© 2017. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2017) 220, 787-795 doi:10.1242/jeb.146597

RESEARCH ARTICLE

Ovarian control of growth and sexual size dimorphism in a male-larger gecko

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ABSTRACT

Sexual size dimorphism (SSD) reflects sex-specific solutions to the allocation of energy among growth, reproduction and survival; however, the proximate mechanisms behind these solutions are still poorly known even in vertebrates. In squamates, sexual differences in body size used to be attributed to direct energy allocation to energetically demanding processes, largely to reproduction. In addition, SSD is assumed to be controlled by specific endogenous mechanisms regulating growth in a sex-specific manner, namely masculinization by male gonadal androgens or feminization by ovarian hormones. We performed a manipulative growth experiment in females of the male-larger gecko *Paroedura picta* in order to test the reproductive cost hypothesis, the male androgen hypothesis and the ovarian hormone hypothesis. Specifically, we investigated the effect of total ovariectomy, prepubertal ovariectomy, unilateral ovariectomy, and total ovariectomy followed by exogenous estradiol, dihydrotestosterone or testosterone treatment, on female growth in comparison to males and reproductively active females. The present results and the results of our previous experiments do not support the hypotheses that SSD reflects direct energy allocation to reproduction and that male gonadal androgens are involved. However, all lines of evidence, particularly the comparable growth of reproducing intact and unilaterally ovariectomized females, were concordant with the control of SSD by ovarian hormones. We suggest that feminization of growth by female gonadal hormones should be taken into consideration as an endogenous pathway responsible for the ontogeny of SSD in squamates.

KEY WORDS: Egg size, Estradiol, Invariant clutch size, Lizards, Testosterone, Unilateral ovariectomy

INTRODUCTION

Sexual size dimorphism (SSD) – the difference in body size between males and females of a single species – is widespread among animals. It is generally agreed that SSD largely reflects adaptations of particular sexes to their specific reproductive or ecological roles (reviewed in Darwin, 1871; Andersson, 1994; Fairbairn et al., 2007; Fairbairn, 2013). As growth is energetically demanding and different body sizes are usually connected with different costs and benefits, SSD probably reflects the sex-specific adaptive solutions of the trade-off between growth, body maintenance, reproduction and survival. Although knowledge of proximate mechanisms is essential for understanding adaptive evolution (for life-history traits and trade-offs see, for example, Flatt and Heyland, 2011), such information regarding body size differences between sexes is still surprisingly incomplete even in such a highly studied group as vertebrates.

Squamate reptiles represent a particularly interesting group for studies of evolutionary changes in SSD, as they include both male-larger and female-larger species, often even among closely related species (Kratochvíl and Frynta, 2002; Cox and John-Alder, 2005; Starostová et al., 2010; see also Cox et al., 2009, for review). Similar to most other vertebrates (Badyaev, 2002), male and female squamates are nearly identical in size at birth/hatching, with SSD being developed only later in ontogeny (Kratochvíl and Frynta, 2002; Taylor and DeNardo, 2005; Frynta et al., 2010; Starostová et al., 2010; Bonnet et al., 2011; Kubička et al., 2013). But what is the nature of the sex-specific growth regulators and modifiers that lead to the ontogeny of SSD?

An intuitively appealing and rather widely accepted hypothesis (hereafter 'reproductive cost hypothesis') is that in lineages with substantial growth after sexual maturation, such as reptiles, sexual differences in growth, and hence SSD, emerge as a direct consequence of a sex-specific split of available energy into growth versus reproduction (e.g. West et al., 2001). This hypothesis suggests that female-biased SSD should occur in species where males are forced to expend energy in demanding activities such as territory defence in order to obtain mating opportunities (e.g. Cox and John-Alder, 2005). In contrast, male-biased SSD should be present in species where females allocate substantially more energy to reproduction than males, and hence it is impossible for them to sustain male-typical growth. The hypothesis that the high cost of reproduction retards growth in females of male-larger species was recently supported by a correlative study in the elapid snake *Notechis scutatus* (Bonnet et al., 2011) and experimentally by ovariectomy ameliorating costs of reproduction in the anole *Anolis sagrei* (Cox and Calsbeek, 2010). However, it was demonstrated in other iguanian lizard, *Sceloporus jarrovii*, that female allocation to reproduction is insufficient to explain SSD (Cox, 2006).

Nevertheless, sexual differences in growth and body size may not be the result of a simple division of available energy; instead, they may be controlled by specific endogenous mechanisms, such as gonadal hormones, directly regulating growth (reviewed in Flatt and Heyland, 2011). One such endogenous mechanism potentially explaining evolutionary shifts in SSD in squamates was proposed by Cox and John-Alder (2005). Based on the results of hormonal manipulations in the iguanian genus *Sceloporus*, these authors suggested that male gonadal androgens can masculinize growth and have positive effects on growth in male-larger species, but a negative influence in female-larger species. Cox et al. (2009) summarized phylogenetically wide evidence on the effect of hormonal manipulations on male growth in squamates, supporting the previous findings and hence the 'male androgen hypothesis' on the control of SSD (for a critical review of this evidence, see Starostová et al., 2013).

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Received 1 August 2016; Accepted 7 December 2016

Journal of Experimental Biology (2017) 220, 787-795 doi:10.1242/jeb.146597

In contrast, our series of previous experiments in lizards produced little support for the male androgen hypothesis. We repeatedly found that the removal of male gonads had no effect on final body length or growth trajectory in the male-larger gecko *Paroedura picta* (Starostová et al., 2013; Kubička et al., 2015), or in the female-larger gecko *Aeluroscalabotes felinus* (Kubička et al., 2013). However, and at first sight paradoxically, the induction of male-typical levels of circulating testosterone in females led to a 'masculinization' of growth in both female-larger and male-larger lizard species. Non-ovariectomized (non-OVX) females treated by exogenous testosterone attained larger, male-like final snout–vent length (SVL) in the male-larger lizard species (Starostová et al., 2013; Cox et al., 2015), while exogenous testosterone acted negatively on the growth and final SVL in females of the female-larger gecko (Kubička et al., 2013). We suggest that the discrepancy between the effects of testosterone in males and females can be explained by the scenario where the development of SSD does not require masculinization by male gonadal androgens, but rather feminization by female gonadal hormones. Under this 'ovarian hormone hypothesis' (Kubička et al., 2013; Starostová et al., 2013), the exogenous testosterone in females would not cause growth masculinization but would negatively affect normal ovarian function and hence lead to defeminization. This hypothesis would also explain another paradox: in agreement with the reproductive cost hypothesis, ovariectomized lizard females exhibited enhanced growth and/or attained larger final body size than reproducing females in male-larger species (Cox and Calsbeek, 2010; Starostová et al., 2013); nevertheless, avoiding the enormous female reproductive effort of egg production by precluding egg laying through the isolation of sham-operated virgin females from males resulted in comparable final SVLs of non-reproducing and regularly reproducing females (Starostová et al., 2013). This observation suggests that ovariectomy does not affect growth simply by the redirection of energy allocation from reproduction to growth but also by the exclusion of ovarian hormone signalling.

Although the ovarian hormone hypothesis can explain some otherwise seemingly paradoxical phenomena in lizards not resolved by the reproductive cost hypothesis and male androgen hypothesis, it has not yet been completely explored or directly supported experimentally. Here, we investigated the effect of different manipulative treatments, including total ovariectomy, total prepubertal ovariectomy, unilateral ovariectomy, and total ovariectomy followed by exogenous estradiol (E_2), dihydrotestosterone (DHT) or testosterone treatment, on female growth in comparison to that of males and reproductively active females in the highly studied gecko species *Paroedura picta* (Peters, 1854). Regularly reproducing females with unilateral ovariectomy (1/2 OVX) were used to test the effect on growth of the reduced, but not totally removed, energy allocation to reproduction, when a more or less typical female hormonal reproductive cycle should be preserved. In this case, the reproductive cost hypothesis predicts that 1/2 OVX females should exhibit intermediate growth and body size between regularly reproducing intact females and fully ovariectomized (OVX) females, while the ovarian hormone hypothesis predicts that 1/2 OVX females and reproductively active intact females should reach comparable body size and exhibit similar growth and that only the growth of the OVX females should be defeminized. Comparison of the growth of OVX females and OVX females treated with exogenous testosterone was carried out in order to test whether there is a direct masculinization effect of testosterone as predicted by the male androgen hypothesis, or whether the effect of exogenous testosterone on female growth in non-OVX females seen in previous experiments (e.g. Starostová et al., 2013, and citations therein; Kubička et al., 2013; Cox et al., 2015) was a result of the interference of testosterone with normal ovarian function, as predicted by the ovarian hormone hypothesis. Oestrogens have been experimentally shown to be the major factor involved in bone growth control in mammals in both males and females (e.g. Weise et al., 2001; see Cutler, 1997, and Grumbach, 2000, for evidence in humans). Elevated levels of testosterone may help restore concentrations of E_2 via aromatization in testosterone-treated OVX females, so we also included a group of OVX females treated with DHT, a non-aromatizable androgen, to test whether elevated testosterone would affect growth indirectly via conversion to E_2. The OVX group with experimentally induced female-like levels of E_2 was used to examine the potential feminizing effect of E_2 on female growth directly. In mammals, it was shown that low, prepubertal levels of ovarian hormones have a positive effect on skeletal growth in humans, while high levels lead to growth inhibition (Cutler, 1997). The comparison of the effect of earlier and later ovariectomy on female growth was used to test whether the period of exposure to ovarian hormones affects final structural body size in a gecko.

MATERIALS AND METHODS

Paroedura picta is a medium-sized, male-larger gecko inhabiting large and diverse areas of the Madagascar lowlands (Schönecker, 2008). It has genotypic sex determination and hence sex chromosomes (Blumberg et al., 2002; Kratochvíl et al., 2008), although sex chromosomes in this species are only poorly differentiated and have not been identified yet (Koubová et al., 2014). This species breeds easily in the laboratory and matures at an early age (usually around 4 months) during the rapid growth phase in both sexes. The growth plateau is reached after 12 months of age; however, this trait is temperature dependent (Starostová et al., 2010). As in other geckos (Kratochvíl and Frynta, 2006), this species possesses the so-called invariant clutch size, with females typically laying two, but sometimes only one, highly calcified eggs within a single clutch. This species has become a well-studied laboratory reptile for developmental biology, genetics, physiology and behavioural and evolutionary ecology (e.g. Kratochvíl et al., 2006, 2008; Noro et al., 2009; Main et al., 2012; Zahradníček et al., 2012; Golinski et al., 2014; Tadashi et al., 2015).

Experimental design

The experiment was conducted with the approval of the Ethical Committee of Charles University and the Central Commission for Animal Welfare and the Environment of the Czech Republic (permit number MSMT-30657/2013-4).

Mated females from our captive breeding colony were individually housed in a climatic chamber maintained at 27°C with a 12 h light:12 h dark cycle. All the animals of the founding population were either imported from the wild or were their F1 progeny, ensuring considerable genetic variability. Eggs obtained from unrelated pairs were individually positioned and incubated at 27°C in the same type of climatic chamber. Upon hatching, animals were housed singly in standardized plastic boxes (20×20×10 cm) with sand substrate, shelter and a water dish in the same climatic chambers in which they were incubated. A constantly maintained temperature of 27°C has been seen to generate the largest SSD (allowing males to reach their maximal dimensions; Starostová et al., 2010) and females at this temperature are highly fecund (Kubička et al., 2012; Starostová et al., 2012); we thus selected this temperature as a very suitable one for our experiment. Geckos were

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 787-795 doi:10.1242/jeb.146597

fed crickets (*Gryllus assimilis*) dusted with vitamins (Roboran, Univit, Czech Republic) twice weekly to satiety. Water enriched with calcium (Vitacalcin, Zentiva, Czech Republic) was always available but was replaced once every 2 weeks with water supplemented with vitamins A, D_3 and E (Hydrovit, Pharmagal, Slovakia).

Experimental animals

Experimental animals were weighed and their SVL measured every month from hatching. At the age of ca. 3–4 months (i.e. the peripubertal age), the sex of the hatchlings was determined based on external morphology (enlarged hemipenal sacs present in males, follicles visible through the abdominal wall in females). At this time, six groups of 12 females selected from the progeny of 26 females were sorted evenly with respect to size, age and mother identity (i.e. no siblings in the same group). Groups of females were then randomly selected as: sham-operated females (Sham) allowed to reproduce regularly; 1/2 OVX females allowed to reproduce regularly; OVX females; OVX females treated with E_2 (E_2-OVX females); OVX females treated with DHT (DHT-OVX females); and OVX females treated with testosterone (T-OVX females). Moreover, to observe the effect of ovariectomy on female growth prior to puberty, we also introduced a group of 12 females ovariectomized at a smaller body size and younger age (median 78 days; Early-OVX females). An additional group of six Intact males originating from the same offspring cohort and maintained under the same conditions as experimental females served as controls of male-typical growth and were used to mate with Sham and 1/2 OVX females at regular monthly intervals. Because of genotypic sex determination and small clutch size, having a large number of experimental females of the same age at the same time was not logistically possible and so the surgery was carried out over a period of 7 weeks, with females recruited when they reached a body mass of 3–4 g (average of 3.43 g) in the case of the Early-OVX group, and 5–6 g (average of 5.37 g) in the remaining treatment groups. For surgery, the females were anaesthetized using a combination of an intramuscular injection of ketamine (Narkamon 5%, Spofa a.s., Prague, Czech Republic; 130 µg g^{-1} body mass) and cold immobilization. The ovaries were exposed via a medial ventral incision. Bilateral or unilateral ovariectomy was performed by ligating the ovary blood supply with surgical silk (Catgut GmbH, Markneukirchen, Germany), prior to its ablation. For the Sham females, surgery was performed in which ventral incisions were made to expose and manipulate the ovaries while leaving them unharmed. The incisions were closed using Maprolen® surgical sutures (Catgut GmbH) and were covered with Glubran ®2 surgical glue (GEM S.r.l., Viareggio, Italy). The experimental females were returned to their enclosures immediately after they recovered from anaesthesia.

The stitches were removed once the wound had healed sufficiently (within 3 weeks in all experimental animals). At this time, regular mating or hormonal treatment commenced. Previously, we have shown that a clutch consisting of a maximum of two eggs (one produced by each ovary) can occur every 7 days (Kubička and Kratochvíl, 2009) and so the presence of eggs in the enclosures of Sham females and 1/2 OVX females was checked once a week. When a clutch was found, egg mass and female SVL were measured. In order to increase the circulating levels of DHT, E_2 and testosterone in the three groups of OVX females, we used a method adapted from the cutaneous application of oil-diluted stress hormones in lizards (Meylan et al., 2003; Trompeter and Langkilde, 2011). A crystalline steroid hormone (DHT, E_2 or testosterone; Sigma Aldrich) dissolved in pharmaceutical quality sunflower oil was applied to the skin between the shoulders of each experimental individual twice a week at regular intervals (every 3–4 days). The mixture was absorbed into the skin within several hours. Based on 14 day preliminary tests in other individuals, 2.4 µg of DHT and testosterone and 0.25 µg of E_2 was applied per gram of body mass (the mass of each animal was measured weekly). Unadulterated sunflower oil was regularly applied to the other treatment groups, similar to hormone-treated females.

After the growth of all experimental animals had slowed considerably (after a year in most cases), the last measurements of body dimensions were taken before the subjects were euthanized by rapid decapitation in order to obtain the maximum amount of blood for further analyses. During the following necropsy, we inspected the internal organs, specifically for the presence of re-grown ovaries in surgically treated females. Females with regrown ovaries (two in the 1/2 OVX group, one in the OVX group and two in the Early-OVX group) were excluded from all analyses. To determine whether our animals had already decelerated growth considerably, we plotted SVL against time for each individual after each measurement. We also compared growth trajectories of control males and reproductively active females with those already known from our previous growth experiments at the same temperature (Starostová et al., 2010, 2013) and we monitored whether these two treatment groups approached the known asymptotic values. We terminated the experiment when the overwhelming majority of individuals notably decreased their growth rate.

Unfortunately, negative effects (body fluid accumulation, overall apathy and loss of appetite) followed by an unexpectedly high level of mortality started to develop several weeks after the beginning of the application of E_2 in the above-mentioned preliminary test of E_2 application. As this occurred when the E_2-OVX females were already involved in the E_2 treatment and although the levels of applied E_2 were low, to prevent potential suffering of the experimental animals we ended the growth experiment in this group after 6–13 weeks of treatment. In addition, we had to euthanize three Sham females prematurely because of an unspecific paralysis observed in these females. One Intact male died 4 days prior to the end of the experiment without any obvious reason. As his growth pattern did not differ from that of other males, we decided to use his growth data for subsequent analyses, although his exclusion would not change the significance of any results.

Hormone level validation

Plasma hormone levels were used as a measurement of the responses of all animals to the experimental treatments. Whole-blood plasma was analysed for levels of E_2, DHT, testosterone and progesterone at the Institute of Endocrinology (Prague, Czech Republic). For the detection of progesterone and testosterone, the protocol for liquid chromatography-tandem mass spectrometry (LC-MS/MS) after Sosvorova et al. (2015) was used. However, the protocol for LC-MS/MS following Vitku et al. (2015) was applied to measure E_2. Briefly, the methods consist of plasma extraction with diethyl ether followed by the appropriate derivatization step (to enhance detection responses of steroids in the MS) and separation using the ultra-high performance liquid chromatography Eksigent ultraLC 110 system (Redwood City, CA, USA). Detection of analytes was performed on an API 3200 mass spectrometer (AB Sciex, Concord, ON, Canada) with the electrospray ionization probe operating in a positive mode. Analyte quantification was determined using calibration curves based on known concentrations. The limits of detection were 0.005 ng ml^{-1} for progesterone and testosterone and 0.004 ng ml^{-1} for E_2.

For DHT, the standard radioimmunoassay (RIA) protocol after Hampl et al. (1990) was used. The method consists of extracting plasma with diethyl ether followed by a RIA using rabbit polyclonal antiserum to dihydrotestosterone-7-(carboxymethyloxime) bovine serum albumin conjugate, and [^{3}H]DHT. Selective oxidation with potassium permanganate was applied to the sample to eliminate testosterone because of its cross-reaction with this antiserum. Intra-assay and inter-assay coefficients of variation for the analyses are typically 17.1% and 17.7%, respectively. The limit of detection of the assay was 0.001 ng ml^{-1}.

As levels of hormones were measured in three independent ways (two LC-MS/MS, one RIA), it required a relatively high volume of blood plasma. In some cases, the whole plasma volume of a single individual was not sufficient to perform all three analyses, which resulted in a different number of measurements between E_2, DHT and testosterone with progesterone. These cases are highlighted in the results.

Statistical analysis

All statistical analyses were conducted using Statistica (version 10.0; StatSoft, Tulsa, OK, USA) or GraphPad Prism (version 6.07; GraphPad Software, San Diego, CA, USA). The Shapiro–Wilk test was applied to test for any departure from normal distribution. When the null hypothesis of normal data distribution was rejected at α=0.05, the non-parametric test was performed for group comparison. Parametric tests were used for variables not significantly violating normality. Sample size of each treatment group was maximized to robustly reveal differences and trends in traits compared within the space limitation of our climatic chambers.

SVL and body mass at the time of hatching were compared among all treatment groups using Kruskal–Wallis ANOVA and ANOVA. Similar comparison of age, SVL and body mass among the female groups was performed at the time of surgery. The plasma hormone levels were compared using Kruskal–Wallis ANOVA and Mann–Whitney *U*-test. Most squamates live for a relatively long time after growth deceleration or cessation and therefore asymptotic or final size is important for the pattern of SSD within a population (Stamps, 1993; Kratochvíl and Frynta, 2002). Computing of asymptotic SVL allows the comparison of animals differing in age (e.g. the youngest group Early-OVX females) and in treatment duration (group of E_2-OVX females or prematurely euthanized Sham females). Moreover, as asymptotic size is an estimation based on fitting of the growth curve to multiple measurements, it is much less sensitive to measurement errors of a single measurement. Because of these benefits, we applied the expression of the asymptotic von Bertalanffy model to our raw data:

$$\mathrm{SVL} = a(1 - e^{-k(t-t_0)}), \quad (1)$$

where a is the asymptotic SVL (mm), e is the base of the natural logarithm, k is the rate of approach to asymptotic SVL, t is age (days) and t_0 is the hypothetical time at length zero. This model is suitable for lizard growth to describe the growth pattern of each experimental individual (St Clair, 1998; Kratochvíl and Frynta, 2002; Kubička and Kratochvíl, 2009; Kubička et al., 2013, 2015). We also applied the linear regression between asymptotic SVL and the SVL at the last measurement in the experiment across all experimental animals in order to reveal a relationship between these values. A close relationship would suggest that the experimental animals already significantly decelerated growth and that asymptotic SVL is a good expression of their size for comparison among treatment groups. Subsequently, we used one-way ANOVA for comparison of asymptotic SVL between all treatment groups and successive *post hoc* Fischer LSD tests helped to reveal differences between treatment groups. We compared body condition among groups to test the potentially detrimental effect of manipulations on body condition using full-factorial ANCOVA with log-transformed body mass as the dependent variable, log-transformed SVL as the continuous predictor and group identity as the categorical predictor. We compared the reproductive effort of Sham females and 1/2 OVX females based on the rate of egg production (number per day) by non-parametric Mann–Whitney *U*-test and mean egg mass by general linear model with 'female identity' as a random categorical predictor nested in the categorical predictor 'female group' with female SVL as the continuous predictor. The rate of egg production was defined as the total number of eggs divided by the time between the first and last clutch of each female. Only eggs found intact were used for the comparison of egg size between groups. Female SVL and egg mass were log-transformed prior to the test. Means and 95% confidence intervals generated by the model were back-transformed and used in the corresponding figure.

RESULTS

At the time of hatching, treatment groups did not significantly differ in SVL (Kruskal–Wallis ANOVA: $H_{7,N=85}$=6.63, P=0.47) and body mass (ANOVA: $F_{7,77}$=0.85, P=0.55; see Table S1 for summary statistics). At the time of surgery, female treatment groups, with the exception of Early-OVX females, did not significantly differ in age (Kruskal–Wallis ANOVA: $H_{5,N=69}$=5.16, P=0.40), SVL (ANOVA: $F_{5,63}$=0.97, P=0.44) or body mass (ANOVA: $F_{5,63}$=0.84, P=0.53). On average, Early-OVX females were 19% younger, 12% shorter and 36% lighter at the time of surgery and the difference was statistically significant (Kruskal–Wallis ANOVA: $H_{6,N=79}$>24.07, P<0.001 for all three cases; see Table S2 for summary statistics).

The hormone assays verified treatment of all individuals (Fig. 1). In some cases, hormone levels were below the limit of detection; in comparisons of hormone levels among treatment groups, we assigned to these animals the value of the limit of detection for a given hormone. Testosterone plasma levels differed significantly between treatment groups (Kruskal–Wallis ANOVA: $H_{7,N=84}$=51.69, P<0.001; Fig. 1A). The testosterone levels of T-OVX females were comparable to the testosterone levels of the Intact males (Mann–Whitney *U*-test: U=22.0, P=0.40) and were within the previously reported range for males of this species (Starostová et al., 2013). In the other treatment groups, testosterone levels were close to the low testosterone levels measured in reproductively active Sham females (Fig. 1A). The plasma levels of DHT also significantly differed among treatment groups (Kruskal–Wallis ANOVA: $H_{7,N=79}$=56.28, P<0.001; Fig. 1B). Here, however, the sample size was smaller, as there was not enough plasma to accurately measure this hormone in five animals (two Sham females, one OVX female, one Early-OVX female and one T-OVX female). The levels of DHT were much higher in DHT-OVX females than in Intact males (Mann–Whitney *U*-test: U=2.0, P=0.003; Fig. 1B), but comparable between T-OVX females and Intact males (Mann–Whitney *U*-test: U=23.0, P=0.61; Fig. 1B). DHT levels in the rest of the treatment groups were similarly low (Kruskal–Wallis ANOVA: $H_{4,N=51}$=1.45, P=0.84; Fig. 1B). In the case of E_2, there were also significant differences among treatment groups (Kruskal–Wallis ANOVA: $H_{7,N=82}$=36.76, P<0.001; Fig. 1C). Here, there was not enough plasma to accurately measure this hormone in two animals (one 1/2 OVX female and one T-OVX female). E_2-OVX females possessed the highest levels of E_2 (Fig. 1C), which were significantly higher than in Sham females (Mann–Whitney *U*-test: U<0.01, P<0.001). The other groups,

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 787-795 doi:10.1242/jeb.146597

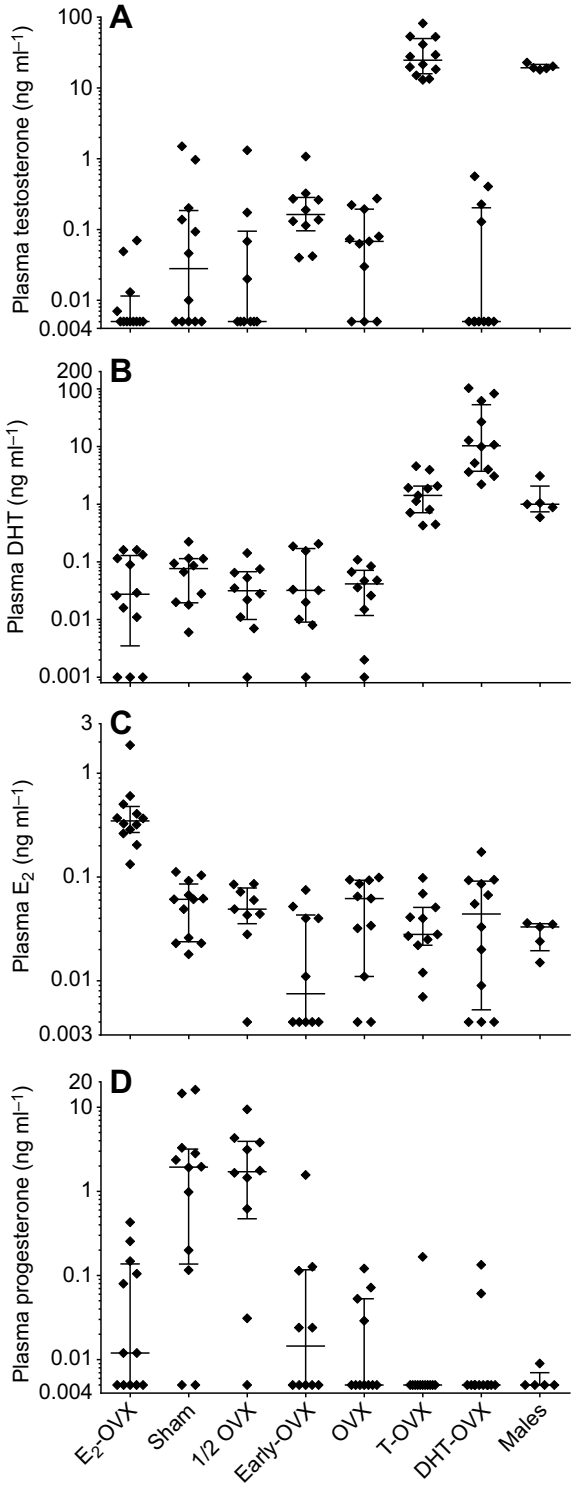

Fig. 1. Plasma hormone levels in experimental animals at the termination of the experiment. (A) Testosterone, (B) dihydrotestosterone (DHT), (C) estradiol (E_2) and (D) progesterone. Experimental groups were as follows: E_2-OVX, ovariectomized females treated with estradiol; Sham, sham-operated reproducing females; 1/2 OVX, unilaterally ovariectomized reproducing females; Early-OVX, early ovariectomized females; OVX, ovariectomized females; T-OVX, ovariectomized females treated with testosterone; DHT-OVX, ovariectomized females treated with dihydrotestosterone; Males, Intact males. Each point represents a single individual; points with testosterone levels of 0.005 ng ml^{-1}, DHT levels of 0.001 ng ml^{-1}, E_2 levels of 0.004 ng ml^{-1} and progesterone levels of 0.005 ng ml^{-1} represent individuals with hormone levels below the limit of detection. Median and inner quartiles are shown.

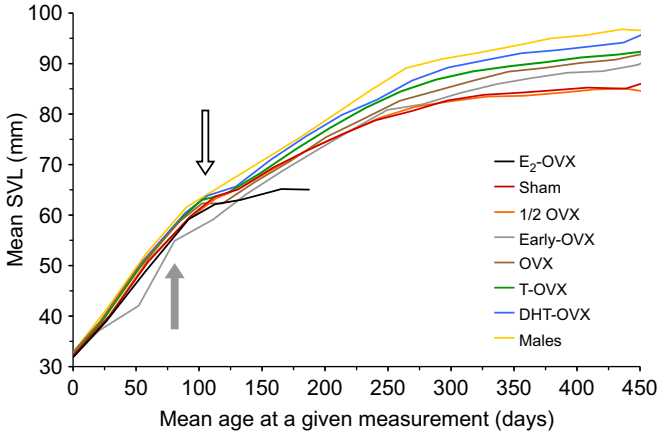

Fig. 2. Growth trajectory for each experimental treatment group during the whole experiment. Note that mean snout–vent length (SVL) and mean age of each treatment group at a given measurement were used. The grey arrow indicates the time of surgery for Early-OVX females, whereas the open arrow estimates the time of surgery for the rest of the female treatment groups. Experimental groups as in Fig. 1.

including Sham females, showed comparable levels of E_2 (Kruskal–Wallis ANOVA: $H_{6,N=70}$=8.86, P=0.18; Fig. 1C). The levels of progesterone also differed among treatment groups (Kruskal–Wallis ANOVA: $H_{7,N=84}$=39.90, P<0.001; Fig. 1D). The Sham and 1/2 OVX females had similar, high levels of progesterone (Mann–Whitney U-test: U=59.0, P=0.95; Fig. 1D), while significantly lower progesterone levels were shared by the rest of the groups (Kruskal–Wallis ANOVA: $H_{5,N=62}$=10.18, P=0.07; Fig. 1D).

The asymptotic von Bertalanffy model explained 96.3–99.8% of the total variability in the growth pattern in each individual, demonstrating the applicability of this growth model. The linear regression between asymptotic SVL and the SVL at the last measurement in the growth experiment across all experimental animals showed a very close relationship between these two variables: asymptotic SVL=1.017×(SVL at the last measurement) +4.25; R^2=0.91. The slope of the regression was not significantly different from 1.0 (95% confidence interval, 0.95 to 1.09) and the intercept was not significantly different from 0 (95% confidence interval, −1.90 to 10.40), demonstrating proportional increase of these two variables and overall similarity of their values. Moreover, the experimental geckos showed substantial deceleration of growth in all treatment groups before the termination of the growth experiment (visualized in Fig. 2; for more details, see Fig. S1), which further supports the applicability of the asymptotic SVL as a measure of size for comparison among the treatment groups in this study.

The treatment groups differed significantly in asymptotic SVL (ANOVA: $F_{7,77}$=13.45, P≪0.001; Fig. 3). OVX females, Early-OVX females, DHT-OVX females, T-OVX females and Intact males reached comparable asymptotic SVL (*post hoc* Fisher LSD: P>0.18 in comparisons between these groups) and were significantly larger than the remaining groups (*post hoc* Fisher LSD: P<0.048 in all comparisons). Sham and 1/2 OVX females reached comparable intermediate asymptotic SVL (*post hoc* Fisher LSD: P=0.76 for comparison between these two groups) and E_2-OVX females attained the smallest asymptotic SVL of all treatment groups (*post hoc* Fisher LSD: P<0.001 in all comparisons). This pattern did not change when Intact males or E_2-OVX females were excluded from the analysis with the exception of a marginally non-significant result in the *post hoc* comparison between Sham and

Journal of Experimental Biology (2017) 220, 787-795 doi:10.1242/jeb.146597

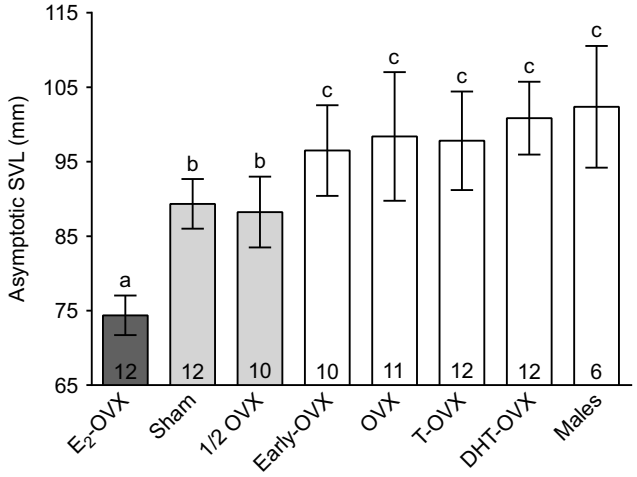

Fig. 3. Asymptotic SVL in experimental animals. Experimental groups as in Fig. 1 (*N* values shown in the bars). Data are means and 95% confidence intervals. Significant differences between treatment groups were confirmed by ANOVA ($F_{7,77}$=13.45, P≪0.001). Different letters and column shading indicate statistically different groups revealed by Fisher LSD *post hoc* test. When E_2-OVX females were excluded from the analysis, the differences between Sham and Early-OVX females became marginally non-significant in the *post hoc* comparison (P=0.062).

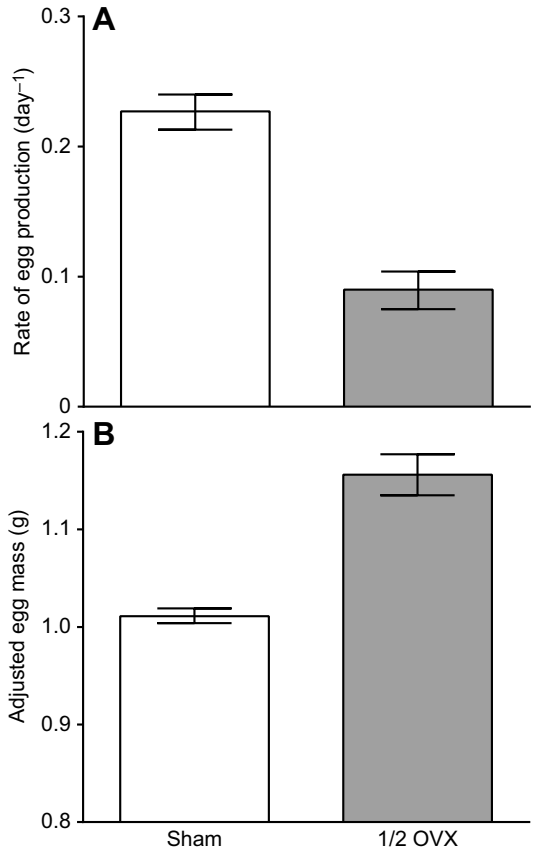

Fig. 4. Comparison of the rate of egg production and egg mass between Sham and 1/2 OVX females. (A) Rate of egg production for Sham females (N=12) and 1/2 OVX females (N=10; Mann–Whitney U-test: U<0.01, P≪0.001). (B) Mass of eggs produced by Sham and 1/2 OVX females during the experiment (N=785 eggs of known mass), statistically adjusted for female identity and female SVL at oviposition (general linear models, comparison between treatment groups: $F_{1,20}$=16.68, P≪0.001). Data are means and 95% confidence intervals.

Early-OVX females (P=0.062) in the case of E_2-OVX exclusion. We report these results both with and without the E_2-OVX group, because they can be affected by potential negative effects of exogenous E_2 on animal health as observed in the preliminary test. At the time of cessation of the experiment, body condition was comparable among all treatment groups (full-factorial ANCOVA: differences neither in interaction nor in factor group, $F_{7,69}$<0.81, P>0.59 in both cases).

Altogether, Sham and 1/2 OVX females laid 911 eggs (785 unbroken eggs of known mass) during the experiment. The two groups had similar asymptotic SVL, but differed dramatically in reproductive output. The rate of egg production was twice as high in Sham females as in 1/2 OVX females (Mann–Whitney U-test: U<0.01, P≪0.001; Fig. 4A). However, adjusted egg mass of 1/2 OVX females was 14% heavier than in Sham females (general linear models, comparison between treatment groups: $F_{1,20}$=16.68, P≪0.001; Fig. 4B).

DISCUSSION

Our hormonal and surgical manipulations had a significant effect on female growth and reproduction in this male-larger gecko species (Figs 2–4; Fig. S1). The induction of male-typical size in certain treatment groups of females suggests that in this species with genotypic sex determination, SSD is probably not controlled by the linkage of growth-controlling genes to the sex-specific parts of sex chromosomes, but to different expressions of autosomal or pseudoautosomal genes. Out of the three competing hypotheses on the proximate mechanism controlling the ontogeny of SSD, i.e. the reproductive cost hypothesis, the male androgen hypothesis and the ovarian hormone hypothesis, only the latter was supported by the results of our experiments in all aspects.

One could argue that the reproductive cost hypothesis is supported by the larger, male-typical asymptotic SVL in OVX and Early-OVX females compared with regularly breeding females (Fig. 3). We observed the same trend in our previous experiment (Starostová et al., 2013), but when we applied the additional technique of preventing reproduction, we found little support for the reproductive cost hypothesis. The socially isolated virgin females with intact gonads did not lay eggs, but reached the same final SVL as the regularly reproducing females (Starostová et al., 2013). However, both these all-or-nothing techniques for the prevention of reproduction have limitations. The function of the ovaries is not only to produce eggs – they are also very active organs hormonally and their removal can thus have severe side-effects. At the same time, the isolation of sham-operated females leads to the prevention of follicular, and hence hormonal, cycling (Weiser et al., 2012), which is also certainly an unnatural condition with potential side-effects on growth. One of the important features of this study was therefore the inclusion of 1/2 OVX females with a highly reduced, but not totally removed, energy allocation to reproduction, along with the preserved reproductive cycles and circulating levels of ovarian hormones (Figs 1C,D and 4). The regularly reproducing Sham females and 1/2 OVX females were comparable in asymptotic SVL (Fig. 3), which demonstrates that at least under the conditions of the experiment, the amount of energy allocated to reproduction does not considerably affect structural growth. This conclusion is also in accord with our previous experiment on the manipulation of food levels in *P. picta* females, which showed that the allocation to structural growth is at least to a certain degree independent from the allocation to reproduction (Kubička and Kratochvíl, 2009). The experiment conducted in parallel in *A. sagrei* also supports the

Journal of Experimental Biology (2017) 220, 787-795 doi:10.1242/jeb.146597

results described here (Cox et al., 2014). In this anole, 1/2 OVX and intact females, i.e. both reproductively active groups, formed a statistically homogeneous group in the comparison of the increment of SVL after surgery, and significantly increased growth was exhibited only in the OVX females of the anole. The consistent results of 1/2 OVX in these two species suggest that the direct energy allocation to reproduction by females is not the major driver of the ontogeny of SSD in lizards.

In three previous independent experiments, we documented that male castration did not affect male growth in the male-larger *P. picta* (Starostová et al., 2013; Kubička et al., 2015) or in the female-larger gecko *A. felinus* (Kubička et al., 2013), which does not support the male androgen hypothesis. However, the induction of male-typical growth by exogenous testosterone in non-OVX females in these two geckos (Kubička et al., 2013; Starostová et al., 2013) as well as in other squamates (mostly studied in female-larger species: reviewed in Starostová et al., 2013; but also recently in the male-larger anole *A. sagrei*: Cox et al., 2015) still leaves open the possibility that testicular androgens can cause growth masculinization. This suggestion was based on the parsimonious expectation that elevated levels of androgens in females should have the same effect in both sexes and therefore influence growth in males and females in the same way. However, we instead suggest that exogenous testosterone may interfere with normal ovarian function and hence normal hormone production, and that it could influence growth by preventing the feminizing effect of female gonads. This hypothesis was indirectly supported by the considerably reduced size and obvious non-functionality of ovaries (i.e. absence of vitellogenic follicles) in testosterone-treated non-OVX females (Starostová et al., 2013). The current study presents a more direct test. In support of the hypothesis that androgens interfere with the ovarian effect on growth but do not have an effect on growth themselves, we found that both T-OVX and DHT-OVX females had a very similar growth to that of OVX females. Moreover, as DHT is a non-aromatizable androgen, we have additionally shown that the aromatization of the circulating testosterone to oestrogens is also not involved in the control of SSD in this species (Figs 2, 3; Fig. S1).

The reproductive cost and male androgen hypotheses could thus be excluded as explanations for the mechanism of the proximate control of the ontogeny of SSD in this species, while the ovarian hormone hypothesis seems to be supported by several lines of evidence. Most importantly, OVX and Early-OVX females showed male-typical growth, which indicates that ovarian hormones influence female growth. The results do not support the possibility that the period of exposure to ovarian hormones affects final structural body size in gecko females, as OVX and Early-OVX females shared similar asymptotic SVL even though the surgery was performed on average 1 month earlier in the latter group (Figs 2, 3; Fig. S1). Nevertheless, the true differences in exposure to ovarian hormones between these two groups were not directly determined and the ontogeny of the levels of ovarian hormones influencing female growth should be determined in future studies. Moreover, it is not known which ovarian hormone(s) is involved in the ontogeny of SSD and how it affects female growth in *P. picta*. The experimental increment of E_2 levels in OVX females had a dramatic negative effect on asymptotic SVL (Figs 1C, 2 and 3), which could be taken as direct support for the negative effect of E_2 on female growth. However, despite only low doses of E_2 being applied in this study, this hormone seems to have had a rather detrimental cumulative effect and hence the indirect effect of exogenous E_2 on growth through the general negative effect on animal health cannot be excluded. Although the levels of E_2 in E_2-OVX females were significantly higher than in other treatment groups, including reproductively active females (Fig. 1C), they fit (with the exception of one outlier) into the physiological range we previously described in reproductively active females (Weiser et al., 2012). It is therefore not clear why these levels of E_2 had such a detrimental effect in only one treatment group. One possible cause of this observed cytotoxicity of E_2 could be the insufficient cycling of E_2 levels in our experimental E_2-OVX females, which is typical for reproducing females of *P. picta* (see Weiser et al., 2012). Future direct testing of the E_2 effect on growth should be based on protocols that allow the cycling of E_2 levels in OVX females. Moreover, our monitoring of the hormonal profile of the experimental groups suggests another ovarian hormone candidate that influences female growth: Sham and 1/2 OVX females formed a homogeneous group reaching female-typical asymptotic SVL, and these two groups differed from the others in having high levels of progesterone (Fig. 1D). In mammals, it was shown that progesterone has a stimulatory effect on bone formation in female rats and thus has the potential to influence skeletal growth in vertebrates (Schmidt et al., 2000). Direct testing of the effect of progesterone on female growth in squamates should therefore be pursued in the future. Additionally, non-ovarian hormones could also be directly involved in sex-specific growth and they could act indirectly via interactions with other hormones. Evidence already exists that, for example, stress and thyroid hormones (Sävendahl, 2012; Williams, 2013), which might be influenced by levels of ovarian hormones, also have growth effects.

Our experimental study has provided an interesting insight into the alternative solutions of the trade-offs concerning the division of resources within a single clutch, current and future reproduction, and the potential of compensatory energy allocation to reproduction among vertebrates. Vertebrates possessing variable numbers of progeny in a clutch or litter react to unilateral ovariectomy by compensatory recruitment of additional follicles in the remaining ovary (lizard: Jones et al., 1977; fish: Tyler et al., 1994; mammal: Greenwald, 1961; Gosden et al., 1989). However, animals such as anoles and geckos, with an invariant clutch size typified by a more or less fixed number of offspring per clutch and ovulating just a single egg per ovary at time (Jones et al., 1976; Kratochvíl and Frynta, 2006; Kratochvíl and Kubička, 2007; Weiser et al., 2012; Meiri et al., 2015), do not have this possibility and can only compensate for unilateral ovariectomy by increasing the rate of clutch laying or by increasing egg size. The rate of egg production was reduced by half in 1/2 OVX females in comparison to Sham females (Fig. 4A), which suggests that females in both groups laid eggs at a maximum possible rate and 1/2 OVX could not further shorten interclutch intervals. However, 1/2 OVX females produced about 14% heavier eggs than Sham females of the same structural size (Fig. 4B), inferring that egg size is not maximized in Sham females and that they compromise size with number and still have the capacity to produce larger and/or heavier eggs. In contrast, similar manipulation in the small iguanian lizard *A. sagrei* with an invariant clutch size also resulted in the generation of approximately half the number of progeny per month in 1/2 OVX females in comparison to sham females, but the hatchling size in the two groups was comparable (data in fig. 2 of Cox et al., 2014). These results suggest that in this anole, females are not able to compensate for unilateral ovariectomy, perhaps because non-manipulated females already produce eggs of maximum possible size. Nevertheless, the differences between the gecko in our study and this anole might be attributed to differences in clutch formation, leading to a different potential for compensatory egg size increase

in 1/2 OVX females. Anoles and geckos evolved invariant clutch size independently (Kratochvíl and Kubička, 2007; Meiri et al., 2015). In most geckos, two eggs forming a clutch are made in parallel, one in each ovary, while anole clutches consist of a single egg and the ovaries alternate in follicle ovulation between subsequent clutches (Jones et al., 1975, 1976).

In conclusion, our long-lasting and complex experiment largely supports the hypothesis that ovarian hormones are the major contributors in the ontogeny of SSD in the studied gecko and we have summarized the evidence that this might also apply for other reptiles (Starostová et al., 2013; this study). In the context of vertebrates, our conclusions are not so surprising. The effects of both E_2 and progesterone on skeletal growth have been well documented (Cutler, 1997; Schmidt et al., 2000; Weise et al., 2001). Of particular interest is that, depending on its concentration, E_2 might have a positive as well as a negative effect on bone growth (Cutler, 1997; Weise et al., 2001), which might explain why ovarian hormones could control female growth and lead to SSD in both female- and male-larger reptiles (Starostová et al., 2013). The independence of SSD from male gonadal androgens explains why SSD is so evolutionarily plastic among lizards including geckos of the genus *Paroedura* (Starostová et al., 2010), although males of all species are very likely to possess high circulating levels of gonadal androgens a long time before growth becomes sexually dimorphic in ontogeny. Nevertheless, further direct studies involving ovarian hormones in phylogenetically broader members of squamate reptiles should be carried out in the future in order to test these hypotheses.

Acknowledgements
We thank Marie Altmanová and Adam Tureček for their help with the experimental procedures, and Christopher Johnson for language revision. We also thank Robert Cox and two anonymous reviewers for critical comments.

Competing interests
The authors declare no competing or financial interests.

Author contributions
L. Kubička and L. Kratochvíl developed the main idea of the experiment. L. Kubička, J.Č. and T.S. conducted the experimental procedures. L. Kubička and L. Kratochvíl performed the statistical analysis and wrote the first draft of the manuscript. All authors then reviewed the manuscript.

Funding
This research was supported by the Czech Science Foundation (Grantová agentura České republiky; project number GP13-19767P to L. Kubička and GA16-24619S to all authors).

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.146597.supplemental

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