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# Prenatal exposure to testosterone increases ectoparasite susceptibility in the common lizard (*Lacerta vivipara*)

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High levels of testosterone can benefit individual fitness, for example by increasing growth rate or ornament size, which may result in increased reproductive success. However, testosterone induces costs, such as a suppressed immune system, thereby generating trade-offs between growth or mate acquisition, and immunity. In birds and reptiles, females allocate steroids to their eggs, which may be a mechanism whereby females can influence the phenotype of their offspring. To our knowledge, only the benefits of early androgen exposure have been experimentally investigated to date. However, to understand this phenomenon, the costs also need to be evaluated. We manipulated testosterone levels in eggs of the viviparous common lizard and monitored growth, endurance and post-parturient responses to ectoparasites of the offspring. Testosterone-treated individuals had significantly higher growth rates than controls, but suffered a significant decrease in growth rate when exposed to ticks, whereas the corresponding difference for controls was non-significant. There was no difference in observed parasite load or leucocyte count between manipulated and control offspring. Thus, our results suggest that high testosterone levels during embryonic development have detrimental effects on immune function resulting in reduced growth rate, and that this must be taken into consideration when evaluating the potential adaptive value of maternal androgen allocation to eggs.

**Keywords:** testosterone; yolk; immunocompetence; maternal effects

## 1. INTRODUCTION

Studies of steroid-mediated effects on organismal fitness have recently been placed in the forefront of research in evolutionary biology. For example, by constituting a negative link between secondary sexual characteristics and immune response, honest signalling may be ensured in 'good genes' processes of sexual selection (Folstad & Karter 1992). However, although testosterone has been shown to have a negative impact on immune response in some vertebrates (e.g. Olsson *et al.* 2000; Peters 2000; Casto *et al.* 2001; Hughes & Randolph 2001), other studies have failed to show such effects, or have even found the reversed pattern (Hasselqvist *et al.* 1999; Evans *et al.* 2000; Bilbo & Nelson 2001; Lindström *et al.* 2001).

There are at least two potential pathways in which increased steroid levels can influence parasite load in free-ranging animals. First, testosterone may increase movement, display rates and aggression, which can lead to higher exposure to parasites (Klein 2000). Second, testosterone may increase susceptibility to infection or infestation by directly lowering the immunocompetence of the individual, via suppression of the immune system (Hillgarth & Wingfield 1997). The relative importance of these two factors is unknown (Klein 2000).

During the last decade, researchers have become increasingly aware of the potential impact of prenatal steroid exposure on fitness-related traits in offspring. In canaries (*Serinus canaria*), for example, maternal allocation of yolk steroids increases chick growth rate (Schwabl 1996) and, in some viviparous animals, increased levels of steroids produced by siblings *in utero* can have important and long-term effects on sexually dimorphic traits (Clark & Galef 1998; Uller & Olsson 2003*a*). Because immunocompetence is also a sexually dimorphic trait (Alexander & Stimson 1988; Schuurs & Verheul 1990), prenatal exposure to steroids may influence offspring immune function in juveniles and, perhaps, in adults (Martin 2000). To what degree early exposure to steroids influences immunological traits is virtually unknown, however, and studies on non-mammalian species are completely lacking (Martin 2000).

To look for effects of yolk hormones on offspring immunocompetence, we experimentally manipulated steroid levels in eggs of the viviparous common lizard (*Lacerta vivipara*). We exposed both manipulated and control offspring to a natural ectoparasite (*Ixodes ricinus*) under controlled laboratory conditions and monitored growth rates, physiological performance and aspects of immune response in the offspring.

## 2. MATERIAL AND METHODS

The common lizard is a small (snout–vent length (SVL) of 50–70 mm, mass of 4–6 g) lizard, occurring from northern Spain to northern Scandinavia, and from Ireland to the Ochotic Sea. In all but a few of the southernmost populations, the common lizard is live-bearing, with a reported mean clutch size of four to six young. Males emerge from hibernation in early spring with females emerging one to two weeks later, whereafter the mating season begins.

Forty-five gravid females from three populations in southern Sweden (Asketunnan, Sandsjöbacka and Öjersjö) were captured in late March–early April 2002, and brought to facilities at the Department of Zoology, Göteborg University. The lizards were

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weighed to the nearest 0.01 g, SVL and total length were measured to the nearest mm with a ruler, and head width and head length measured to the nearest 0.01 mm with a pair of callipers. All females had mated in the field, as evident from copulation marks on the belly (inflicted by the male during copulation; Bauwens & Verheyen 1985). From palpation, it was concluded that all females were pre-ovulatory or had recently ovulated. To avoid infertility, females captured prior to ovulation were allowed to copulate in the laboratory. Four to five females were kept in each cage (500 mm × 400 mm × 350 mm), with peat and bark as substrate and rocks and tiles as shelters. A 40 W spotlight allowed thermoregulation for 10 h daily. Water, crickets and mealworms were provided *ad libitum*.

Fifteen females were randomly selected for manipulation of yolk hormone levels. At the time of surgery, all females had recently ovulated. The females were anaesthetized for *ca*. 40 min using 0.0083 mg $g^{-1}$ body mass of Brietal (Lilly, VL-660), wiped with 70% ethanol on the abdomen, and taped to a sterile surgical board. An 8–12 mm-long incision was made 2–3 mm laterally of the mid-ventral line using a pair of surgical scissors. Using a sterile blunt probe bent into desired shape, one oviduct at the time was carefully lifted out of the incision. A custom-made testosterone-column inserter, loaded with 50 µg dihydrotestosterone compacted with 0.9 NaCl, was carefully inserted in the oviduct and the testosterone placed on top of, or between, the yolked eggs (depending on the number of eggs in the oviduct). The oviduct was gently closed and folded around the eggs after the incision and no signs of complications owing to the insertion of the testosterone-column could be observed. The females were sutured using surgical thread (HS 15, Catgut, Germany) and allowed to wake up in a sterilized cage with paper as substrate and a bowl of water. All females were from then on kept individually with paper as substrate (sterilized and changed every second day), and water and food (mealworms) *ad libitum*. From twenty-four hours post-surgery onwards, manipulated females showed no difference in behaviour compared with females that had not undergone surgery. As females progressed through gestation, cages were checked at least twice daily for hatchlings.

At parturition, the neonates were weighed to the nearest mg, SVL was measured and total length ascertained to the nearest 0.5 mm with a ruler, and head width and head length to the nearest 0.01 mm with a pair of callipers. Unmanipulated hatchlings were sexed by hemipenis eversion (Harlow 1996), but because of the enlargement of the hemipenes owing to the elevated testosterone levels in the manipulated group (Dufaure 1966; T. Uller, personal observation), they were re-sexed at maturity. Physiological performance was tested by letting the neonates swim in a 700 mm × 400 mm × 350 mm thermally insulated aquarium filled with water and heated to 30 °C (the preferred body temperature of *L. vivipara* (Van Damme *et al.* 1986; Uller & Olsson 2003*b*)), which gives a repeatable measure of endurance in juvenile common lizards (Olsson *et al.* 2002; T. Uller and M. Olsson, personal observation). When the lizard stopped swimming, it was encouraged to continue by a slight tap on its body side. The trial was interrupted when the lizard did not resume swimming after three consecutive taps. All juveniles from the control group, and a random subset of juveniles from the manipulated group (with respect to family and locality) were put in individual cages (180 mm × 180 mm × 90 mm), with paper as substrate and tiles as shelter. The cages were sprayed with water once daily with crickets fed daily *ad libitum*. A heating cable in the back of the cages provided opportunities for thermoregulation for 10 h daily in a thermal gradient from 20 °C to 38 °C, and with an ambient light cycle set to 12 L : 12 D. Ultraviolet light was provided for 4 × 15 min per day by Ultravitalux light bulbs placed above the cages to facilitate vitamin D4–D3 conversion and corresponding calcium uptake. Fifteen days post-parturition, all juveniles were weighed and measured as described above. For unmanipulated offspring, each family was split into two groups, one control and one experimental. In the experimental group, each cage was 'infested' with five ticks (*I. ricinus*; size *ca*. 1.5 mm) collected from lizards in natural populations. Because not all families were represented by more than one offspring in the manipulated group, the offspring were randomly allocated to one of the two treatments. To prevent ticks from climbing out of the cages, each cage had flypaper glued to its upper circumference, making it possible to quantify tick loss. However, only a few ticks ever left the bottom substrate during the experiment. Ticks that fed on the lizards or were observed engorged with blood in the cages were monitored. Five days into the experiment, two more ticks were added to cages exposed to ticks.

After a second period of 15 days, the lizards were again measured, weighed and tested for endurance as described above. Blood smears were obtained by taking 3 µl blood from a sinus in the upper jaw of each lizard (Olsson 1994). The smears were air-dried and stained with Hemacolor (JT Baker, Holland). From each smear, three pictures were randomly taken from a homogenous area of the film, using a Leica microscope camera, and all leucocytes and erythrocytes were counted manually in Adobe Photoshop v. 7.0.

Because of unequal clutch sizes, nested ANOVA could not satisfactorily be used, and all analyses are therefore based on family means. The number of leucocytes and the ratio betweeen leucocytes and erythrocytes were log transformed to achieve normality.

## 3. RESULTS

There was no difference in body size (mass, SVL and total length) or endurance at parturition between testosterone-manipulated and control offspring (*t*-tests: $p > 0.05$ for all variables). There was, however, a trend for testosterone-manipulated offspring to grow faster than controls during the first 15 days (*t*-test: $t = 1.78$, $p = 0.08$, d.f. = 39), and a significant effect of testosterone on growth for the total duration of the experiment, i.e. 30 days (using only offspring not exposed to ticks, *t*-test: $t = 3.51$, $p = 0.0027$, d.f. = 17). An ANOVA with treatment (ticks versus controls), manipulation status (testosterone versus control) and the interaction term between the two factors, showed that both treatment and the interaction were statistically significant, whereas manipulation status fell short of significance (treatment: $F_{1,37} = 13.17$, $p < 0.001$; manipulation status: $F_{1,37} = 2.87$, $p = 0.098$; interaction: $F_{1,37} = 7.03$, $p = 0.012$; figure 1). Separate *t*-tests showed that offspring exposed to high levels of testosterone during gestation had a significantly decreased growth rate when exposed to ticks (*t*-test: $t = 3.46$, $p = 0.0035$, d.f. = 15; figure 1), whereas control offspring did not show retarded growth response to tick treatment (paired *t*-test: $t = 1.05$, $p = 0.32$, d.f. = 11; figure 1). Both groups showed reduced endurance after exposure to ticks, although this was statistically significant only for testosterone-manipulated offspring (manipulated:

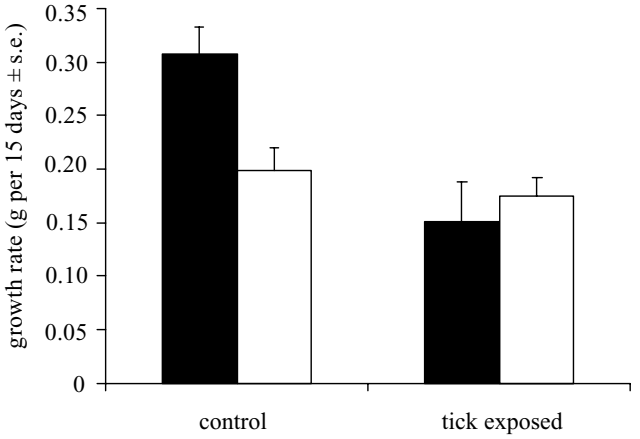


Figure 1. Growth rates for testosterone-manipulated (filled bars) and control (open bars) juvenile common lizards under tick exposure and control conditions. See § 3 for test statistics.

37.2 s versus 48.3 s, tick treatment versus controls, *t*-test: $t = 2.21$, $p = 0.044$, d.f. = 15; unmanipulated: 40.3 s versus 47.2 s, tick treatment versus controls, paired *t*-test; $t = 2.03$, $p = 0.07$, d.f. = 11).

Neither the number of feeding or engorged ticks, nor the size of the fed ticks, differed between manipulated and control offspring (Wilcoxon two-sample test: $Z = 0.92$, $p = 0.36$; *t*-test: $t = 0.12$, $p = 0.91$, d.f. = 8, respectively). Furthermore, there was no effect of parasite treatment on the number of red or white blood cells or the ratio between the two (ANOVA; $p > 0.05$ for all variables).

## 4. DISCUSSION

Sex steroids are central for the understanding of many evolutionary problems, particularly when they form the proximate link between immunobiology, development and lifetime fitness. Sex steroids deposited in the yolk of birds and reptiles have been suggested to be an adaptive maternal strategy for increasing the growth rate of the offspring (Schwabl 1996). However, if testosterone has detrimental effects on immunological traits, benefits of increased maternal allocation of steroids may be counteracted in terms of long-term fitness. In the present study, we show that, under controlled laboratory conditions, prenatal exposure to high testosterone levels *in utero* not only increased offspring growth rate, but also susceptibility to a common ectoparasite (*I. ricinus*) in the common lizard, *L. vivipara*. Testosterone-manipulated lizards grew significantly worse when exposed to ticks, whereas control offspring did not show impaired growth in the tick treatment (figure 1). Furthermore, lizards exposed to high androgen levels during embryonic development also had decreased endurance after tick exposure, confirming a systemic physiological cost of androgen exposure.

This study is important for at least two reasons. First, it shows that there is indeed a trade-off between testosterone and immunocompetence, with immune function being compromised at experimentally elevated levels of testosterone. Because this study was conducted under controlled laboratory conditions, increased susceptibility to ectoparasites cannot be linked to movement and associated exposure to ectoparasites. Thus, our results most probably reflect a negative impact of testosterone on immune function itself. However, in spite of testosterone effects on growth and performance, we could not confirm downstream effects on ectoparasite load or relative or absolute number of leucocytes and erythrocytes. The non-significant difference in tick load suggests that high testosterone levels do not compromise the primary defence against ticks (i.e. active removal and consumption of ticks; T. Uller, personal observation). Thus, the significant decrease in growth rate of testosterone-treated young should plausibly impact negatively on immune function, resulting in lower leucocyte counts, but this could not be confirmed. However, information regarding the proximate links between testosterone and immune function is lacking, particularly in reptiles, and although leucocytes are involved in immune defence against ticks, their actual number may poorly reflect a triggered immune response. For example, chemicals in tick saliva inhibit leucocyte responsiveness and haemostasis (Wikel 1996), and possibly such responses of the immune system are compromised when animals are exposed to high levels of androgens. Furthermore, in chickens treated with testosterone during development, the bursa of Fabricus fails to mature, with low numbers of B lymphocytes as a result (Glick 1986). Thus, a higher increase in leucocytes in the manipulated group may have been masked by lower numbers of white blood cells at parturition. In addition to potential effects on immune function itself, testosterone may also increase basal metabolic rate (Buchanan *et al.* 2001), thereby making it relatively more costly for manipulated lizards to replenish blood volumes removed by ticks.

Second, to the best of our knowledge, this is the first study in any taxa to show that prenatal exposure to testosterone in egg yolk influences both growth rate and immune function after hatching/parturition. The increased growth rate in response to testosterone treatment is consistent with studies on birds, where chicks hatching from testosterone-treated eggs have higher growth rates, partly because of increased begging behaviour (Schwabl 1996). In this study, food was provided *ad libitum*, suggesting that testosterone during development either increases food intake or food conversion, or both, in hatchlings. The negative effect of tick exposure in manipulated lizards can probably be linked to steroid-mediated differentiation of the immune system during embryogenesis, which is likely to be partly responsible for the common pattern of sex differences in immune function found in many species (Martin 2000). As a net result of these two effects, growth rate under tick exposure was no longer higher in testosterone-manipulated lizards compared with controls (figure 1), adding support to the notion that the benefits of high levels of steroids are environment-dependent.

Although difficult to assess because of the potential leakage of testosterone from the oviduct, the steroid levels used in this experiment are probably unnaturally high. Unfortunately, natural variation in testosterone levels in eggs of the common lizard is unknown. There was, however, no pre-parturient mortality linked to testosterone treatment and, at parturition, manipulated offspring did not differ in body size or condition compared with normal offspring. Thus, these results suggest that the levels of

testosterone were not high enough to be pharmacological or to impair embryonic development. Because steroids are allocated to the egg yolk in reptiles (as in birds), differential allocation also within the natural variation is likely to show effects on offspring growth and immunocompetence similar to those revealed in our experiment. In canaries, androgen levels increase with increasing laying order, which has been suggested to be an adaptive maternal response to reduce within-clutch asymmetry in hatchling growth. Although no study has investigated the impact of increased testosterone in eggs on immune defence (but see Glick 1986), our present study suggests that chicks from later eggs should have relatively poorer immunocompetence. In reptiles, however, all eggs are ovulated at the same time and differential maternal allocation within a clutch is unlikely. Thus, all offspring in a clutch are presumably exposed to equal levels of testosterone in the yolk (but levels may differ owing to steroid leakage between foetuses; Uller & Olsson 2003*a*). Still, adjustment of steroid investment may provide mothers with a potential mechanism to manipulate offspring traits depending on, for example, degree of parasitism. Because both individual and nest ectoparasite load are relatively easy to manipulate, experimental studies of ectoparasitism on offspring immunocompetence may provide opportunities for testing this adaptive scenario.

In conclusion, exposure to testosterone during gestation increased growth rate in juvenile common lizards. However, when exposed to ticks, testosterone-treated lizards showed significantly impaired growth, leading to similar growth rates as control offspring. Thus, this study supports steroid-mediated costs and benefits on offspring fitness.

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