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

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Summary statement

We demonstrate that sexual size dimorphism in a male-larger lizard is influenced neither by male gonadal androgens, nor by female reproductive allocation, but that it is controlled by ovarian hormones.

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 did not support the hypotheses that SSD reflects direct energy allocation to reproduction and that male gonadal androgens are involved. On the other hand, 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.

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Introduction

Sexual size dimorphism (SSD), understood as the differences 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 e.g. 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 e.g. 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), males and females in 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 costs 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). On the other hand, 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 driven by a simple division of available energy, but may be controlled by specific endogenous mechanisms such as gonadal hormones, driving not just energetically demanding processes, but directly regulating growth (reviewed in Flatt and Heyland, 2011). A single 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).

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), nor in the female-larger gecko *Aeluroscalabotes felinus* (Kubička et al., 2013). On the other hand, and at first sight paradoxically, the induction of male-typical levels of circulating testosterone (T) in females led to a "masculinization" of growth in both female-larger and male-larger lizard species. Non-ovariectomized females treated by exogenous T attained larger, male-like final snout-vent lengths (SVL) in the male-larger lizard species (Starostová et al., 2013; Cox et al., 2015), while exogenous T 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 T effects 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 T 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 costs hypothesis, ovariectomized lizard females exhibited enhanced growth and/or attain 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 intact 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 control hypothesis can explain some otherwise seemingly paradoxical phenomena in lizards not resolved by the "reproductive costs hypothesis" and "male androgen hypothesis", it has not yet been completely explored nor 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 T treatment, on female growth in comparison to 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 costs 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 control 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 T was carried out in order to test whether there is a direct masculinization effect of T as predicted by the male androgen hypothesis, or whether the effect of exogenous T on female growth in non-OVX females seen in previous experiments (e.g. Starostová et al., 2013 and citation therein; Kubička et al., 2013; Cox et al., 2015) was a result of interference of T with normal ovarian function, as predicted by the ovarian control hypothesis. Estrogens 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; Cutler, 1997 and Grumbach, 2000 for evidence in humans). Elevated levels of T may help restore concentrations of E_2 via aromatization in T-treated OVX females so we also

included a group of OVX females treated with DHT, a non-aromatizable androgen, to test whether elevated T would affect growth indirectly via its 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 if the period of the 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 four 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 possess 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

Mated females from our captive breeding colony were individually housed in a climatic chamber maintained at 27 °C with a 12L:12D light 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 chambers. Upon hatching, animals were housed singly in standardized plastic boxes (20 x 20 x 10 cm) with sand substrate, shelter, and a water dish in the same climatic chambers where they were incubated. A constant 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 fed crickets (*Gryllus assimilis*) dusted with vitamins (Roboran, Univit, Czech Republic) twice weekly to satiety. Water enriched by calcium (Vitacalcin, Zentiva, Czech Republic) was always available but was replaced once every two 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. three to four 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 in 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 T (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 smaller body size and at younger age (median 78 days) (Early OVX females). An additional group of six intact males (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 Sham and1/2 OVX females at regular monthly intervals. Due to 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 the females recruited when they reached a body mass of 3-4 grams (average of 3.43 grams) in the case of Early OVX group, and 5-6 grams (average of 5.37 grams) in the remaining treatment groups. For surgery, the females were anesthetized using a combination of an intramuscular injection of ketamine (Narkamon 5%, Spofa a.s., Prague, Czech Republic; 130 μg/g 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, “sham” surgeries were performed, in which ventral incisions were made to expose and manipulate the ovaries while leaving them unharmed. The incision was closed using Maprolen® surgical sutures (Catgut GmbH, Markneukirchen, Germany) and 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 the anesthesia.

The stitches were removed once the wound had healed sufficiently (within three 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 seven 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 T 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 T; 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 T and 0.25 μg of E_2 were 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 similarly to hormonally 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 terminated using 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 females) were excluded from all analyses. To determine whether our animals already decelerated growth considerably, we plotted SVL on time for each individual after each measurement. We also compared growth trajectories of control males and reproductively

active females to 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 our 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 the potential suffering of the experimental animals we ended the growth experiment in this group after 6 to 13 weeks of treatment. In addition, we had to euthanize three Sham females prematurely as well due to an unspecific paralysis observed in these females. One control male died four days prior the end of the experiment without any obvious reason. As his growth pattern did not differ from other males, we decided to include his growth data for subsequent analyses, although this exclusion did not change the significance of any results.

Hormone levels validation

Plasma hormonal levels were used as measurements of the responses of all animals to the experimental treatments. Whole blood plasma was analysed for levels of E_2, DHT, T and progesterone (P) at the Institute of Endocrinology (Prague, Czech Republic). For the detection of P and T 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 liquid

chromatography Eksigent ultraLC 110 system (Redwood City, CA, USA). Detection of analytes was performed on an API 3200 mass spectrometer (AB Sciex, Concord, Canada) with the electrospray ionization probe operating in a positive mode. Their quantification was determined using calibration curves based on known analyte concentrations. The limits of detection were 0.005 ng per ml for P and T and 0.004 ng per ml 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 radioimmunoassay 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 T present due to its cross-reaction with used 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 per ml.

As levels of hormones were measured in three independent ways (two LC-MS/MS, one RIA), it required 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 different number of measurements between E_2, DHT and T with P. These cases are highlighted in the results.

Statistical analysis

All statistical analyses were conducted using Statistica version 10.0 (StatSoft, Tulsa, USA) or GraphPad Prism (version 6.07; GraphPad Software, San Diego, 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 $\alpha = 0.05$, 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 were maximalized 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 female 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 sacrificed 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 measurements errors of a single measurement. Due to these benefits, we applied the expression of the asymptotic von Bertalanffy model to our raw data:

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

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 the description of growth in lizards 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 both 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 (day^{-1}) by nonparametric 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" and 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: $F_{7, 77} = 0.85$, $p = 0.55$; please 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 in body mass (ANOVA: $F_{5, 63} = 0.84$, $p = 0.53$). On average, Early OVX females were of 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; please see Table S2 for summary statistics).

The hormone assays verified treatment of all individuals (Fig. 1). In some cases the hormonal 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. The T plasma levels differed significantly between treatment groups (Kruskal-Wallis ANOVA: $H_{7, N = 84} = 51.69$, $p < 0.001$, Fig. 1A). The T levels of T OVX females were comparable to the T 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, all T levels were close to the low T 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). The 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, 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 P 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 P (Mann-Whitney U test: $U = 59.0$, $p = 0.95$; Fig. 1D), while significantly lower P 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 to 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 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 (visualised in Fig. 2, for more details please see the Fig. S1), which further supports the applicability of the asymptotic SVL as a measure of size for the comparison among the treatment groups in this study.

The treatment groups differed significantly in the 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 body size (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 only exception of marginally non-significant result in the post-hoc comparison between Sham and Early OVX females ($p = 0.062$) in the case of E_2 OVX exclusion. We report these results both with and without the group of E_2 OVX females, 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 the 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. Both groups have similar asymptotic SVL, but differed dramatically in reproductive output. The rate of egg production was twice as high in Sham females than in 1/2 OVX females (Mann-Whitney U test: $U = 0.0$, $p << 0.001$; Fig. 4A). However, adjusted eggs 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 (Fig. 2, 3, 4 and S1). The induction of male-typical size in certain treatment groups of females suggests that in this species with genotypic sex determination, SSD is likely 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 tested competing hypotheses on the proximate mechanism controlling the ontogeny of SSD, i.e. the reproductive costs hypothesis, the male androgen hypothesis, and the ovarian control 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 both OVX treatment groups compared to regularly breeding females (Fig. 3). We observed the same trend in our previous experiment, but when we have applied the additional technique of preventing reproduction, we have 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 of the prevention of reproduction have limitations. The function of the ovaries is not only to produce eggs, but they are also very active organs hormonally and their removal can thus have severe side-effects. At the same time, the isolation of intact 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

circulation levels of ovarian hormones (Fig. 1C, 1D 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 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 homogenous 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) nor 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 T 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 the possibility open 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 T 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 T-treated non-OVX females (Starostová et al., 2013). The current study presents a more direct test. In support for 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 and DHT OVX females have 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 T to estrogens is also not involved in the control of SSD in this species (Fig. 2, 3 and S1).

The reproductive costs and androgen control 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 control hypothesis seems to be supported by several lines of evidence. Most importantly, OVX and Early OVX females reached male-typical growth, which shows 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 both OVX and Early OVX females share similar asymptotic SVL even though the surgery was performed on average one month earlier in the latter group (Fig. 2, 3 and 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 does 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 (Fig. 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 compared to 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 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 which 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 which influences female growth: Sham and 1/2 OVX females formed a homogeneous group reaching female-typical asymptotic SVL and these were the only two groups which differed from the others in high levels of P (Fig. 1D). In mammals, it was shown that P seems to have 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 P 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 or Gosden et al., 1989). However, animals such as anoles and geckos, with an invariant clutch size typified with 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 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 of producing larger and/or heavier eggs. In contrast, similar manipulation in the small iguanid 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 both groups were comparable (data in Fig. 2 in Cox et al., 2014). These results suggest that in this anole, females are not able to compensate for unilateral ovariectomy, maybe 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 the differences in the way of 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 summarize the evidence that this might also apply for other reptiles (Starostová et al., 2013 and this study). In the context of vertebrates, our conclusions are not so surprising. The effects of both E_2 and P 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 on male gonadal androgens explains why SSD is so evolutionary plastic among lizards including geckos of the genus *Paroedura* (Starostová et al., 2010), although males of all species very likely 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.

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Competing interests

No competing interests declared.

Author contributions

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

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Figures

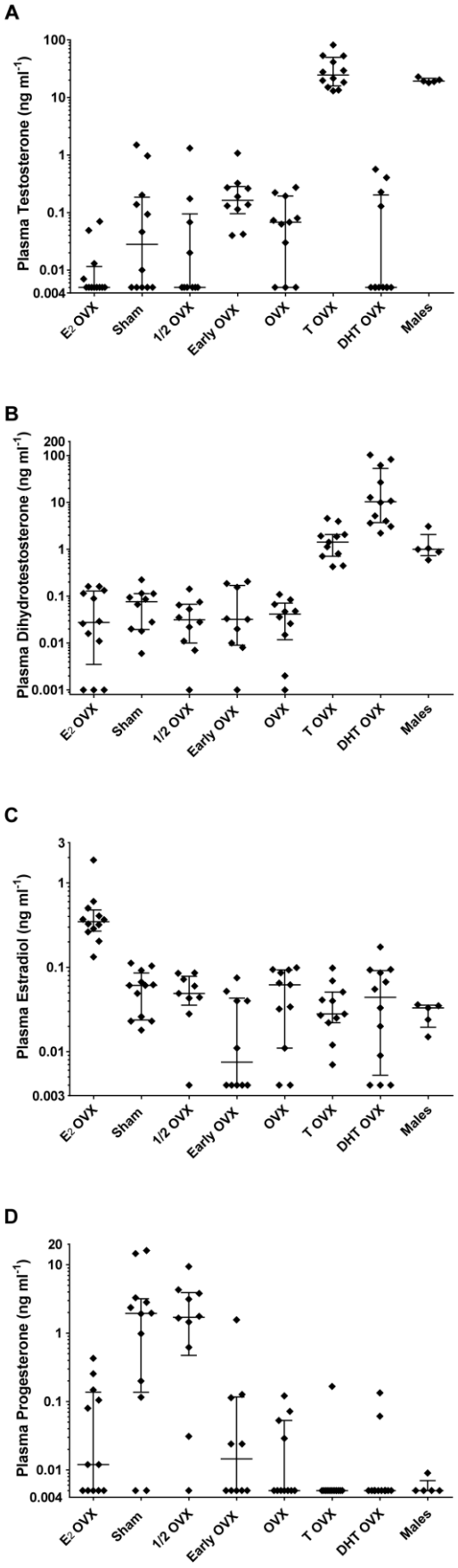


Fig. 1. Concentration of plasma levels of testosterone (A), dihydrotestosterone (B), estradiol (C) and progesterone (D) in experimental animals at the time of experiment termination. Experimental group abbreviations: E_2 OVX, ovariectomized females treated with estradiol; Sham, sham-operated reproducing 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 the testosterone levels 0.005 ng per ml, dihydrotestosterone levels 0.001 ng per ml, estradiol levels 0.004 ng per ml and progesterone levels 0.005 ng per ml represent the individuals with the hormonal levels below the limit of detection. Median and inner quartiles are shown.

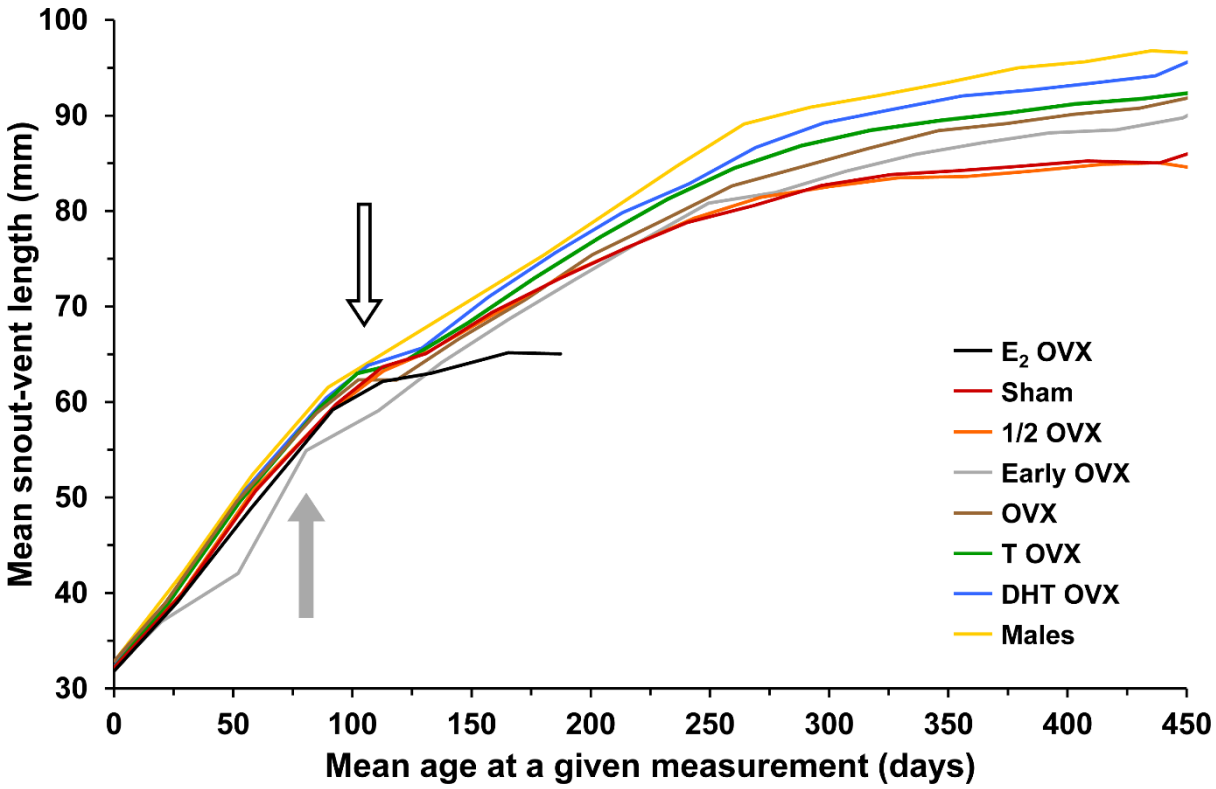


Fig. 2. Growth trajectory for each experimental treatment group during the whole experiment. Note that mean snout-vent length and mean age of each treatment group at a given measurement was used. Grey arrow highlights the time of surgery for Early ovariectomized females, whereas open arrow estimates the time of surgery for the rest of the female treatment groups. Experimental group abbreviations: 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.

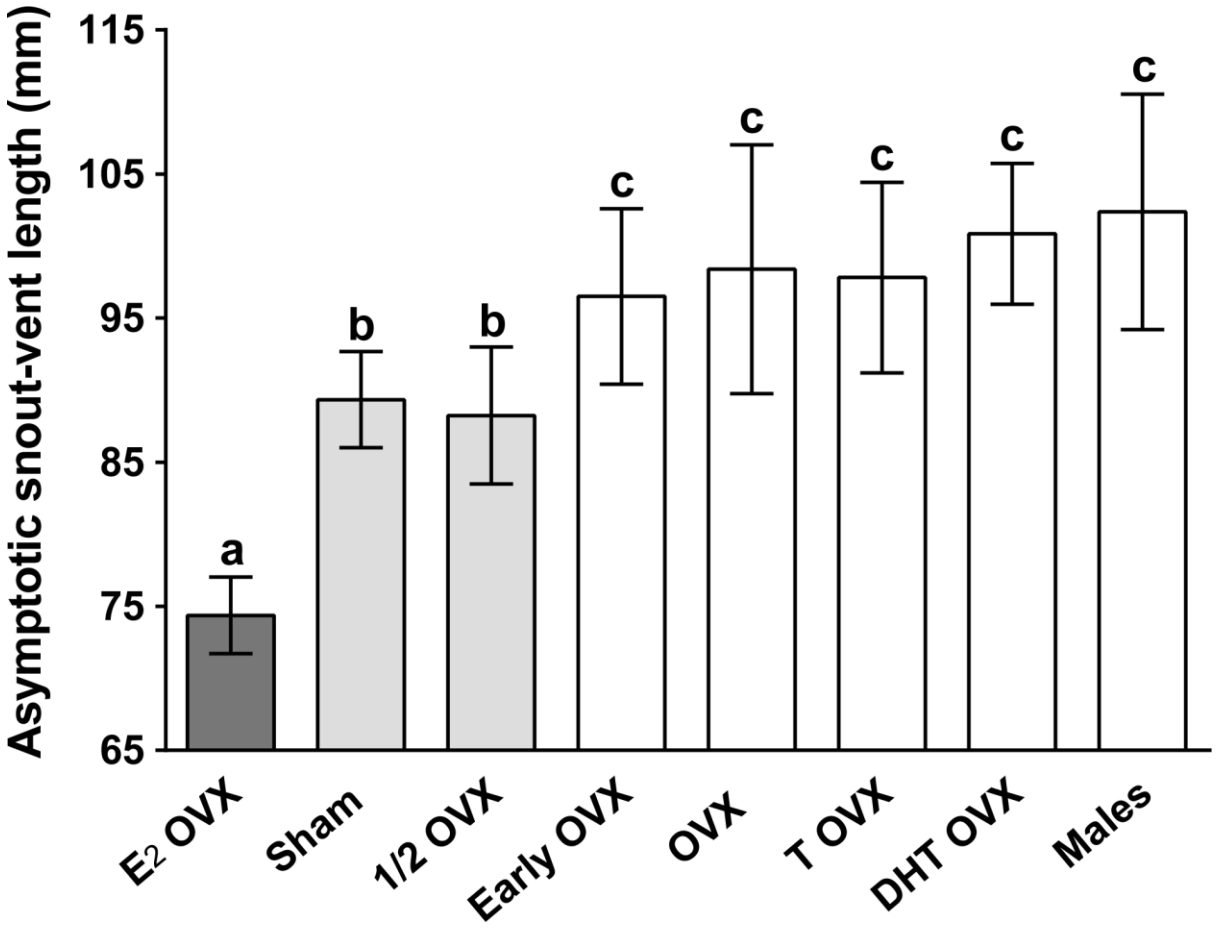
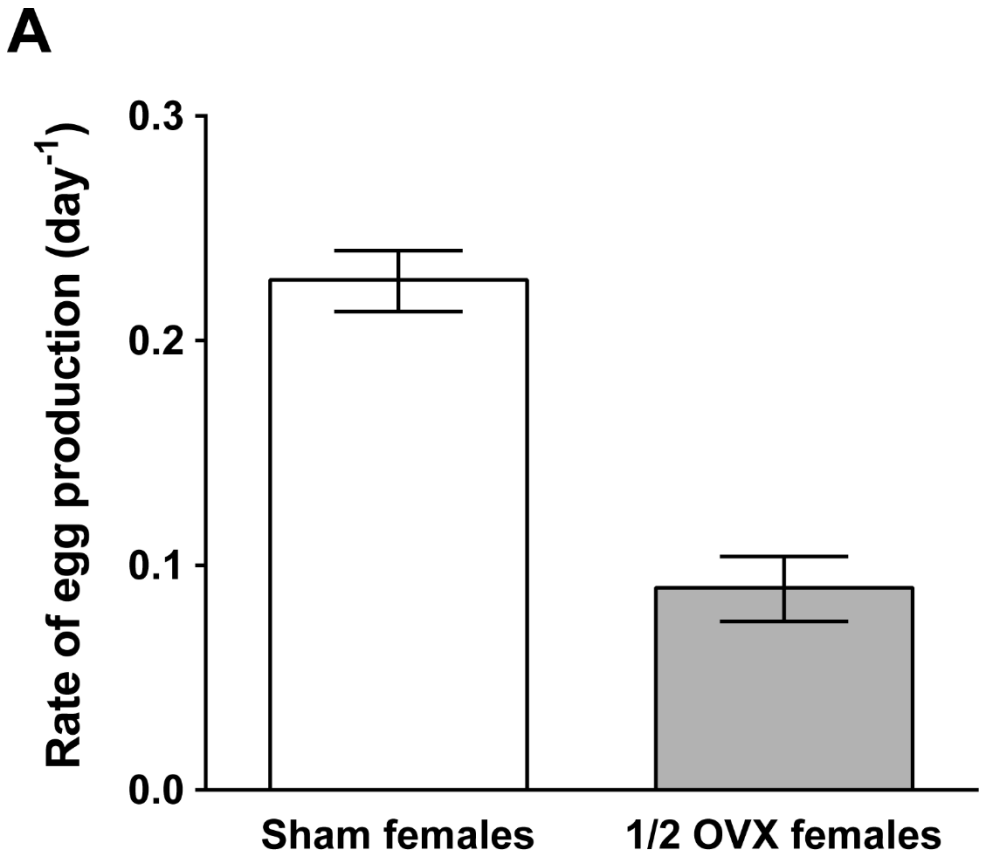


Fig. 3 Asymptotic snout-vent length in experimental animals. Experimental group abbreviations from the left: E_2 OVX, ovariectomized females treated with estradiol (N = 12); Sham, sham-operated reproducing females (N = 12); 1/2 OVX, unilaterally ovariectomized reproducing females (N = 10); Early OVX, early ovariectomized females (N = 10); OVX, ovariectomized females (N = 11); T OVX, ovariectomized females treated with testosterone (N = 12); DHT OVX, ovariectomized females treated with dihydrotestosterone (N = 12); Males, Intact males (N = 6). Means and 95% confidence intervals are given. Significant differences between treatment groups were confirmed by ANOVA ($F_{7, 77} = 13.45$, $p << 0.001$). Letters and column colour pattern denote statistically homogenous 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 ($p = 0.062$).



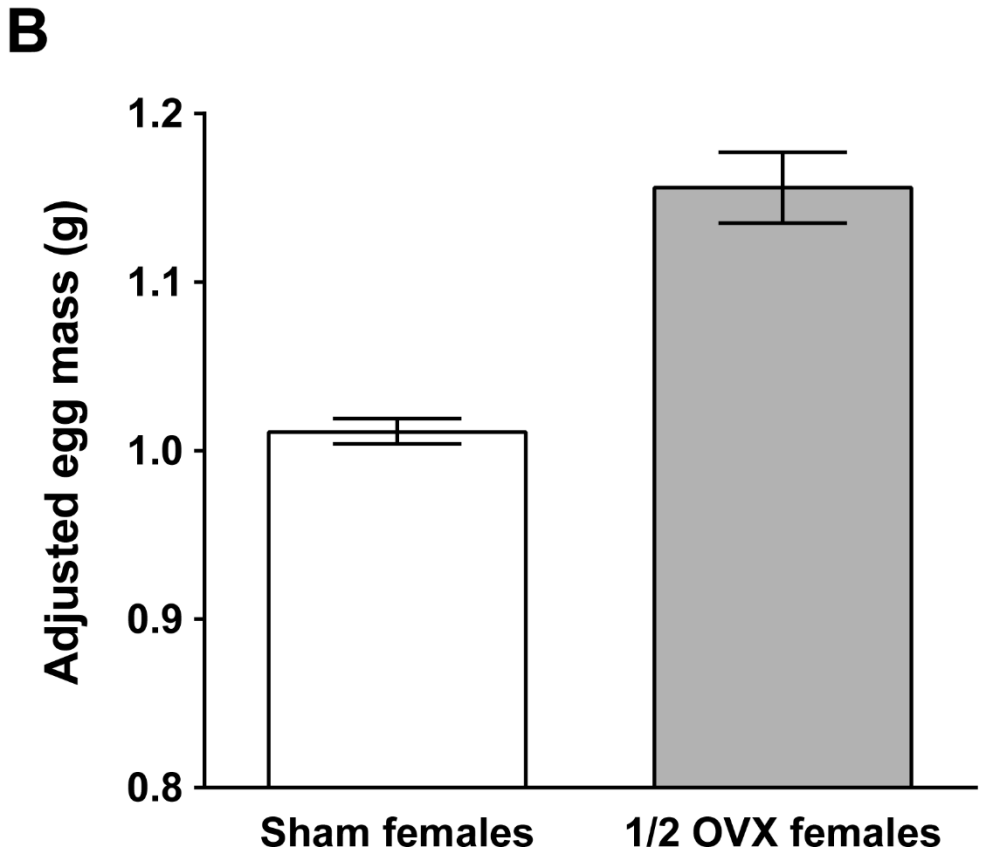
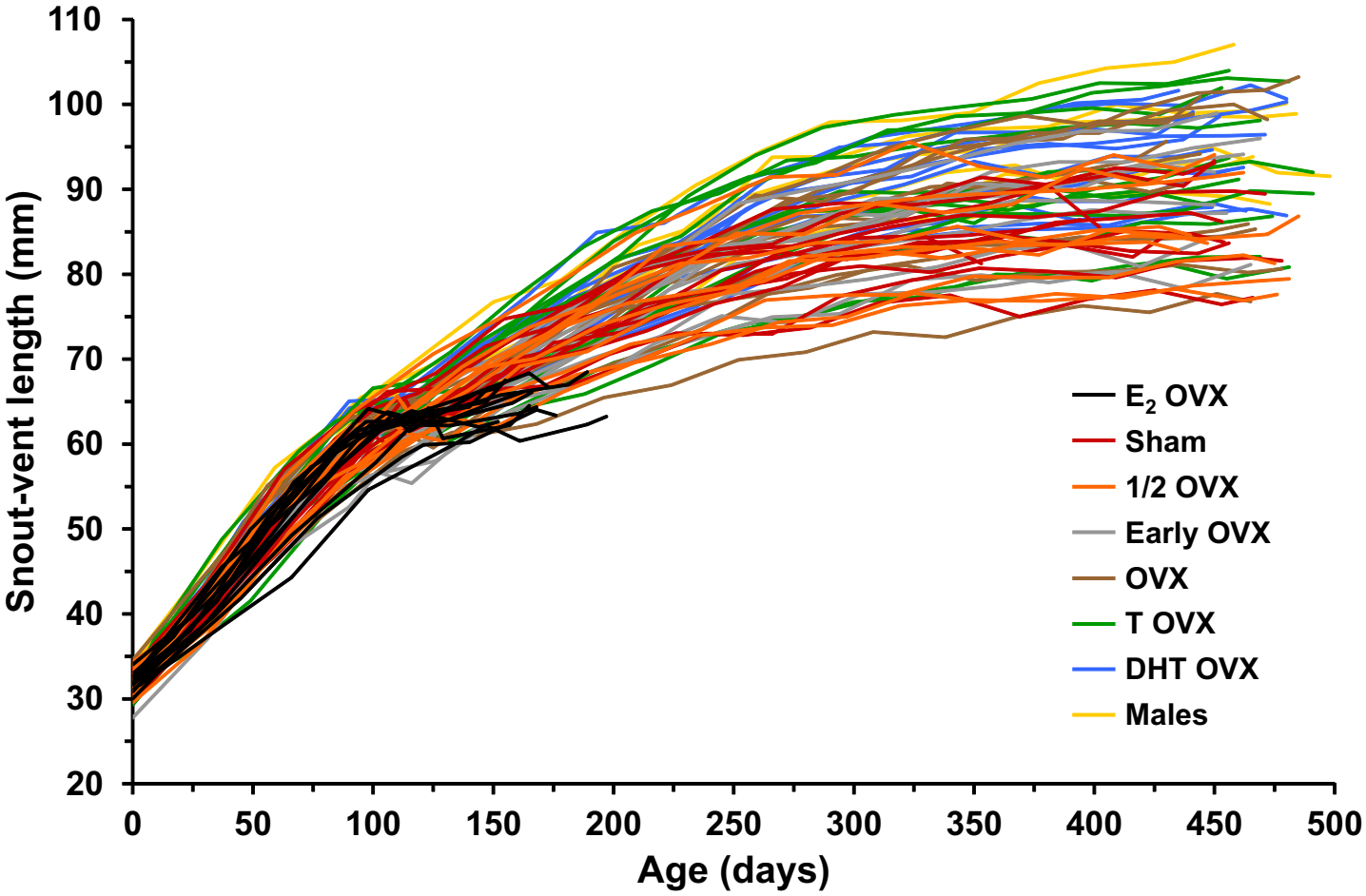


Fig. 4 (A) Comparison of the rate of egg production between sham-operated (Sham females, N = 12) and unilaterally ovariectomized females (1/2 OVX females, N = 10; Mann-Whitney U test: U = 0.0, $p << 0.001$). (B) The mass of eggs produced by Sham and 1/2 OVX females during the experiment (N = 785 eggs of known mass) statistically adjusted for female identity and female SVL at oviposition (General linear models, comparison between treatment groups: $F_{1, 20} = 16.68$, $p << 0.001$). Means and 95% confidence intervals are given.



Supplementary Figure 1. Growth trajectory (snout-vent length increment) of each experimental animal during the whole experiment. Experimental group abbreviations: 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.

Supplementary Table 1. Summary statistic for snout-vent length and body mass in all treatment groups at the time of hatching.

Treatment group	N	Snout-vent length (mm) Median, quartile range	Body mass (g) Mean, 95% confidence interval
E_2 OVX females	12	31.75, 31.34 - 32.46	0.79, 0.72 - 0.87
Sham females	12	32.46, 31.18 - 33.31	0.77, 0.70 - 0.84
1/2 OVX females	10	31.96, 31.19 - 33.50	0.77, 0.66 - 0.88
Early OVX females	10	32.76, 31.12 - 33.42	0.84, 0.74 - 0.94
OVX females	11	33.01, 32.46 - 33.53	0.86, 0.81 - 0.90
T OVX females	12	32.21, 31.86 - 33.13	0.77, 0.71 - 0.83
DHT OVX females	12	32.61, 31.92 - 33.13	0.80, 0.73 - 0.88
Intact males	6	33.26, 31.51 - 34.43	0.81, 0.67 - 0.94

Experimental group abbreviations:

E_2 OVX females	ovariectomized females treated with estradiol
Sham females	sham-operated reproducing females
1/2 OVX females	unilaterally ovariectomized reproducing females
Early OVX females	early ovariectomized females
OVX females	ovariectomized females
T OVX females	ovariectomized females treated with testosterone
DHT OVX females	ovariectomized females treated with dihydrotestosterone

Supplementary Table 2. Summary statistic for age, snout-vent length and body mass of female treatment groups at the time of surgery.

Treatment group	N	Age (days) Median, quartile range	Snout-vent length (mm) Median, quartile range	Body mass (g) Median, quartile range
E_2 OVX females	12	106, 98 - 115	62.39, 61.47 - 63.62	5.33, 5.19 - 5.46
Sham females	12	108, 103 - 115	63.10, 62.26 - 64.16	5.33, 5.29 - 5.47
1/2 OVX females	10	108, 96 - 115	62.55, 61.50 - 63.24	5.38, 5.14 - 5.46
Early OVX females	10	78, 76 - 90	55.30, 54.65 - 56.02	3.45, 3.25 - 3.59
OVX females	11	100, 98 - 110	63.04, 61.37 - 63.7	5.43, 5.17 - 5.52
T OVX females	12	105, 94 - 109	62.71, 62.14 - 63.99	5.47, 5.33 - 5.55
DHT OVX females	12	102, 93 - 107	64.09, 62.79 - 64.26	5.34, 5.17 - 5.53

Experimental group abbreviations:

E_2 OVX females	ovariectomized females treated with estradiol
Sham females	sham-operated reproducing females
1/2 OVX females	unilaterally ovariectomized reproducing females
Early OVX females	early ovariectomized females
OVX females	ovariectomized females
T OVX females	ovariectomized females treated with testosterone
DHT OVX females	ovariectomized females treated with dihydrotestosterone

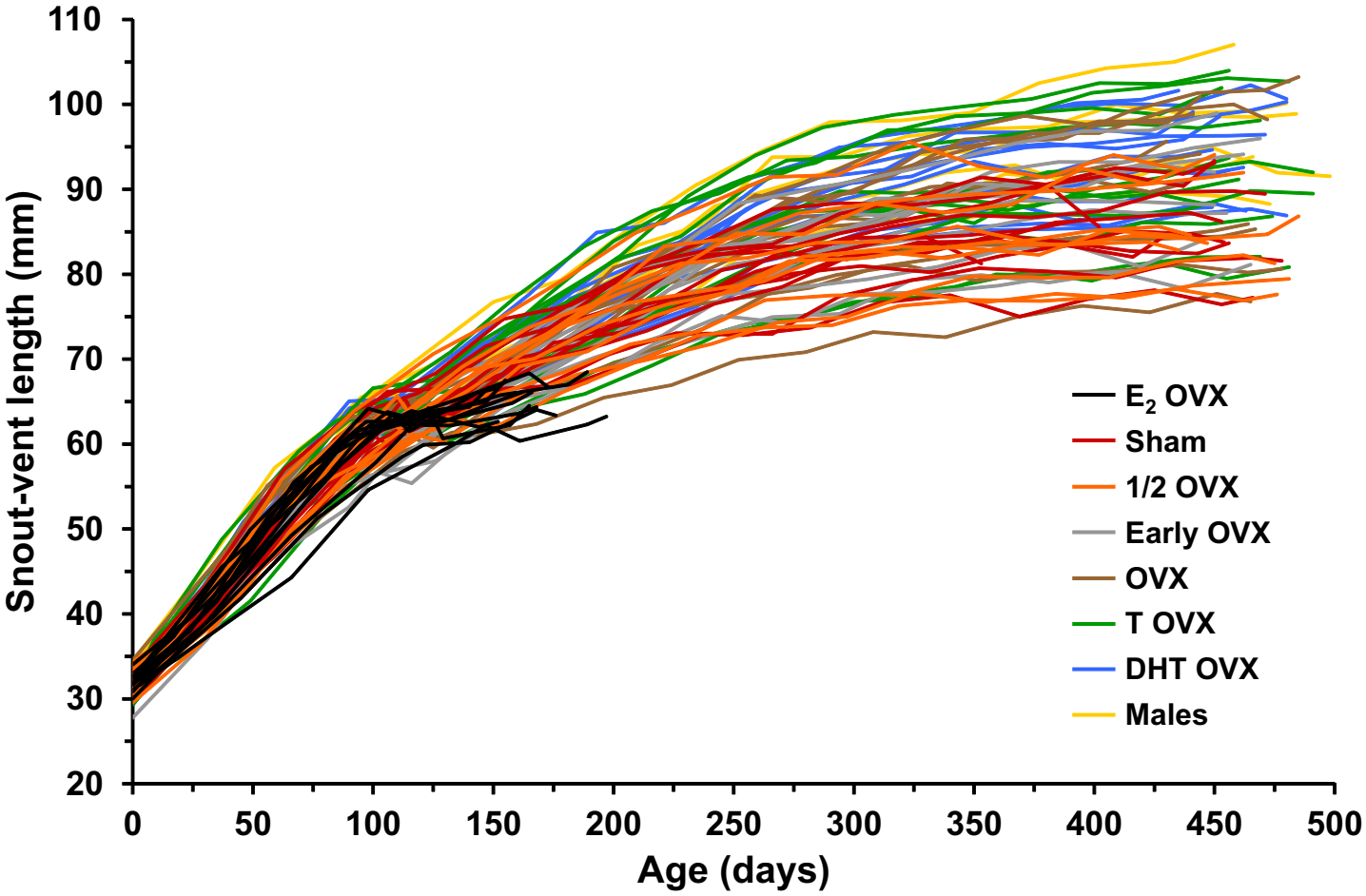


Fig. S1. Growth trajectory (snout-vent length increment) of each experimental animal during the whole experiment. Experimental group abbreviations: E_2 OVX, ovariectomized females treated with estradiol; Sham, sham-operated reproducing females; 1/2 OVX, unilaterally ovariectomized reproducing females; 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.

Table S1. Summary statistic for snout-vent length and body mass in all treatment groups at the time of hatching.

Treatment group	N	Snout-vent length (mm) Median, quartile range	Body mass (g) Mean, 95% confidence interval
E_2 OVX females	12	31.75, 31.34 - 32.46	0.79, 0.72 - 0.87
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Early OVX females	early ovariectomized females
OVX females	ovariectomized females
T OVX females	ovariectomized females treated with testosterone
DHT OVX females	ovariectomized females treated with dihydrotestosterone

Table S2. Summary statistic for age, snout-vent length and body mass of female treatment groups at the time of surgery.

Treatment group	N	Age (days) Median, quartile range	Snout-vent length (mm) Median, quartile range	Body mass (g) Median, quartile range
E_2 OVX females	12	106, 98 - 115	62.39, 61.47 - 63.62	5.33, 5.19 - 5.46
Sham females	12	108, 103 - 115	63.10, 62.26 - 64.16	5.33, 5.29 - 5.47
1/2 OVX females	10	108, 96 - 115	62.55, 61.50 - 63.24	5.38, 5.14 - 5.46
Early OVX females	10	78, 76 - 90	55.30, 54.65 - 56.02	3.45, 3.25 - 3.59
OVX females	11	100, 98 - 110	63.04, 61.37 - 63.7	5.43, 5.17 - 5.52
T OVX females	12	105, 94 - 109	62.71, 62.14 - 63.99	5.47, 5.33 - 5.55
DHT OVX females	12	102, 93 - 107	64.09, 62.79 - 64.26	5.34, 5.17 - 5.53

Experimental group abbreviations:

E_2 OVX females	ovariectomized females treated with estradiol
Sham females	sham-operated reproducing females
1/2 OVX females	unilaterally ovariectomized reproducing females
Early OVX females	early ovariectomized females
OVX females	ovariectomized females
T OVX females	ovariectomized females treated with testosterone
DHT OVX females	ovariectomized females treated with dihydrotestosterone