Direct Exposure to Corticosterone During Embryonic Development Influences Behaviour in an Ovoviviparous Lizard

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Abstract

It is becoming increasingly clear that conditions experienced during embryonic development can be of major importance for traits subsequent to parturition or hatching. For example, in mammals, offspring from stressed mothers show a variety of changes in behavioural, morphological, and life-history traits. The effects of maternal stress on trait development are believed to be mediated via transfer of glucocorticoids, the main hormones released during the stress response, from mother to offspring. However, also other physiological maternal responses during stress could be responsible for changes in offspring phenotype. We investigated the direct effects of corticosterone on offspring development, without other confounding factors related to increased maternal stress, by injection of corticosterone in eggs of the ovoviviparous lizard *Lacerta vivipara*. Corticosterone-manipulated offspring did not show impaired development, reduced body size or body condition at parturition. However, corticosterone-treated offspring showed altered antipredator behaviour, as measured by the time required to emerge from shelter after a simulated predator attack. Differential steroid exposure during development, possibly mediated by maternal stress response, may explain some of the variation in behaviour among individuals in natural populations.

Introduction

Behaviours are generally considered plastic traits and understanding the ontogeny of behaviour is therefore of major importance for understanding their evolutionary significance. Recently, it has become evident that the prenatal environment can have profound effects on offspring traits, both as juveniles and as adults (e.g. Clark & Galef 1998; Dufty et al. 2002). For example, in viviparous vertebrates, maternal physiological status can influence offspring both directly, by limiting energy resources to the foetus, or by diffusion of hormones between mother and offspring. One aspect of maternal effects that has been subject to considerable interest is how maternal stress influences the offspring. In laboratory rats, maternal stress influences offspring morphology, behaviour and immunocompetence [the prenatal stress (PS) syndrome, recently reviewed in Edwards & Burnham 2001; Weinstock 2001; Welberg & Seckl 2001], and evidence for similar phenomena is accumulating in human and non-human primates (e.g. Couzin 2002; Kofman 2002). The major hormones behind the PS syndrome are believed to be glucocorticoids (mainly corticosterone and cortisol) that increase during stress and can cross the placenta to the developing embryo (Zarrow et al. 1970; Edwards & Burnham 2001). Prenatal exposure to relatively high, stress-induced levels of corticosterone could alter the development of the Hypothalamic-Pituitary-Adrenal (HPA) axis (Weinstock 1997; Welberg & Seckl 2001), leading to changes in postnatal behaviour, primarily those related to fear

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and anxiety (e.g. Thompson 1957; Weinstock 2001; see also Korte 2001). However, because many other physiological and behavioural maternal responses occur under stress, and because offspring cannot be independently treated with hormones without affecting maternal tissue in mammals, alternative explanations cannot be ruled out (but see Barbazanges et al. 1996).

Maternal hormonal influence on developing embryos is not restricted to mammals. Many reptiles and fish are viviparous, in the sense that embryos are retained inside the mother during embryonic development (see Bernardo 1996 for a discussion of ovoviviparity vs. viviparity), with the potential for maternal steroids to influence the offspring (e.g. Meylan et al. 2002). Furthermore, eggs of oviparous vertebrates have been shown to contain substantial amounts of maternally derived hormones (e.g. Dickhoff et al. 1990; Schwabl 1993; McCormick 1998; Bowden et al. 2001; Lovern & Wade 2003; Hayward & Wingfield 2004). Thus, maternal effects mediated via hormones may be a common and important factor influencing offspring development. Unfortunately, the direct effects of prenatal steroids on offspring behaviour are still largely unknown. Experimental manipulation of steroid levels, and naturally induced changes in maternal effects (such as maternal stress), are therefore both required to understand the ontogeny of behavioural and other traits.

In the present paper, we exploit the ovoviviparous lizard, *Lacerta vivipara*, to investigate the direct effects of corticosterone on development of embryos and hatchling traits. *Lacerta vivipara* has no well-developed placenta and substantial hormone leakage between mother and offspring and between foetuses is likely to occur (de Fraipont et al. 2000; Meylan et al. 2002; Uller & Olsson 2003a; Uller et al. 2004). Thus, changes in maternal hormonal status are likely to directly influence offspring traits and, consequently, maternal stress has been shown to influence important life-history traits, such as dispersal (de Fraipont et al. 2000; Meylan et al. 2002). To investigate effects of prenatal corticosterone exposure, without confounding factors related to maternal hormone levels, we injected corticosterone directly into ovulated eggs and monitored embryonic development, growth, endurance, and post-parturient behaviour under stress in the offspring.

Materials and Methods

The common lizard (*L. vivipara*) is a small (4–5 g, 50–70 mm snout-to-vent length, SVL), ground-dwelling lizard distributed in Eurasia. *Lacerta vivipara* is viviparous in all but some of the southernmost populations and have a mean clutch size of 4–6 young. Emergence from hibernation occurs in late Mar.–Apr. in southern Sweden, with subsequent mating approx. 2 wk later. Parturition occurs in Aug.–Sep.

Forty female *L. vivipara* from two populations (Asketunnan, Sandsjöbacka) in south-western Sweden were caught during May to early Jun. 2003. All females were mated in the field as evident from copulation scars on the belly (inflicted by the male during copulation, Bauwens & Verheyen 1985). Females were kept separately in cages ($500 \times 400 \times 350$ mm), with peat and bark as substrate, rocks and tiles as shelter, and a 40 W spotlight for thermoregulation. Mealworms (*Tenebrio* larvae), crickets (*Gryllus* spp.), and water were provided ad libitum. For 28 females, half the clutch was corticosterone manipulated using the following procedures. The females were anaesthetized for approx. 40 min by subcutaneous injection of 0.0083 mg/g body mass of Brietal (Lilly, VL-660, Indianapolis, Indiana, USA), 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, one oviduct at a time was carefully lifted out of the incision. All eggs in one of the oviducts (chosen randomly) received an injection of 60 ng corticosterone dissolved in 1 μl of peanut oil. The resulting concentration in the egg is within the natural range of corticosterone concentration in the blood of gravid females (approx. 1–100 ng/ml; Fig. 1 in Meylan et al. 2003) and below the experimentally increased blood levels used in previous studies [Meylan et al. 2002, 2003; calculations of egg volume in vivo based on ellipsoid geometry and data on egg-laying species in the same genus of similar adult and juvenile body size and clutch size (in den Bosch & Bout 1998)]. However, the levels experienced directly by the embryos are unknown as these depend on the degree of leakage between maternal and foetal tissue and the pattern of yolk utilization. We therefore emphasize that our aim was to investigate if corticosterone itself (when compared with maternal stress response) have organizing effects on offspring behaviour during embryonic development, not to look for dose-specific responses (see Discussion). The injections were performed from the opposite side of the embryo, using a Leica micromanipulator (Leica, Göteborg, Sweden) for in vitro fertilization. No leakage of yolk could be

observed in any of the manipulations. Injection of hormones into egg yolk has proved successful for investigating effects of prenatal hormone exposure in other taxa (e.g. Schwabl 1996; Eising & Groothuis 2003; Andersson et al. 2004). Eggs were classified according to their stage in development at the time of manipulation where 1 equals recently ovulated and 2 equals approximately one-third to one-half of total embryonic development (Dufaure & Hubert 1961). The left oviduct was then sealed by tying a loop around it below the eggs using surgical thread (HS 15, Catgut, Markneukirchen, Germany), to ensure that only young from one oviduct would be born at parturition, whereas the other could be delivered under the experimenter's control by Caesarean section. Thus, family effects on size and development could be controlled for and potential interactions between family and corticosterone exposure on offspring development and size at parturition could be investigated. The oviducts were gently pulled back into the body cavity and the females were sutured using surgical thread and allowed to wake up in a sterilized cage with paper as substrate and a bowl of water. Injection of oil into eggs in the other oviduct would have required time-consuming change of experimental set-up during surgery, and we therefore refrained from doing that to minimize complications and risk of mortality. Instead, to be able to control for the injections and surgery per se, six females were sham operated, using the same technique as described above, but injected with vehicle only, and six females were left unmanipulated as an extra control group. All females were from then on kept on paper, with water and mealworms provided ad libitum. Cages were checked three times daily for neonates.

At parturition, the young were individually marked, weighed (to the nearest mg) and measured (SVL and total length to the nearest 0.5 mm with a ruler and head width and head length to the nearest 0.01 mm using a pair of calipers) and sexed by hemipenis eversion (Harlow 1996). Two females gave birth to all young despite the sealing of the oviduct, and they were therefore excluded from the analyses. Offspring from the sealed oviduct were delivered using Caesarean section. However, because full-term offspring not delivered naturally suffered from high mortality of unknown causes subsequent to delivery (see also Saint-Girons 1985), only naturally delivered young were included in the analyses of endurance and stress response (see Results). The young were placed individually in small cages ($180 \times 180 \times 90$ mm) with a heating cable in the back of the cages, providing a thermal gradient of 22–38°C. Crickets were provided ad libitum and water was sprayed in the cages once daily.

Endurance was measured the day after parturition by letting the neonates swim in a thermally insulated aquarium (30°C, within the preferred body temperature of common lizards, Van Damme et al. 1986; Uller & Olsson 2003b). When the lizard stopped swimming, it was encouraged to continue by a light tap on the body side. When it did not resume swimming after three consecutive taps, the trial was interrupted. This method yields a repeatable measure of endurance in juvenile common lizards (e.g. Uller & Olsson 2003b). On day 2 after parturition, stress response of juveniles was measured. Because *L. vivipara* inhabits areas where the ambient temperature often is too low for optimal performance, lizards are dependent on active basking. One of the most common stressors in natural populations is predation during basking, and, consequently, lizards face a trade-off between increasing body temperature to optimal levels and risk of predation (Huey & Slatkin 1976). Thus, risk behaviour during thermoregulation is most likely related to fitness in reptiles. A lizard was gently transferred from its cage to a large cage ($500 \times 400 \times 350$ mm) containing rocks for shelter at one end and a 40 W spotlight at the other end. It was then allowed to acclimatize for 12 min. A predator attack was mimicked by startling and chasing the lizard with a paintbrush, at which it disappeared between the rocks. The time from the simulated attack until the lizard reappeared and resumed basking was used a measure of fear response. The trial was interrupted if the lizard did not reappear after 10 min, whereafter the juveniles were transferred back to their cages. Two lizards did not bask after the given acclimatization time and were therefore excluded from the analysis. All trials were conducted without knowledge of juvenile treatment. Twenty days post-parturition, lizards were again weighed and measured as described above and growth was calculated as mass after 20 d minus birth mass.

Ethical Note

The surgery did not lead to increased female mortality or reduced offspring developmental success (Kruskal–Wallis test, $\chi^2 = 1.94$, $p = 0.38$). However, as noted above, offspring from the sealed oviduct suffered from high mortality. Mortality of normally delivered offspring was very low. Females were kept under standard laboratory conditions subsequent to

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the experiment, whereas all juveniles were released into their natural populations. No recapture of animals from the present experiment was conducted.

Results

In total, 203 young were delivered from the 40 females, with no difference in developmental success between treatments (Kruskal–Wallis test, $\chi^2 = 1.94$, $p = 0.38$). We started by analysing differences between offspring treated with corticosterone and controls within females. Corticosterone did not affect developmental time, as all young within each clutch were at the same stage (full-term) at parturition regardless of offspring treatment. To analyse differences in offspring size (SVL and body mass) and body condition at parturition, we ran ANOVAs with family, corticosterone manipulation, and the interaction between the two as factors. Family was treated as a random effect. Family and the interaction proved significant for all variables, whereas corticosterone was non-significant (e.g. body size: family: $F_{24,58} = 9.50$, $p < 0.001$; corticosterone: $F_{1,19} = 0.06$, $p = 0.81$; family × corticosterone: $F_{19,58} = 2.88$, $p = 0.001$). The developmental stage at manipulation did not influence size at birth [e.g. body mass, ANOVA, egg stage: $F_{1,21} = 0.19$, $p = 0.70$; family (egg stage): $F_{21,43} = 9.02$, $p < 0.0001$].

Studies of rodents suggest that corticosterone levels may influence degree of masculinization/feminization (e.g. Ward 1972; Sachser & Kaiser 1996). To look for similar effects, we used relative head length (based on residuals from a regression on SVL) as a measure, because this is a sexually dimorphic trait, most likely influenced by prenatal hormone exposure (Uller & Olsson 2003a). There was no significant difference between treatments in relative head length (separate analyses for the sexes, males: family: $F_{22,23} = 1.72$, $p = 0.10$; corticosterone: $F_{1,10} = 2.59$, $p = 0.14$; family × corticosterone: $F_{10,23} = 0.93$, $p = 0.52$; females: family: $F_{20,16} = 0.66$, $p = 0.82$; corticosterone: $F_{1,5} = 0.43$, $p = 0.54$; family × corticosterone: $F_{5,16} = 1.54$, $p = 0.23$).

To confirm that the injection per se did not have any effects, and because steroid leakage between foetuses could reduce the difference between manipulated and non-manipulated offspring within females, we reanalysed our data using only corticosteroid-manipulated offspring and offspring from females from both control groups. All analyses yielded the same qualitative results, with no significant difference between prenatally stressed and unstressed offspring (Appendix 1).

In the analysis of behaviour, only naturally delivered, manipulated offspring, and offspring from the control groups were included for reasons described above. There was no significant difference between sham-manipulated and unmanipulated offspring in either endurance or time to emergence from shelter ($p > 0.10$ for all analyses), and we therefore pooled the control treatments for further analyses to increase the power of the tests. Corticosterone-treated offspring did not have significantly reduced endurance compared with controls [ANCOVA, treatment: $F_{1,20} = 0.19$, $p = 0.66$; family (treatment): $F_{20,56} = 2.91$, $p < 0.001$; body size: $F_{1,56} = 10.46$, $p = 0.002$], and there was no difference depending on when in development corticosterone was administered [ANCOVA, egg stage: $F_{1,12} = 1.89$, $p = 0.19$; family (egg stage): $F_{12,21} = 8.96$, $p < 0.0001$; body size: $F_{1,21} = 11.14$, $p = 0.003$]. However, corticosterone-treated animals stayed in shelter for significantly longer time after the simulated predator attack than did controls [$\bar{x} \pm$ SE: 4 min 53 $\pm$ 52 s vs. 3 min 25 $\pm$ 23 s, survival analysis on mean values per female, using PROC LIFEREG in SAS 8.2 (SAS Institute, Cary, NC, USA), $\chi^2 = 7.10$, $p = 0.008$, Fig. 1]. The response did not differ depending on when in development corticosterone was administered (survival analysis, $\chi^2 = 0.41$, $p = 0.52$). Growth was not influenced by prenatal corticosterone exposure [ANOVA, treatment: $F_{1,21} = 0.18$, $p = 0.67$; family (treatment): $F_{21,63} = 3.92$, $p < 0.0001$].

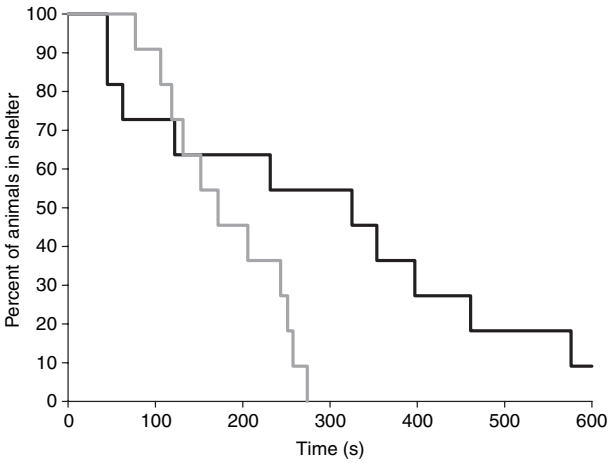

Fig. 1: Offspring that were treated with corticosterone during embryonic development (black line) remained in shelter for significantly longer time after a simulated predator attack than control offspring (grey line). Data represent mean values per family. See text for test statistics

Discussion

Stress during pregnancy can influence offspring in a variety of ways during life history (the PS syndrome, e.g. Welberg & Seckl 2001). By directly manipulating corticosterone levels in the eggs of an ovoviviparous lizard, we were able to show that increased exposure to corticosterone is likely to be responsible for some, but not all, of these effects. Corticosterone-manipulated lizards did not show reduced embryonic development, reduced birth weight, body condition, or endurance. Manipulated lizards did, however, show altered anti-predator behaviour, as measured by the time required to emerge from a shelter after a simulated predator attack.

Many studies in mammals have reported decreased birth weight of neonates under maternal stress (Weinstock 1997), and similar patterns have also been found in studies of the common lizard (Meylan et al. 2002; Meylan & Clobert 2004, in press; but see de Fraipont et al. 2000). Earlier studies have manipulated gravid females either by using some kind of stressor, or by administration of glucocorticoids to the mother. In contrast, we manipulated corticosterone levels directly and found no support for decreased body size or condition of juveniles. This suggests that increased glucocorticoids are not directly responsible for decreased size at birth, but rather that reduced offspring size is a result of decreased maternal food intake, decreased energy allocation to the offspring, early parturition, or other physiological processes linked to maternal stress response. Importantly, there was a significant interaction between family and corticosterone treatment on juvenile traits, suggesting variation in the effects of corticosterone on offspring development due to genetic variation or differences in maternal effects (e.g. initial hormone levels). A genetic component of stress sensitivity has been demonstrated by selective breeding for lines with high or low stress response in rainbow trout (*Oncorhynchus mykiss*, e.g. Pottinger & Carrick 1999; Fevolden et al. 2002). Contrary to the results on offspring body size, the lack of effect of prenatal corticosterone on postnatal endurance agrees with results obtained by hormone administration to gravid females (Meylan & Clobert 2004). Some mammalian studies have found masculinizing and feminizing effects of PS on offspring morphology and behaviour (Ward 1972; Sachser & Kaiser 1996). We found no such effect in our study, adding support to the notion that these effects are not caused by glucocorticoids, but from correlated changes in plasma testosterone of stressed mothers (Ward 1972; Ward & Weisz 1980).

One of the most important results from studies of PS is its effect on behavioural traits, in particular those related to emotions such as fear and anxiety (reviewed in Braastad 1998; Weinstock 2001). Such prenatal programming of offspring phenotype could also lead to associated changes in physiological traits (e.g. sprint speed) by affecting motivation (Meylan & Clobert 2004). In the present study, corticosterone-treated individuals spent significantly longer time in shelter after a simulated predator attack than did control offspring, indicating a stronger response to the stressor. Our study therefore suggests that the effects on behaviour found in earlier studies are due to direct organizational effects of corticosterone rather than other aspects of maternal stress. Furthermore, it also supports the generality of the effect of embryonic exposure of corticosterone on behaviour across taxa (earlier studies have almost exclusively dealt with mammals, e.g. Thompson 1957; Weinstock 2001; Griffin et al. 2003; but see de Fraipont et al. 2000; Belliure et al. 2004; Meylan & Clobert 2004, in press), and suggests that early hormone exposure could be an important factor generating variation in behaviour in vertebrates. The effects on behaviour associated with stress are most likely due to changes in development of the HPA axis under corticosterone exposure (Weinstock 1997; Welberg & Seckl 2001). In reptiles, stress has been found to influence thermal regulation (Cree et al. 2003; Belliure & Clobert 2004; Belliure et al. 2004), which could confound results from predator avoidance experiments such as the present one. We find this unlikely, however, because in *L. vivipara*, PS led to an increase in the time spent basking (Belliure et al. 2004). Thus, potential effects on thermal preference would act in the opposite direction from the one found in the present study. Nevertheless, there may be a general decrease in activity in prenatally stressed animals, including lizards (Braastad 1998; Belliure et al. 2004; Meylan & Clobert 2004) that could be linked to a more cautious behaviour under predation risk.

Studies suggest that there is a time specificity for the PS syndrome (the programming window, Welberg & Seckl 2001). Naturally, hormones can only have a programming effect on an organ during the development of the tissue. This suggests that administration of corticosterone should have different effect during different stages in the development. We did not find such patterns in our study, however, but admittedly, sample sizes were not large

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enough to provide a conclusive answer to this question. Furthermore, yolk utilization patterns may influence when in the development the offspring are exposed to the experimentally increased hormone content of the yolk. Future studies should investigate dose-dependent responses and base their manipulations on the natural variation in foetal exposure to corticosterone as there may be threshold effects of prenatal hormone exposure. However, variation in foetal hormone exposure under natural conditions is not easily obtained in viviparous animals (but see Painter et al. 2002). Nevertheless, further studies of the degree of leakage between maternal and foetal tissue (Painter et al. 2002), and of yolk and hormone utilization in reptilian embryos will be important for the interpretation of experimental studies of hormone injection into eggs or administration of hormones to gravid females.

Most studies aimed at understanding the PS syndrome have been conducted on laboratory rodents, and their evolutionary implications have been largely neglected. However, studies of mammals, lizards and fish from natural populations suggest that PS has important consequences for fitness-related traits such as body size, sprint speed, sexual attractiveness, dispersal, survival and risk behaviour (McCormick 1998, 1999; de Fraipont et al. 2000; Meylan et al. 2002; Marchlewska-Koj et al. 2003; Meylan & Clobert 2004, in press; this study). Some authors have suggested that the PS syndrome is not merely a side-effect of maternal stress, but rather an adaptive modification of offspring to prevailing conditions (e.g. de Fraipont et al. 2000; Meylan & Clobert in press). This view may be difficult to reconcile with reduced birth weight, impaired learning ability and increased susceptibility to disease, at least from the offspring's perspective. On the contrary, a risk-averse behaviour could be adaptive if maternal stress is indicative of an unsafe environment or if PS makes offspring particularly sensitive to, for example, predation. A recent study on *L. vivipara* suggested that prenatally stressed offspring have higher survival but reduced growth (Meylan & Clobert in press), which is likely if the changes in behaviour that were observed in the present study occur also in natural populations. However, the duration of the prenatally induced changes in offspring behaviour under natural conditions remains to be investigated. The mammalian placenta contains a corticosterone-metabolizing enzyme, protecting the foetus from excessive steroid levels (Welberg & Seckl 2001). Thus, regulation of offspring steroid exposure could be possible, but increased exposure during stress could also be a side-effect of imperfect metabolism of glucocorticoids by the placenta. In *L. vivipara*, non-adaptive explanations would perhaps seem even more likely, as viviparity in this species has evolved relatively recently (Heulin et al. 1999), and maternal hormones probably leak directly to the offspring without sophisticated regulation. However, Painter et al. (2002) have shown that regulation of hormone transfer between mother and offspring can evolve relatively rapidly in viviparous lizards.

In conclusion, we found no effect of direct administration of corticosterone on offspring development or size at parturition. However, manipulated offspring showed less risky behaviour indicative of increased fear or anxiety. Early hormone exposure, mediated via maternal stress, could be an important evolutionary factor generating variation in behaviour in natural populations of animals.

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Appendix 1: Results from nested ANOVAS with corticosterone-manipulated offspring vs. offspring from control females

Dependent variable	Source	F	df	p
Body mass	Treatment	0.31	1, 31	0.58
	Family (treatment)	13.19	31, 93	0.04
Relative head length (males)	Treatment	3.46	1, 24	0.08
	Family (treatment)	1.84	24, 32	0.05
Relative head length (females)	Treatment	1.21	1, 23	0.28
	Family (treatment)	1.55	23, 37	0.12