female (Grubbs' test: $p = 0.016$; Dixon test: $p = 0.011$) and a 255-day-old male (Grubbs' test: $p < 0.0001$; Dixon test: $p = 0.004$). In addition, triplicates for the measurements of the expression of the gene *IGF1* were too variable in a 307-day-old male. In total, we excluded 7 out of 350 total measurements (2.0%). The statistics with the inclusion of the outliers would lead to the same interpretation of the results with the exception of *ESR1* which has no significant trend with the presence of its outlier.

Gene expression only differed significantly in genes *IGF1, ESR1,* and *AR* between males and females of age 42, 255, and 512 days. For other genes there was no significant difference using ANOVA tests: *IGF1R* ($F_{5,22} = 2.589$; $p = 0.055$), *IGF2* ($F_{5,24} = 0.748$; $p = 0.596$), *IGF2R* ($F_{5,24} = 0.448$; $p = 0.811$).

A significant difference was found in *IGF1* gene expression in 255 days old males compared to all-female groups and the youngest male group, as pointed by the ANOVA ($F_{5,24} = 6.106$; $p = 0.0008$) and *post hoc* Tukey tests (significant adjusted $p < 0.041$; non-significant adjusted $p > 0.106$; Figure 2) and prompted us to add two age groups (211 days old and 307 days old individuals) to further verify this trend. After the addition of these four sex/age groups, the overexpression found in 255 days old males was still maintained through ANOVA ($F_{9,38} = 5.221$; $p = 0.0001$) and *post hoc* Tukey tests (significant adjusted $p < 0.036$; non-significant adjusted $p > 0.078$; Figure 3).

The significant differences in expression for genes *ESR1* and *AR* are not as substantial as for *IGF1*. The ANOVA for *ESR1* test showed a significant difference between age/sex groups ($F_{5,23} = 2.882$; $p = 0.036$) while the *post hoc* Tukey tests only showed a trend of slightly higher expression in the oldest males compared to the youngest males (adjusted $p = 0.037$; Figure 2) while its expression was comparable among other comparisons (adjusted $p > 0.128$; Figure 2). Also, gene *AR* shows according to ANOVA a significant difference between groups ($F_{5,22} = 4.45$, $p = 0.005$) but according to the *post hoc* Tukey tests it is only slightly higher expression in 255 days old females compared to the youngest and oldest males (adjusted $p = 0.018$; adjusted $p = 0.026$; Figure 2) while the other *post hoc* comparisons were non-significant (adjusted $p > 0.083$; Figure 2).

Spearman's correlation coefficient analysis indicated no correlation between ln-transformed *IGF1* gene expression and ln-transformed mean mass of testicles ($r = 0.363$, $n = 23$, $p = 0.089$) while the correlation between ln-transformed body mass and ln-transformed mean testicle mass was strongly significant ($r = 0.887$, $n = 23$, $p << 0.001$). Testosterone plasma levels were higher in males than females already well before the separation of male and female growth curves, while estradiol plasma levels were comparable in both sexes before the breakpoint but higher after that in females as described in Kubička et al. (2022) and showed in Supplementary Figures S1, S2. All raw data used in Kubička et al. (2022) are available in the Mendeley database (doi:10.17632/fxcdd6j4sh.1).

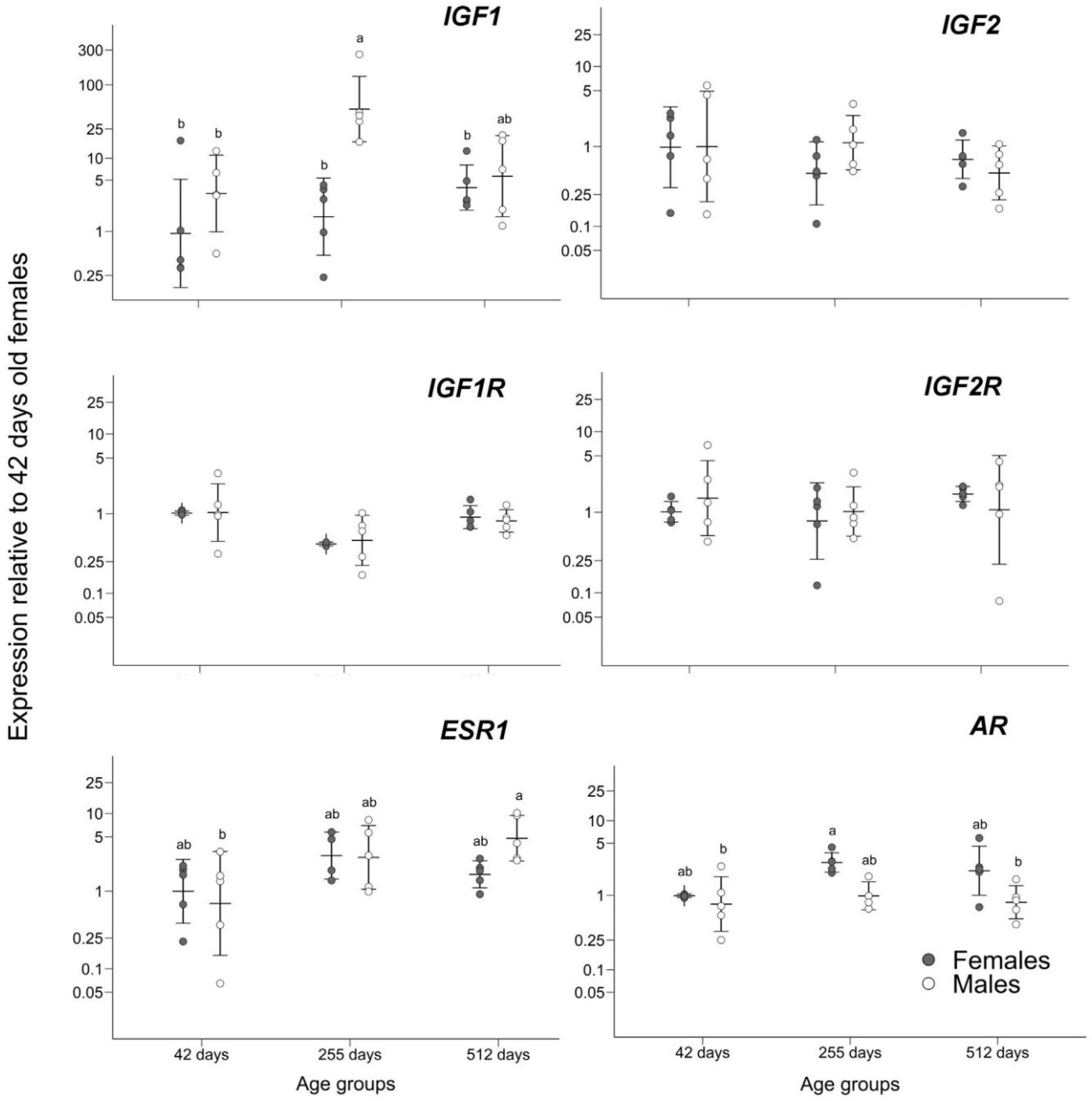

FIGURE 2
Gene expression relative to the group of 42 days old females. Means ± standard deviations are depicted. Statistically homogenous groups as identified by the *post hoc* Tukey test are depicted where there were significant differences among sex/age groups.

Discussion

We followed the expression of candidate genes that could contribute to sex-specific growth at the stage when SSD develops. Three (*AR, ESR1,* and *IGF1*) out of six monitored genes were found to significantly differ in the expression between the sexes and growth phases. In fact, only one of them (*IGF1*) gave a pattern consistent with its role in sexually dimorphic growth. The overexpression trend in males for gene *IGF1,* especially the noticeable spike in males around the time of departure of male and female growth trajectories, suggests that this gene has a role in SSD development (Figure 3).

As well documented in humans, IGF1 has an important role in bone elongation and growth plate closure (Yakar et al., 2018; Racine and Serrat, 2020). In *P. picta*, the male-specific spike of *IGF1* might delay the senescence of bone growth plates in males,

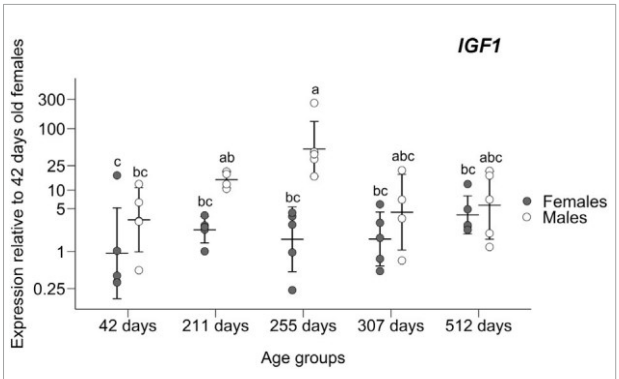

FIGURE 3
Expression of the insulin-like growth factor-1 gene in livers relative to the group of 42 days old females. Means ± standard deviations are depicted. Statistically homogenous groups identified by the *post hoc* Tukey test are depicted.

which is translated to male-larger SSD. But what drives the peak in *IGF1* expression in gecko males and why is it missing in females?

In humans, IGF1 levels have been considered to be associated with puberty and occur in both sexes during the so-called pubertal growth spurt (Cole et al., 2015; Juul and Skakkebæk, 2019). A small spike of IGF1 circulating plasma levels was also found in brown house snakes (*Lamprophis fuliginosus*) which could indicate its role in sexual maturity (Sparkman et al., 2010). In *P. picta* the breakpoint and overexpression of *IGF1* in males come a long time after sexual maturity and no correlation between *IGF1* levels and gonad size could be drawn. This further highlights the findings of Kubička et al. (2022) that growth changes in *P. picta* are not associated with sexual maturation and that castration does not alter structural growth in males (Starostová et al., 2013; Kubička et al., 2015). In agreement, circulating levels of testosterone are already sexually dimorphic at the age of 42 days in *P. picta* (Supplementary Figure S2). The size of testicles correlates with male body mass and no significant changes in these parameters coincide with the peak of *IGF1* in males and the onset of sexually dimorphic growth.

As castrated males and ovariectomized females of *P. picta* follow the growth trajectory of control intact males (Starostová et al., 2013; Kubička et al., 2015; Kubička et al., 2017), we expect that the peak of *IGF1* appears without a direct activation by male gonads. *IGF1* expression in livers is controlled by somatotropin (GH) produced by the pituitary gland (e.g., Schwartz and Bronikowski, 2016). GH production in humans increases during childhood and peaks during the pubertal growth spurt (Meinhardt and Ho, 2007). The situation in *P. picta* suggests that it is possible to disconnect the peak of GH/IGF1 from sexual maturation during evolution and this observation deserves further study. As stated previously, growth commonly continues in ectotherms after sexual maturation which is not the case for endotherms. This phenomenon is quite interesting and highlights the novelty of the spike in *IGF1* expression we found. This spike of *IGF1* happens after sexual maturation and gives an insight into how in ectotherms growth mechanisms are disconnected from sexual maturation and also how is the ontogeny of SSD detached from reproduction.

Previous growth experiments in *P. picta*, particularly the defeminization of growth by total but not by unilateral ovariectomy and the negative growth effect of exogenous estradiol (Kubička et al., 2017), suggest that the peak in *IGF1* in females of this species can be inhibited by ovarian hormones. This is also consistent with the observation that an increase and a start of cycling of estradiol levels in females roughly coincides with the closure of their bone growth plates, which happens at a smaller body size than in males of *P. picta* (Kubička et al., 2022). We found only a significant but small difference in expression to be higher in the oldest males than in the youngest males in *ESR1* and no differences with the other groups. Also, the pattern of expression found in *ESR1* is not sexually dimorphic as only young and old males differ significantly. We cannot answer why old males have somewhat elevated *ESR1* expression or young males have lower levels compared to each other. Both these groups do not differ in *ESR1* expression from the other experimental groups (Figure 2). However, it does not exclude circulating estrogens as candidates for the modifiers of GH/IGF1 expression as the relationship between hormone and receptor levels and their action is not straightforward (Moore and Evans, 1999). Even more importantly, increased female-typical estrogen levels can influence growth via their effect on the GH production/releasing pathway in the brain, which should be tested in the future. Moreover, the estrogen and its receptors can act directly in the growing bones (although this effect would not directly explain the lack of *IGF1* peak in females). In mice, skeletal differences between males and females corresponding with higher mass in males were correlated to IGF1 while both androgens and estrogens were found to have stimulatory effects in males and inhibitory in females, respectively (Callewaert et al., 2010). The role of estrogen receptors in sexual dimorphism has also previously been supported by the report of higher expression of *ESR2* in the cranial skeleton in *Anolis carolinensis*, suggesting its inhibiting role on bone growth (Sanger et al., 2014).

The pattern of *AR* expression through ontogeny in livers does not correspond with the testosterone level difference found between the sexes (Kubička et al., 2022; Supplementary Figure S2). We also know from Kubička et al. (2017) that testosterone increment has no effect on growth in ovariectomized *P. picta* females. The revealed pattern of slightly higher expression of *AR* in 255 days old females compared to the youngest and the oldest males does not seem to be of any biological impact if we also consider that either of these groups shared a similar expression with the remaining groups (Figure 2). Both expression patterns in genes *ESR1* and *AR* seem to be likely not biologically relevant or at the very least not relevant to SSD and could be explained by independent hormonal fluctuations that are not linked to SSD.

IGF2 did not show any significant differences in the expression between sexes and age cohorts in *P. picta*. The previously assumed divergence in the role of IGF2 asa pre-natal growth factor and IGF1

as a post-natal growth factor has been challenged in reptiles (Schwartz and Bronikowski, 2016; Beatty and Schwartz, 2020) resulting in a necessity to understand their exact role in the ontogeny of growth in vertebrates. If IGF2 has an important role in postnatal growth in *P. picta*, it would be constant throughout development (or at least for the tested age groups) and not associated with SSD.

Future studies should look at the proximate cause of SSD in different lizard groups and test whether the pattern started to be uncovered here for *P. picta* is more general. It would be fascinating to compare ontogeny of growth and *IGF1* expression in monomorphic and female-larger species of the genus *Paroedura* (Starostová et al., 2010). We predict that monomorphic/female-larger species will lack the male-specific peak of *IGF1* expression. A recent study focused on a short-term growth experiment found comparable hepatic expression of *IGF1* in adult males and females in the female-larger iguana *Sceloporus undulatus* (Duncan et al., 2020); however, more systematic studies comparing *IGF1* expression throughout the whole ontogeny from hatching till growth cessation are needed.

Conclusion

We compared the expression levels of candidate genes and circulating steroid hormones for sex-specific growth modifiers in liver samples collected at different phases of growth in a male-larger gecko species and interpreted the results in the light of previous growth experiments. We found that the ontogeny of sexual dimorphism in this species is likely not connected with male sexual maturation or with plasma circulating levels of male gonadal androgens and expression of *AR,* and *ESR1* in livers. On the other hand, the higher activity of bone growth plates before their closure in males (Kubička et al., 2022) seems to be connected with a significant increase in the expression of *IGF1* at that growth stage. The peak could be inhibited by ovarian hormones produced by active reproductive organs in females.

Genetic variation of the gene *IGF1* played for example a large role in the evolution of body size among dog breeds (Sutter et al., 2007), and variation in *IGF1* is an important mediator of life-history variation among vertebrates (Lodjak and Verhulst, 2020). We demonstrated that the time-specific and sex-specific expression of this gene may play an important role in the development of SSD in reptiles. We hope that future studies monitoring growth patterns and *IGF1* expression across ontogeny in both male-larger and female-larger species will help clear the role of IGF1 in SSD. Further investigation should also uncover if this sex-specific growth mechanism is shared or specific to certain lineages.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The study was conducted with the approval of the Ethical Committee of the Faculty of Science, Charles University, Prague, and the Ministry of Education, Youth and Sports of the Czech Republic (permit number 35484/2015-11).

Author contributions

ZS, LKr, and LKu designed the study. BM designed RT-qPCR assays, collected and analyzed the data. BM, LKr, and ZS drafted the first version of the manuscript. All authors discussed the results, contributed to the later stages of the manuscript and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.917460/full#supplementary-material

SUPPLEMENTAL FIGURE S1
Variation in estradiol plasma levels among groups. Note that estradiol levels generally increased and started to fluctuate in females after they started reproduction (they were mated around the age of four months). Age groups with measured gene expression are marked.

SUPPLEMENTAL FIGURE S2
Testosterone plasma levels across sex and age groups. The levels of the androgen became sexually dimorphic quite early in the ontogeny. They stayed low in females and vary a lot but are higher among males. Age groups with measured gene expression are marked.

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III

Complex ontogeny of sexual size dimorphism in a female-larger gecko with determinate growth.

Meter Brandon, Kratochvíl Lukáš, Starostová Zuzana, Kučera Tomáš and Kubička Lukáš

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Complex ontogeny of sexual size dimorphism in a female-larger gecko with determinate growth

Brandon Meter[1], Lukáš Kratochvíl[2*], Zuzana Starostová[1], Tomáš Kučera[3], Lukáš Kubička[2]

[1]Department of Zoology, Faculty of Science, Charles University, Viničná 7, Prague, 12844, Czech Republic

[2]Department of Ecology, Faculty of Science, Charles University, Viničná 7, Prague, 12844, Czech Republic

[3]Institute of Histology and Embryology, First Faculty of Medicine, Charles University, Albertov 4, Prague, 12801, Czech Republic

* Corresponding author: Lukáš Kratochvíl, email address: lukas.kratochvil@natur.cuni.cz

ORCID

Brandon Meter: 0000-0001-8845-7651

Lukáš Kratochvíl: 0000-0002-3515-729X

Zuzana Starostová: 0000-0002-5251-1617

Tomáš Kučera: 0000-0003-2134-4657

Lukáš Kubička: 0000-0002-4392-2553

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Research highlights

Ontogeny of sexual size dimorphism (SSD) is complex in a gecko *Paroedura vazimba* with determinate growth: females grow faster, but close vertebrae growth plates earlier than males. Development of SSD in male-larger and female-larger geckos is compared.

Abstract

Ectothermic vertebrates such as reptiles were assumed to be indeterminate growers. In recent years, evidence of the determinate nature of growth in lizards has been accumulating, which necessities to re-examine models on the ontogeny of sexual size dimorphism (SSD) in this group. We monitored postembryonic growth throughout 15 months in the female-larger gecko *Paroedura vazimba*. After hatching, females grew faster than males but also attained their final body size, i.e. closed growth of their vertebrae, earlier than males. Closure of bone growth in females is correlated with the onset of reproductive maturation. We compared the pattern with previously minutely studied, male-larger species *Paroedura picta*, where we earlier documented determinate growth as well. We suggest a model explaining evolutionary switches in the direction of SSD in lizards based on bipotential effects of ovarian hormones on growth. In this model, male growth is assumed to be a neutral state, while growth in females is feminized by ovarian hormones. Low levels of ovarian hormones can promote bone growth, but high levels connected with maturation of reproductive organs promote senescence of bone growth plates and thus cessation of bone growth. We suggest that future models on growth, life-history and evolution of body size in many lizards should acknowledge their determinate nature of growth.

Introduction

In contrast to endotherms, ectothermic vertebrates such as reptiles and fish were assumed for a long time to be indeterminate growers (Charnov, Turner and Winemiller, 2001; Vitt and Caldwell, 2014; Frýdlová et al., 2020). However, evidence for predominance of determinate growth, which was verified in several species by bone growth plate analysis (Frýdlová et al., 2019; Kubička, Tureček, Kučera and Kratochvíl, 2022), is being accumulated at least in lizards (Frýdlová et al., 2020). The determinate nature of growth in lizards has broad implications for the evolution of body size and growth regulation across, as well as within species, which necessitates a revision and potentially also a reformulation of evolutionary hypotheses on body size evolution in lizards. Under determinate growth, selection can operate not only on the regulation of growth rates through the ontogeny as in indeterminate growers, but also on final body size, i.e. on the timing of growth termination.

The most common and notable differences in body size within species are related to sexual size dimorphism (SSD). The discourse about SSD in lizards has been dominated for a long time by the role of energetical trade-off between growth and reproduction, the so-called "cost of reproduction hypotheses". This hypothesis expects that growth is plastic with respect to energy expenditure, e.g. that gamete production is costly (Hayward and Gillooly, 2011), and states that the sex allocating less to reproduction should be the larger sex (Cox, 2006; Cox and Calsbeek, 2010; Cox, McGlothin and Bonier, 2016). It is based on an assumption of indeterminate growth, i.e. that energy expenditure to reproduction influences growth rate, and does not assume that growth can be canalized to a final size in a sex-specific manner. Observations in agreement with the cost of reproduction hypothesis was presented for example in iguanian *Anolis sagrei* and *Sceloporus jarrovii* and gekkotan *Eublepharis macularius*, where ovariectomized females attained larger body length (Tousignant and

Crews, 1985; Cox, 2006; Cox and Calsbeek, 2010; Cox R.M., Wittman and Calsbeek, 2022). However, the positive effect of ovariectomy on growth in females could be explained by a removal of a source of ovarian hormones, instead of the removal of the cost of reproduction. This would be a similar effect of ovariectomy resulting in higher bone expansion and bone mass as described in rodents (Callewaert et al., 2010; Zhongbo, Subburaman and Shoshana, 2016). Little support for the development of SSD due to differences in energy allocation to reproduction was found in the male-larger gekkonid lizard *Paroedura picta*, where socially isolated non-reproducing females and reproducing females with removal of only a single ovary did not differ in growth from control, reproductively active females. Only total ovariectomy induced male-like growth in body length in this species (Kubička, Schořálková, Červenka and Kratochvíl, 2017).

Another hypothesis postulates that the ontogeny of SSD is controlled by circulating levels of male gonadal androgens. In this case, testosterone would trigger enhanced growth in male-larger species and inhibit growth in female-larger species (Cox, Skelly and John-Alder, 2005; Cox, Stenquist and Calsbeek, 2009; Duncan, Cohik and John-Alder, 2020). Most of the support came from the observed effects of exogenous androgens on growth rates in iguanian lizards. In such manipulations, it is always a danger that the doses are unnatural; monitoring direct plasma levels throughout growth can give a clearer picture of the role of these hormones. Moreover, in most of the tests, experimental animals were followed for only a relatively short time and thus only had an effect on growth rate, not on final body size. Final body size was not evaluated, and the social environment potentially affecting growth was in most cases not controlled (reviewed in Starostová, Kubička, Golinski and Kratochvíl, 2013). The hypothesis on the control of SSD by male gonadal androgens was not supported by longer-lasting manipulations in males kept in social isolation in the male-larger gecko *P. picta*, female-larger gecko *Aeluroscalabotes felinus* and male-larger iguanian *Chamaeleo*

calyptratus (Starostová et al., 2013; Kubička, Golinski, John-Alder and Kratochvíl, 2013; Kubička,Starostová and Kratochvíl 2015; Bauerová, Kratochvíl and Kubička 2020; see Starostová et al., 2013 for a review of older studies), where castrated and control males exhibited similar growth rates and attained comparable body length. Moreover, monitoring of androgen levels through the postembryonic development in the rattlesnake *Crotalus atrox* and in the gecko *P. picta* showed that their elevation in males does not correlate with the departure of male and female growth trajectories (Taylor and Denardo, 2005; Kubička et al., 2022).

Accumulating evidence suggests a role of ovarian hormones in the proximate control of SSD in at least some squamates. The rise of estradiol levels in the ontogeny showed a correlation with the deceleration of female growth in the male-larger gecko *P. picta* (Kubička et al., 2022). Furthermore, full ovariectomy induced male-like growth and final body length, and the application of exogenous estradiol retarded growth in females of this species (Starostová et al., 2013; Kubička et al., 2017). Moreover, the application of exogenous testosterone in females led to male-like growth in both male-larger (*P. picta*) and female-larger (*Aeluroscalabotes felinus*; *Sceloporus undulatus; Sceloporus virgatus*) lizards (Abell, 1998; Starostová et al., 2013; Kubička et al., 2013; Duncan, Cohick and John-Adler, 2020), which can be explained by the inhibition of normal ovarian hormone secretion by this manipulation (reviewed in Starostová et al., 2013). The role of ovarian hormones in the development of SSD is well supported in mammals, where they influence activity of growth plates mainly through direct effect of the insulin-like growth factor 1 (IGF1), a key hormone of the insulin-like growth network (Ohlsson et al., 2009; Zhongbo et al., 2016; Yakar et al., 2018). The insulin-like growth network impact on bone growth is largely mediated by hepatic IGF1 which is guided by growth hormone (GH) that targets mainly the liver (Ohlsson et al.2009; Schwartz and Bronikowski, 2016; Yakar et al., 2018), but the influence of estrogen

on growth plates can be independent of GH (Callewaert et al., 2010; Zhongbo et al., 2016; Yakar, Werner and Rosen, 2018). Estrogens were found to possibly trigger growth plate senescence also directly (Nilsson, Marino, de Luca, Phillip and Baron, 2005; Nilsson et al., 2014; Zhongbo et al., 2016).

IGF1 plays a role in cell growth and proliferation (Schwartz and Bronikowski, 2016), but also in longevity and reproduction (Sparkman, Byars and Bronikowski., 2010; Lodjak and Verhulst, 2020). In reptiles, the role of IGF1 in growth has been confirmed (Reding, Addis, Palacios, Schwartz and Bronikowski, 2016; Beatty and Schwartz, 2020), with evidence for an influence of nutrition (Duncan, Jetz, Cohick and John-Alder, 2015; Marks, Beatty, Schwartz, Sorlin and Lailvaux., 2021). The role of IGF1 in SSD has been supported by the comparison of its expression in young and adult individuals of the male-larger iguanian lizard *Anolis sagrei* and in adults of the monomorphic species *Anolis apletophallus*. Adult males of *A. sagrei* had higher levels of *IGF1* than females, and the male bias in adults and juveniles was stronger in *A. sagrei* than in adults of *A*. *apletophallus* (Cox C.L.et al. 2022). In male-larger gecko *P. picta*, *IGF1* expression was monitored throughout the postembryonic growth, and a notable spike of hepatic levels of *IGF1* was found in males around the departure of male and female growth trajectories (Meter, Kratochvíl, Kubička and Starostová et al., 2022). This evidence in *P. picta* suggests a role of *IGF1* in SSD through more intense bone elongation in males; however, fully grown adults of both sexes after growth cessation did not differ in the hepatic *IGF1* expression (Meter et al., 2022).

The genus *Paroedura* is an ideal candidate for studying growth and SSD, as *P. picta* has become a model species for growth studies in recent years (reviewed in Meter, Starostová, Kubička and Kratochvíl 2020) and the genus contain both male-larger and female-larger species (Starostová, Kubička and Kratochvíl 2010). A systematic study of growth plate activity through development in *P. picta* highlighted the dynamics of

determinate growth related to its male-biased SSD (Kubička et al., 2022). Sexes grow similarly after hatching and SSD develops later in the ontogeny (Starostová et al., 2010; Starostová et al., 2013; Kubička et al., 2017, 2022). The divergence in growth between males and females occurs during female onset of reproduction due to more abrupt closure of bone growth plates in females, although growth continues long after sexual maturity. However, this growth pattern cannot be universal, and it should differ in monomorphic and female-larger species of the genus. In this paper, we study the growth pattern of the female-larger species *Paroedura vazimba.* We performed the novel growth pattern analysis together with monitoring states of growth plates of vertebrae, activity of female gonads, sexual hormone levels and gene expression of hepatic *IGF1* through postembryonic ontogeny in *P. vazimba.* The data presented in this paper for female-larger species combined with the previous experiments with male-larger *P. picta* (Kubička et al., 2022; Meter et al., 2022) allows for a comparison of two closely related species with opposite SSD.

Material and methods

Experimental animals

The genus *Paroedura* is a group of geckos endemic to Madagascar and Comoros Islands. As best represented by *P. picta*, their frequent egg laying and somewhat rapid growth makes them an ideal object for growth experiments in lizards. Little known *P. vazimba*, living in northwest Madagascar, is a much smaller species than *P. picta* with maximum snout-vent length (SVL) 49 mm (Nussbaum and Raxworthy 2000). The whole genus *Paroedura* reflects Rensch's rule, i.e. small species are female-larger, while large species are male-larger (Starostová et al., 2010). A laboratory stock of *P. vazimba* was established from legal imports from Madagascar. Individually housed mated females were regularly checked for eggs, which were incubated at 28 °C (± 0.2). The hatched individuals were kept at the same constant

temperature and 12 h light:12 h dark cycle in 18 x 13 x 7 cm boxes containing dry sand substrate, a shelter and a water dish. The geckos were fed *ad libitum* with crickets (*Gryllus assimilis*) dusted with vitamin powder (Roboran H, UniVIT, Czech Republic). Water with calcium was also available *ad libitum* with the calcium water being exchanged every two weeks for water with vitamins A, D_3 and E (Kombisol AD_3E, Trouw Nutrition Biofaktory Ltd., Czech Republic) once every two weeks. Individuals were kept in social isolation during the growth experiment.

To follow the growth and take samples for the hormonal analyses and *IGF1* expression assay, 233 individuals of both sexes were euthanized in 11 age groups covering the age since hatching till the age of 15 months. Before euthanasia by rapid decapitation all animals were weighed, and their SVL was measured with digital calipers. Blood and liver samples were collected (with the exception of the earliest age group of age 0). Chirurgical gonadal inspection was conducted after decapitation to confirm the sex of all individuals and mass of ovaries and oviducts was collected in all females with the exception of the earliest age group of age 0. Furthermore, vertebrae were collected for examination of growth markers by histology. From this pool of individuals a limited number were selected for each analysis as described below. The study was conducted with the approval of the Ethical Committee of the Faculty of Science, Charles University, Prague, and the Ministry of Education, Youth and Sports of the Czech Republic (permit number 1479/2019-3).

Statistical analysis of growth

The classical modeling of growth in lizards used an asymptotic curve, mostly the von Bertalanffy growth model (e.g.,von Bertalanffy, 1957; St Clair, 1998; Kubička & Kratochvíl, 2009; Kubička et al. 2013, 2015, 2017). However, such models use preconceived assumptions about a mechanistic understanding of growth and their fitting might force the growth to a presumed pattern. In contrast, general additive models (GAMs; Pedersen, Miller,

Simpson and Ross, 2019) allow for the modeling of growth without specific assumptions on a pattern or a mechanism behind and can be used for direct testing of the influence of factors such as sex on growth.

We employed GAMs to establish the relationship between SVL (response variable), sex and age (predictors) using the "mgcv" package in R (Wood S, 2017). We constructed a series of GAMs fitted by the Restricted Maximum Likelihood (REML) using all 233 individuals. The Akaike Information Criterion (AIC) was used to compare the different models to find the best solution of the trade-off between model complexity and goodness of fit. The model with the lowest AIC value was preferred. At $\Delta AIC < 2$, the models were considered equivalent and the more parsimonious model was preferred; the more complex model was considered supported at $\Delta AIC > 2$. We tested varying values of the smoothing parameter (k) for GAM function exploring the values up to 16 and used AIC again to select the best performing smoothness.

Examination of growth plates activity

Growth plate activity was explored through previously established methods (Kubička et al. 2022) in three males and three females from 10 different age groups (72, 114, 156, 198, 240, 282, 324, 366, 408 and 450 days old). We collected presacral vertebrae for analysis as vertebrae are best related to the lizard body length. The presacral vertebrae were rinsed in water and decalcified in 10% formic acid solution. After decalcification they were rinsed in water and embedded in paraffin. Bone sections were stained using the hematoxylin-eosin staining technique and mounted in Canada balsam. Growth plates images were taken with Leica DMLB microscope and MC170 HD camera (Leica Microsystems) and with microscope Olympus BX53 equipped with a 21-megapixel high-resolution digital DP74 color camera (Olympus, Shinjuku City, Japan) and DP manager imagining software (Olympus). All

slides were checked for any signs of presence or absence of proliferating chondrocytes within the vertebral growth zones which serves as a marker of active bone growth.

Gene expression analysis

The livers were chosen for gene expression analysis as hepatic levels of *IGF1* have a role in bone elongation and growth (Yakar et al., 2018; Racine & Serrat, 2020) and *IGF1* had higher expressions in the liver than other tissues in reptiles (Reding et al., 2016; Duncan et al., 2020; Marks et al., 2021). Also, liver tissue was used in the previous paper on the sexually dimorphic growth in *P. picta* (Meter et al., 2022). We targeted six age groups, five (72, 114, 156, 198 and 240 days old individuals) during pronounced growth and one (408 days old individuals) at the end of the growth curves. For each age group we included five individuals of each sex for a total of 60 individuals analyzed. The liver samples were chopped into small pieces and stored in RNAlater (Sigma-Aldrich Inc., Missouri, United States) at -20 °C. RNA was extracted according to the manufacturer's protocol using the HighPure RNA tissue kit (Roche, Germany) and then treated with DNase with a standardized amount of about 5 mg of liver tissue for each extraction. RNA concentration was measured using the Quantus fluorometer (Promega Corporation, Madison, United States).

RNA integrity was assessed with the Agilent 2,100 Bioanalyzer and Agilent RNA 6000 Nano Kit (Agilent Technologies, California, United States). Usually, samples with RNA integrity number (RIN) samples with a RIN between 3 and 5 are considered partially degraded (Farrell 2017). Our samples had a RIN range from 7.9 to 10 and were therefore considered non-degraded. The purity of RNA measured as A260/A280 ratios was determined with the NanoDrop One (Thermo Fisher Scientific Inc., Massachusetts, United States) as ratios around 2 are considered pure RNA (Farrell, 2017), only samples with acceptable ratios (between 1.9 and 2.1) were kept. Once extracted, RNA samples were diluted to 13 ng/µl, divided into aliquots and stored at -80 °C. The RNA samples were reverse transcribed using

the TATAA GrandScript cDNA synthesis kit (TATAA Biocenter, Sweden) with a standard amount of 195 ng of total RNA in each sample.

Primer pairs for *IGF1* and the two reference genes (YWHAZ & 18S) were previously used in *P. picta* (supplementary information 1; Meter et al., 2022). The primer pairs were verified for *P. vazimba* with sequencing of amplified products. The primer pairs were used in standard curves using the mix of all samples in dilution series at different annealing temperatures to assess primer efficiency and best annealing temperature. The thermal cycle selected for all the assays was as follows: 95 °C for 30 s of activation, then 40 cycles of 5 s at 95 °C followed by 60 °C for 15 s and 72 °C for 10 s of amplification cycles. Finally, a melting curve of 60–95 °C was done after the final cycle to assess for possible secondary products.

We measured samples in triplicates in each assay (or primer pair). For each assay, triplicates of no template controls (wells with nuclease-free water instead of cDNA—NTC) were used to assess for contaminations in reagents. 5 µl of the TATAA® SYBR® green master mix (TATAA Biocenter, Sweden), 2.6 µl of nuclease-free water, 0.4 µl of forward and reverse primer (10 µM) respectively of the assay and finally 2 µl of the sample cDNA for a total of 10 µl in each well. An interplate calibrator (TATAA IPC kit, TATAA Biocenter, Sweden) was used to ensure comparisons between plates.

The relative gene expression was calculated using a previously verified method scripted on R (Meter et al., 2022). Briefly, mean values were calculated from triplicates with the efficiencies of all primer pairs used before the normalization and calibration steps. The calibrator being the mean of the group of 72 days old females and the normalization used the two reference genes. We calculated the relative expression by using this calibrator for ΔCq calculations. We then calculated the geomean of efficiency to the power of ΔCq of both reference genes and used to divide the efficiency to the power of ΔCq of *IGF1*.

$$Relative\ expression = Ln\left(\frac{E^{Cal_{IGF1}} - {}^{CQ_{IGF1}}}{Geomean\ (E^{Cal_{18S}} - {}^{CQ_{18s}} + E^{Cal_{YWHAZ}} - {}^{CQ_{YWHAZ}})}\right)$$

We transformed the data with the natural logarithm before subsequent analyses. Biological outliers of the groups for each gene were assessed through statistical tests using the mean Cq of each sample. Triplicate outliers were verified from the raw Cq values in accordance with their coefficient of variation. Coefficients of variation above 30% were deemed unacceptable and the replicates were redone.

Hormone level analysis

Levels of testosterone, dihydrotestosterone, progesterone and estradiol were measured from the plasma. Hormone levels were measured for individuals if possible, but especially in younger animals, blood samples had to be pooled to attain enough plasma for analysis. Hormonal levels were analyzed in the Laboratory of Mass Spectrometry at BIOCEV Research Center; Faculty of Science, Charles University.

The standard calibration curve for testosterone, dihydrotestosterone and progesterone was prepared as described previously in charcoal-treated plasma (Sosvorova, Vitku, Chlupacova, Mohapl and Hampl, 2015; Gorityala et al., 2018; Reichard, Douda, Blažek and Janovská, 2022) where standards and deuterated internal standards were used for the quantification of each hormone. Briefly, 100 pg of internal standards in methanol were pipetted to 2 ml glass vials and methanol was evaporated. Subsequently, 20 µl of charcoal-treated gecko plasma was added. Hormones were extracted with 1.3 ml of ice-cold tert-butylmethylether. The organic phase was transferred to a glass vial and evaporated. To enhance sensitivity of mass spectrometry detection, the hormones were derivatized using 2-hydrazinopyridine (Higashi,Nishio,Hayashi and Shimada, 2007). The final sample was resuspended at 20 µl of 50% methanol with 0.1% formic acid.

The standard calibration curve for estradiol was prepared as described previously in charcoal-treated plasma (Vitku et al., 2015, Gorityala, Yang, Montano and Xu, 2018) where standard and deuterated internal standard were used for its quantification. In this case 100 pg of internal standard in methanol was pipetted to 4 ml glass vials and methanol was evaporated. Subsequently, 20 µl of charcoal-treated gecko plasma was added. Estradiol was extracted with 2 ml of ice-cold chloroform. The organic phase was transferred to a glass vial and evaporated. To enhance sensitivity of mass spectrometry detection, estradiol was derivatized using dansyl chloride (Anari et al., 2002). The final sample was resuspended at 20 µl of 50% methanol with 0.1% formic acid.

The samples were analyzed on a Dionex Ultimate 3000RS liquid chromatography system coupled to a TSQ Quantiva mass spectrometer (ThermoScientific). Chromatographic column Kinetex® EVO C18 (150 mm × 2.1 mm, 5 µm, 100 Å) coupled with SecurityGuardTM ULTRA EVO-C18 (Phenomenex) was used for a separation of analytes. The column temperature was held at 40 °C and injection volume of the sample was 10 µl. The buffer composition was A: 2% methanol, 0.1% formic acid; B: 95% methanol, 0.1% formic acid. For estradiol measurement, the flow rate was 200 µl/min and total run time 20 minutes. The elution gradient (A/B) started at 0 to 1.5 minutes: constant 95% A, from 1.5 to 3 minutes: decreased to 10% A, from 3 to 8.9 minutes: decreased to 0% A, followed by an equilibration phase from 9 to 20 minutes: constant 95% A. For androgen and progesterone measurement, the flow rate was 300 µl/min and total run time 20 minutes. The elution gradient (A/B) started at 0 to 1.5 minutes: constant 95% A, from 3 to 6 minutes: decreased to 1% A, from 6 to 8.9 minutes: constant 1% A, followed by an equilibration phase from 9 to 20 minutes: constant 95% A. Analytes were detected through a mass spectrometric detection using electrospray ionization in positive mode followed by a selective reaction monitoring (SRM). Ion source parameters were set as follows: ion transfer tube temperature 300 °C,

vaporizer temperature 300 °C, spray voltage 3500 V, sheath gas 40 and aux gas 10. Data was processed in Skyline software. The limits of detection were 0.05 ng per ml for estradiol, dihydrotestosterone and testosterone and 0.25 ng per ml for progesterone.

Statistical analysis

Statistical tests for the gene expression analysis and GAM of the growth curves were done with the R program and the interface R studio (RStudio Team, 2019; Core Team, 2020). Biological outliers for each age and sex group were assessed using both Dixon and Grubbs' tests using the package "outliers" (Komsta, 2011). Shapiro-Wilk test was used to check for normal distribution in the relative gene expression. Statistically significant differences between groups were assessed using ANOVA after rank transformation due to the non-normal distribution of gene expression. For all statistical tests, the significance threshold was set at $p = 0.05$.

Results

Generalized additive model of growth

The best GAM model ($n = 233$; marginal $r^2 = 0.95$; Figure 1) on the relationship between age and SVL in *P. vazimba* included the factor sex, showing that growth is sexually dimorphic. The null model and models with and without sex had much higher AIC scores (Supplementary information 1 and 2).

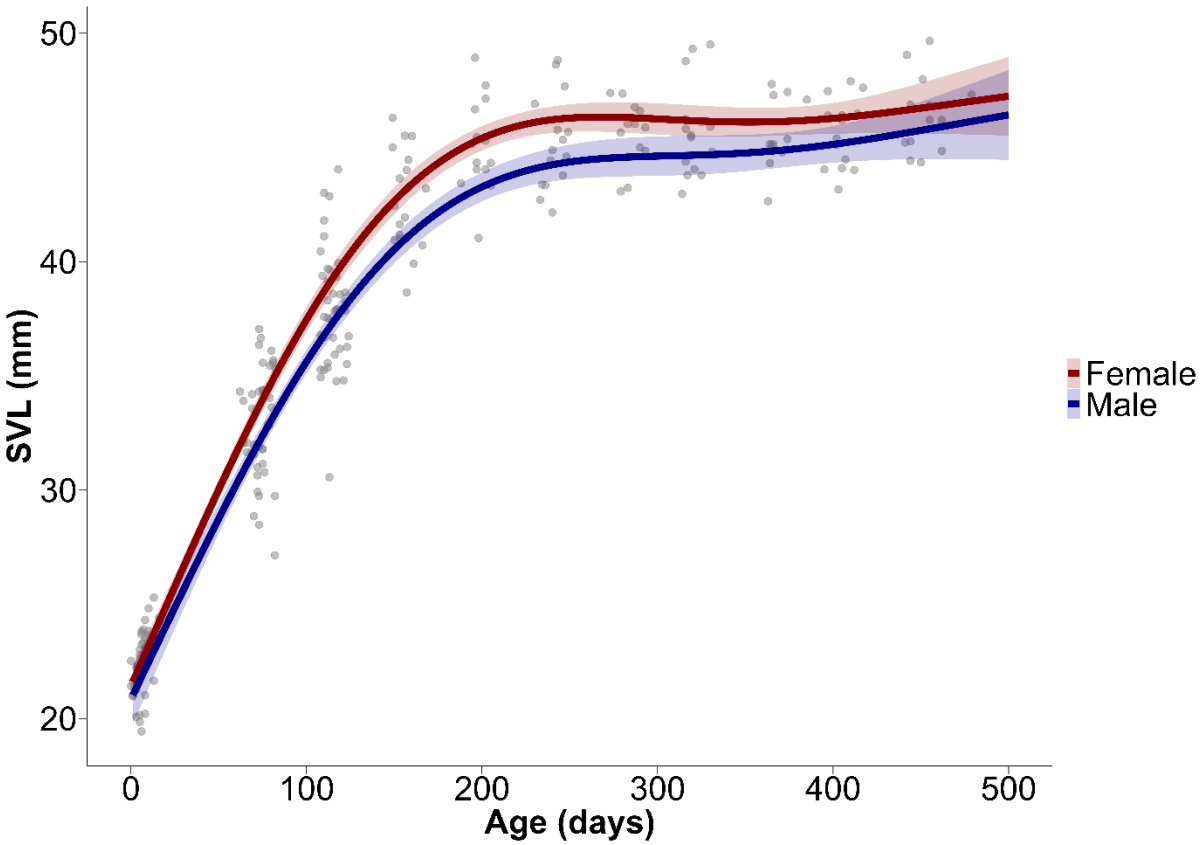

Figure 1: GAM model according to 233 individuals of *Paroedura vazimba* depicting sex-specific growth curves and their 95% confidence intervals with females (red line) growing faster and finishing their growth earlier than males (blue line).

The sexes start at hatchling at similar size, however, they diverge in growth very early. The early growth of females is more rapid. Nevertheless, females decelerate growth earlier and males approach them in size later. In contrast, an analogously constructed GAM model for male-larger *P. picta* from previously published data (Kubička et al. 2022) showed the clear differences in the growth curves between the species: the growth in *P. picta* is not sexually dimorphic for quite a long time after hatching and females decelerate growth around the time of sexual maturation (Figure S1).

Growth plate analysis

The analysis of 60 individuals (supplementary information 2) from the selected age groups was used to estimate the state of vertebrae growth plates. In both sexes, vertebral growth zones are losing proliferating chondrocytes with age (Figure 2). Stop of growth is represented

by their complete absence and total calcification of the growth zone. Surprisingly, for a female-larger species, there is no vertebrae growth marker present at the age of 156 days in females, while males vertebrae are still capable of growth at this age. In males, evidence of cessation of growth appears for individuals at 198 days.

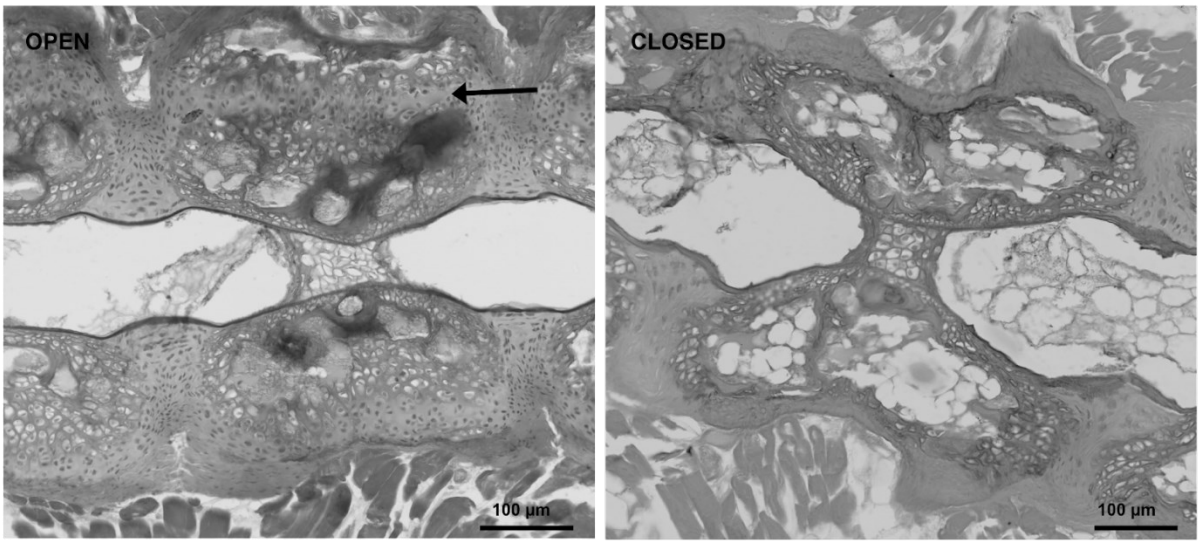

Figure 2: Examples of the growth plate activity of vertebrae. “Open” presacral vertebrae of a young animal (female, 73 days of age) have a notable growth zone (indicated by an arrow), which is lacking in the “closed” vertebrae in older animals (female, 402 days of age).

Ovary and oviduct mass

Ovary and oviduct mass show a noticeable increase already at 156 days of age (Figure 3A and B; supplementary information 2) indicating the possible sexual maturation of females within the 114 to 156 day time frame.

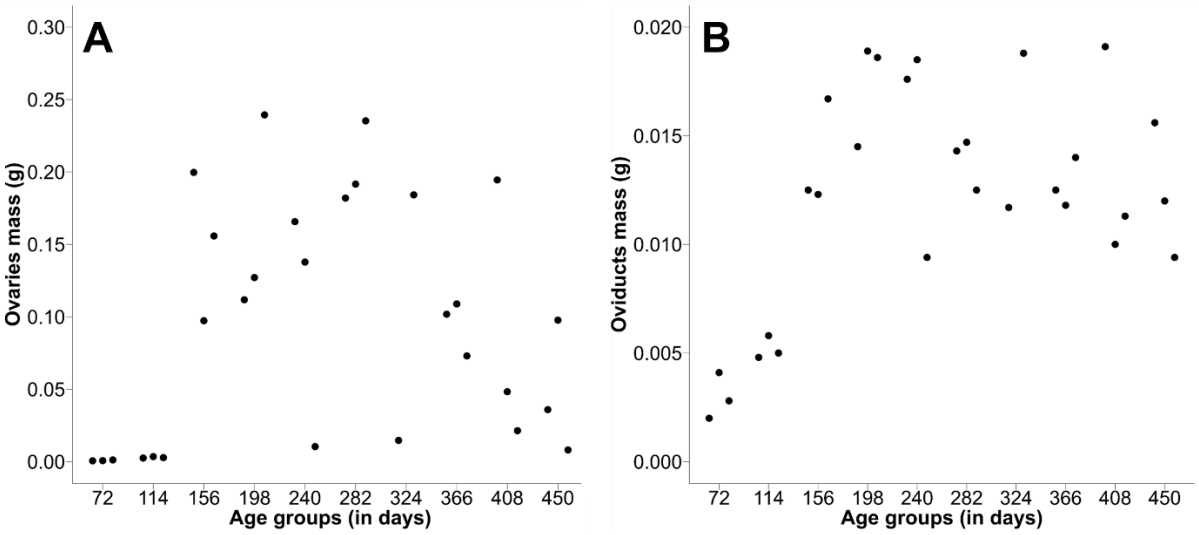

Figure 3: Ovary (A) and oviduct mass (B) throughout the female age groups in Paroedura vazimba

Gene expression

Gene expression of *IGF1* was measured using RT-qPCR (Figure 4; supplementary information 2). No biological outliers were found with the Dixon and Grubb tests within each age/sex group. The Shapiro-Wilk test shows a non-normal distribution of the data for *IGF1* expression ($W = 0.95$; $p = 0.043$) because of this we rank-transformed the data before performing ANOVA analysis.

The lowest AIC had the ANOVA model including the age-sex interaction model (see the supplementary information 1). Paired contrasts between the age/sex groups revealed only marginally significant differences between the sexes for age groups 198 and 408 only (both $F_{1,10} = 5.076$, $p = 0.03$) and in the opposite direction. Considering multiple comparisons and the potential effect of outliers, we suggest that there is generally no clear trend in *IGF1* expression values.

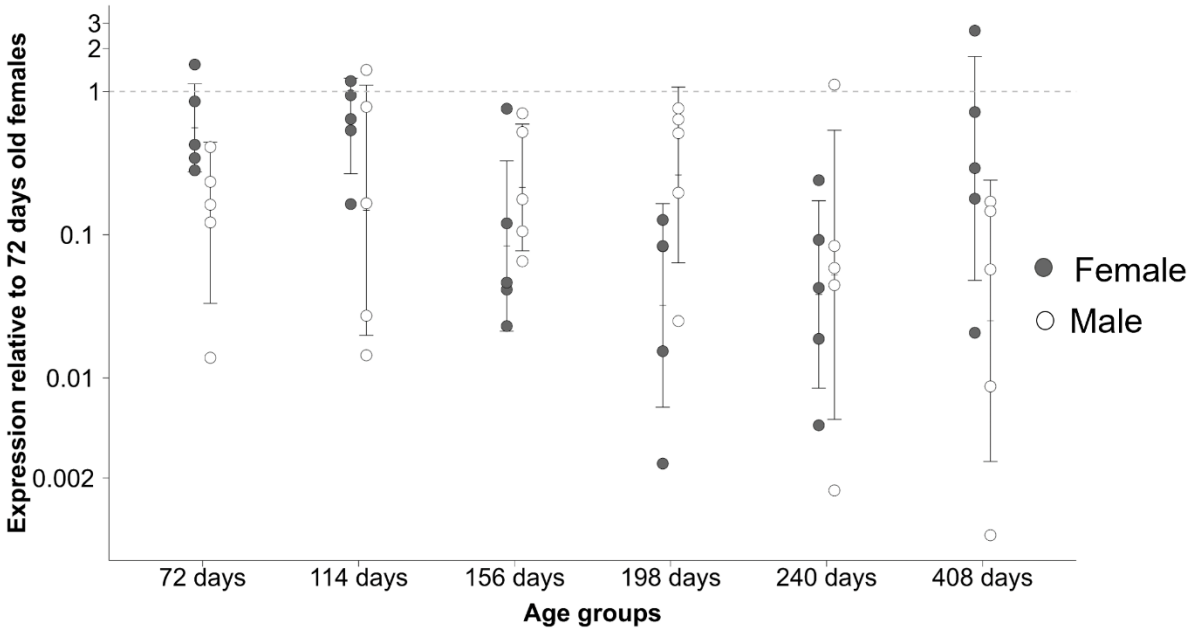

Figure 4: Gene expression of *IGF1* relative to the calibrator (mean of group 72 days old females as presented by the gray line). Mean ± standard deviations are depicted.

Hormone levels

Testosterone and dihydrotestosterone levels were generally higher in males compared to females throughout growth. Testosterone levels were below detection limit in most females, but they were much higher already at the youngest age in males (Figure 5). A rather inverted trend can be found for progesterone, where levels were increased already at the age of 156 days in females (Figure 5 and supplementary information 2). Estradiol levels were too low to be usable in the analysis as most individuals of both sexes had levels under the limit of detection.

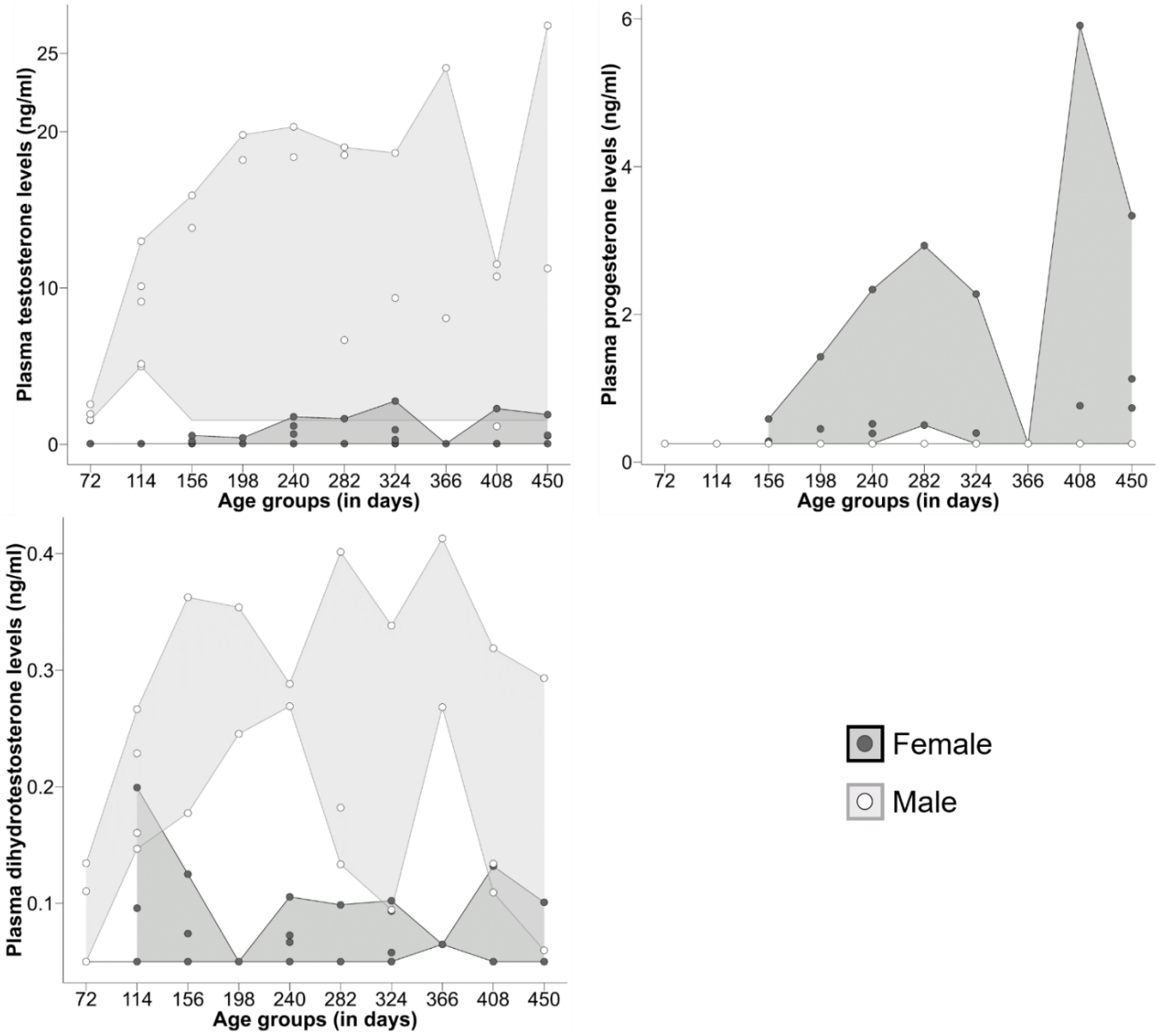

Figure 5: Plasma levels of steroid hormones throughout age groups according to sex.

Discussion

Our long-term growth study confirmed the female-larger SSD in *P. vazimba* as previously recorded by Starostová et al. (2010). We also documented a determinate growth in this species as in closely related male-larger *P. picta* (Kubička et al. 2022) and in most lineages of squamates (Frýdlová et al. 2020). For a long time, it was considered that growth in them is not determinate and studies focused mainly on growth rates. Our studies points that growth rates can be plastic with respects for instance to hatchling mass (Starostová, Pichová,

Bauerová, Kubička, and Kratochvíl, 2024), but that final structural body size driven by closure of bone growth plates can be much more canalized (reviewed in Meter et al., 2020; Starostová et al., 2024).

In vertebrates, growth is generally expected to be similar between the sexes in the earlier stages with the size differences developing only later (Badyaev, 2002). This was also the case in *P. picta*, where the divergence in growth patterns between sexes occurs around sexual maturity by a sex-specific pattern of bone growth zones (Meter et al. 2022; Kubička et al. 2022). The similar growth of both sexes in early ontogeny was found also in other male-larger species (*Anolis sagrei*, Cox R.M., Cox C.L., McGlothlin, Card, Andrew and Castoe, 2017; *Varanus indicus*, Frynta et al., 2010) and a female-larger lizard (*Aeluroscalabotes felinus*, Kubička et al. 2013). In contrast, the ontogeny of SSD in *P. vazimba* is surprisingly complex. The growth between the sexes in this species diverge early after hatching with females growing faster and attaining a final, larger body size earlier than males (Figure 1). Somewhat surprisingly, for a female-larger species, females of *P. vazimba* fused growth plates and thus ceased growth earlier than males (see figure 6 for a schematic overview).

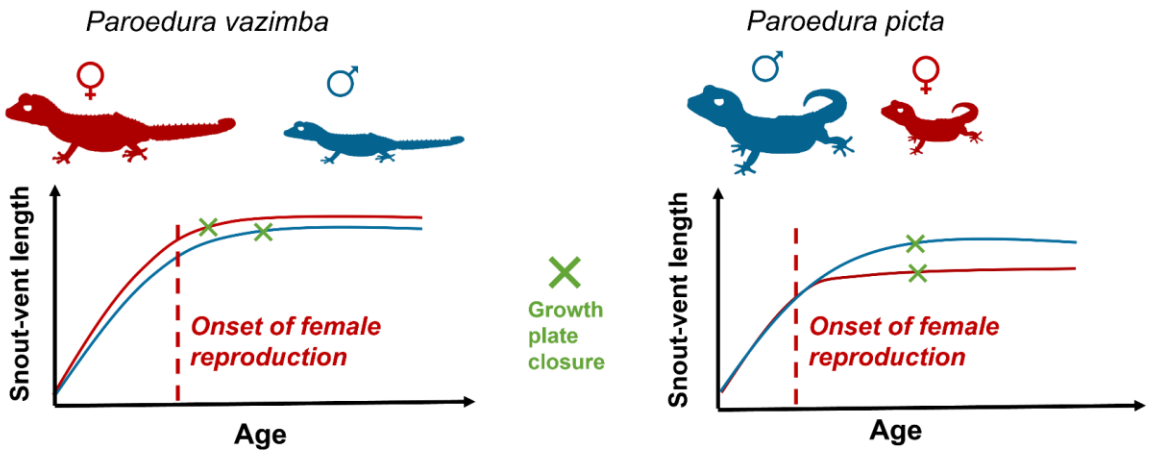

Figure 6: Schematic representation of the model on the ontogeny of sexual size dimorphism in male-larger and female-larger lizards based on data from species of genus *Paroedura*.

The cessation of growth in females of both *P. picta* and *P. vazimba* corresponds to an increase in their progesterone levels and increase of ovary and oviduct mass, which are markers of reproductive activity (Kubička et al. 2017, 2022). In *P. vazimba,* males continue growing longer than females and sexual differences in size are thus slightly smaller in fully grown individuals than around the time of female growth cessation (Figure 1).

Just as in *P. picta* (Kubička et al., 2022), testosterone levels were already increased in males of *P. vazimba* prior to their growth plate closure indicating a less likely role in SSD development. The same is true for dihydrotestosterone. Still, we cannot exclude that growth rate is decreased by androgenes throughout earlier development in *P. vazimba*. Testosterone was reported to retard growth and decreased hepatic *IGF1* levels in another female-larger lizard species *Sceloporus undulatus* (Duncan et al., 2020), while it upregulated hepatic *IGF1* expression in male-larger *A. sagrei* (Hale, Robinson, Cox C.L. and Cox,R.M., 2022). However, the manipulative long-term growth experiments involving gonad removal did not detect any significant changes in body length growth between castrated and control males in both male-larger and female-lager lizards (Bauerová et al., 2020; Kubička et al., 2013, 2015).

In *P. vazimba*, we did not find a notable spike of *IGF1* expression in the larger sex in any age/sex groups. This lack might indicate no role of hepatic *IGF1* in SSD development in *P. vazimba*. This is a stark contrast to *P. picta*, where the spike of expression in males around the time of dimorphism development was around two orders of magnitude higher than the levels in young animals of either sex (Meter et al., 2022). The differences in *IGF1* levels across groups in *P. vazimba* were very small and we cannot exclude that our essay was not sensitive enough to detect more subtle, yet functionally important differences in the expression levels. It is also possible that an expression spike happened earlier in the development, such as before the 72 days of age, and we could not detect it due to the size of the liver of the younger individuals.

In conclusion, we already seem to have a quite well-supported model for the development of SSD in male-larger *P. picta* characterized by common growth of both sexes post hatching and bone growth arrest driven likely by ovarian hormones at the onset of female reproduction (Kubička et al. 2022), likely preventing a spike of IGF1 levels present in males (Meter et al. 2022). On the other hand, the mechanisms driving the development of female-larger growth in *P. vazimba* is much less clear and stays more speculative. Along with the insights obtained in this study and in agreement with experimental evidence in human beings, mammals and other vertebrates, we suggest a model for SSD development in female-larger species such as *P. vazimba*. It is well supported that estrogens at high levels decelerates growth and leads to a decrease activity of bone growth plates (Callewaert et al., 2010; Weise et al. 2001, Nillson et al, 2014; Zhongbo et al., 2016; Yakar et al., 2018). However, slightly enlarged (but still very low) levels of estrogens stimulates growth and prolong the proliferation activity/longevity of bone growth plates leading to growth spurt in females. For example, the clinical study on adolescent humans by Cutler (1997) concluded that the pubertal growth spurt of both sexes is driven primarily by low levels of estrogens and that

shorter body height in women is due to their more rapid epiphyseal maturation before puberty in comparison to men which may be explained by higher estradiol levels in women at this time. Our model expects that the bipotential effect of estrogens is conserved across amniotes. We predict that even in female-larger species, male growth trajectory is a neutral state, with females delaying the first reproduction already have slightly enlarged but still low, growth-stimulating, levels of estrogens at the age when males decelerate growth. These levels of estrogens stimulate female growth; however, equally as in male-larger species, high levels of estrogens increased dramatically around the first reproduction decelerate growth in females considerably. The role of ovarian hormones in female-larger lizards was up to now supported only indirectly – females with exogenous testosterone possessed male-typical growth in the female-larger lizards (reviewed in Starostová et al., 2013). The lack of any effect of castration on male growth suggests that the females with elevated testosterone were not masculinized, but defeminized. One can ask why a prepubertal spurt in female growth is not visible in the comparison of male and female growth trajectories in the male-larger species such as *P. picta*. We admit that we do not know, but as an *ad hoc* explanation we offer that at the stage of stimulating pre-reproductional levels of estrogens both sexes still exhibit their maximal growth rates and that the potential stimulatory effect of estrogens is thus masked.

Conclusions

Our study highlights the necessity of long-term growth studies in comprehensively considering the mechanisms of SSD in lizards. These findings coupled with the previous findings in *P. picta* show the diversity in mechanisms guiding growth and SSD. We hope this study will encourage the use of long-term monitoring of growth combined with histological processing of bones in other sexually dimorphic lizards. More generally, we stress that future studies on development, growth and body size evolution in lizards should take into account the determinate nature of their growth.

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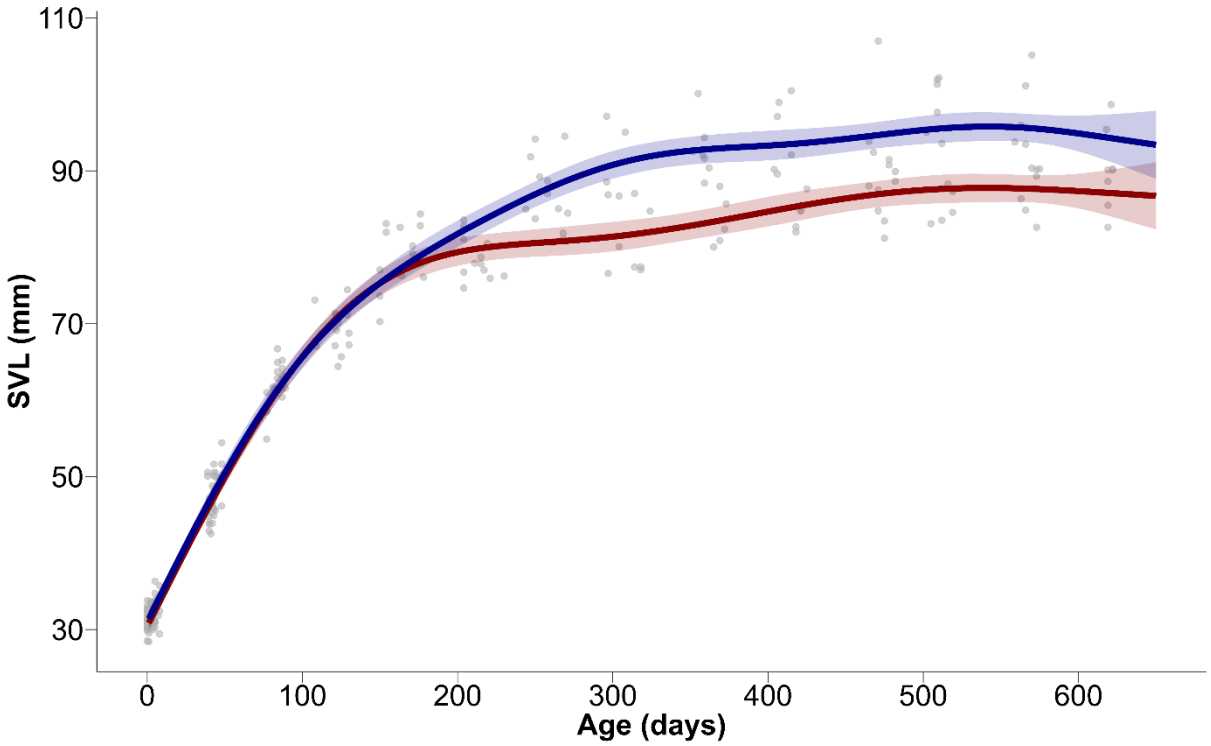
Supplementary material



Supplementary figure S1: In the paper by Kubička et al. (2022), the growth curves for male-larger *P. picta* were modeled by von Bertalanffy growth model, not by GAMs. Using the same dataset, we modeled the growth curves with GAM to make better comparisons between the *Paroedura* species. The selected model SVL ~ sex + s(age, by = sex) with $k = 7$ (n = 256; marginal $r^2 = 0.98$) is presented. Fitted curves and their 95% confidence intervals are depicted. Raw data used in Kubička et al. (2022) are available in the Mendeley database (doi:10.17632/fxcdd6j4sh.1).

Primers used for RT-qPCR

Gene	Forward 5'-3'	Reverse 5'-3'	Amplicon size (bp)	Efficiency (%)
Target				
IGF1	GTGGAGCTGAGCTGGTTGAT	ACAGCTCTGGAAGCAGCATT	140	93
Reference				
18S	GAGGTGAAATTCTTGGACCGG	CGAACCTCCGACTTTCGTTCT	92	94
YWHAZ	TGATGTGCTGTCTCTTTTGG	TTGTCATCACCAGCAGCAA	129	78

Supplementary statistics

Supplementary information – General Additive Models

Paroedura vazimba

For female larger gecko *P. vazimba* 233 individuals were used for modelling. The values of k, the parameter controlling the degrees of freedom or smoothness of the splines used for the smooth terms (s) are given in the Generalized Additive Models (GAM). In total, 16 *k* values were tested for SVL as a response variable and sex and age as predictors. In addition, the null model and models with and without sex were modeled. The best fitted model is in bold, all models and their respective Akaike Information Criterion (AIC) is presented in the table below.

Model formula	***k* value**	**AIC**
SVL ~ 1	4	1678.9
SVL ~ s(age)	4	1003.7
SVL ~ s(age, by = Sex)	4	1004.4
SVL ~ sex + s(age, by = sex)	1	1065.2
SVL ~ sex + s(age, by = sex)	2	1065.2
SVL ~ sex + s(age, by = sex)	3	1065.2
SVL ~ sex + s(age, by = sex)	**4**	**974.5**
SVL ~ sex + s(age, by = sex)	5	977.7
SVL ~ sex + s(age, by = sex)	6	980.4
SVL ~ sex + s(age, by = sex)	7	982.2
SVL ~ sex + s(age, by = sex)	8	979.6
SVL ~ sex + s(age, by = sex)	9	979.6
SVL ~ sex + s(age, by = sex)	10	979.8
SVL ~ sex + s(age, by = sex)	11	980.1
SVL ~ sex + s(age, by = sex)	12	980.3
SVL ~ sex + s(age, by = sex)	13	980.5
SVL ~ sex + s(age, by = sex)	14	980.5
SVL ~ sex + s(age, by = sex)	15	980.6
SVL ~ sex + s(age, by = sex)	16	980.6

Parametric coefficients and approximate significance of smooth terms of the best model are presented below (SVL ~ sex + s(age, by = sex); n = 233; marginal r^2= 0.95)

Coefficient	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(intercept)	38.6	0.16	229.7	<0.001
Sex M	-1.5	0.26	-5.6	<0.001
Approximate significance	**edf**	**Red.df**	**F**	**p-value**
s(age):SexF	2.98	3	1152.6	<0.001
s(age):SexM	2.97	2.9	417.6	<0.001

Paroedura picta

In the same method, GAMs were modeled for male larger *P. picta* using 256 individuals and the best fitted model is presented in the supplementary material.

Model formula	***k* value**	**AIC**
SVL ~ 1	10	2363.9
SVL ~ s(age)	10	1537.7
SVL ~ s(age, by = Sex)	10	1503.6
SVL ~ sex + s(age, by = sex)	1	1624.7
SVL ~ sex + s(age, by = sex)	2	1624.7
SVL ~ sex + s(age, by = sex)	3	1624.7
SVL ~ sex + s(age, by = sex)	4	1469.4
SVL ~ sex + s(age, by = sex)	5	1390.4
SVL ~ sex + s(age, by = sex)	6	1386.3
SVL ~ sex + s(age, by = sex)	**7**	**1383.8**
SVL ~ sex + s(age, by = sex)	8	1385.9
SVL ~ sex + s(age, by = sex)	9	1386.3
SVL ~ sex + s(age, by = sex)	10	1385.3
SVL ~ sex + s(age, by = sex)	11	1388.2
SVL ~ sex + s(age, by = sex)	12	1387.4
SVL ~ sex + s(age, by = sex)	13	1387.7
SVL ~ sex + s(age, by = sex)	14	1385.7
SVL ~ sex + s(age, by = sex)	15	1385.2
SVL ~ sex + s(age, by = sex)	16	1385.2

Parametric coefficients and approximate significance of smooth terms of the best model are presented below (SVL ~ sex + s(age, by = sex); n = 256; marginal r^2= 0.98).

Coefficient	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(intercept)	61.9	0.3	204.8	<0.001
Sex M	3.4	0.44	7.7	<0.001
Approximate significance	**edf**	**Red.df**	**F**	**p-value**
s(age):SexF	5.67	5.95	918.9	<0.001
s(age):SexM	5.69	5.93	1082.4	<0.001

Supplementary information – ANOVA analysis

AIC for the different ANOVA models describing relative expression, in function of age, sex or their interaction.

ANOVA models	**AIC**
Null model	516.4829
Age	517.1494
Sex	518.0510
Age x Sex	510.2246