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Corticosterone stimulates hatching of late-term tree lizard embryos.

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Abstract

The regulation of hatching in oviparous animals is important for successful reproduction and survival, but is poorly understood. We unexpectedly found that RU-486, a progesterone and glucocorticoid antagonist, interferes with hatching of viable tree lizard (*Urosaurus ornatus*) embryos in a dose-dependent manner and hypothesized that embryonic glucocorticoids regulate hatching. To test this hypothesis, we treated eggs with corticosterone (CORT) or vehicle on Day 30 (85%) of incubation, left other eggs untreated, and observed relative hatch order and hatch time. In one study, the CORT egg hatched first in 9 of 11 clutches. In a second study, the CORT egg hatched first in 9 of 12 clutches, before vehicle-treated eggs in 10 of 12 clutches, and before untreated eggs in 7 of 9 clutches. On average, CORT eggs hatched 18.2h before vehicle-treated eggs and 11.6h before untreated eggs. Thus, CORT accelerates hatching of near-term embryos and RU-486 appears to block this effect. CORT may mobilize energy substrates that fuel hatching and/or accelerate lung development, and may provide a mechanism by which stressed embryos escape environmental stressors.

Keywords

development; glucocorticoids; oviparous; reproduction; *Urosaurus ornatus*; stress

INTRODUCTION

The physiological regulation of parturition and oviposition has been well studied in many viviparous and oviparous taxonomic groups. A key difference between parturition and oviposition is that while the former is the last step of the reproductive event for viviparous species, the latter is not the final step for oviparous species. Oviparous mothers have not successfully reproduced until a viable embryo emerges from the oviposited egg. Thus, the physiological regulation of hatching by full-term embryos is a critical step in the reproduction of oviparous animals, as well as in the survival of an individual from the embryo to the juvenile life stage.

Remarkably little is known about the process of hatching in lizards, and even less is known about the regulation of that process. Based on information from other taxonomic groups, hatching of lizards likely results from a combination of (1) embryonic movements (Oppenheim,

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1973) and (2) the secretion of a proteolytic enzyme, or “hatching enzyme,” that breaks down or softens the egg membranes and thereby assists the embryo with its escape from egg membranes (insects: Kafatos and Williams, 1964; echinoderms: Lepage and Gache, 1989; fish: Yamagami, 1988; amphibians: Katagiri, 1975; birds: Yoshizaki et al., 2000; mammals: Sawada et al., 1990; Quesada et al., 2004). The timing of hatching may be influenced by environmental conditions such as oxygen availability and light levels (e.g., Helvik and Walther, 1992; Helvik and Walther 1993; Downing and Litvak, 2002; Warkentin, 2002). The manner in which these environmental cues are transduced to stimulate or inhibit embryonic movements and hatching enzyme secretion is largely unknown, however the embryonic nervous and/or endocrine systems are surely involved. For example, the release of hatching enzyme in teleosts appears to be under dopaminergic control (Schoots et al., 1983), is inhibited by thyroid hormones (Reddy and Lam, 1991), and is stimulated directly by prolactin and indirectly by glucocorticoids (Schoots et al., 1982).

Glucocorticoids are part of the phylogenetically conserved neuroendocrine stress axis. This group of adrenal steroid hormones coordinates behavioral and physiological responses to environmental conditions, allowing the animal to respond accordingly (Wingfield et al., 1998). Thus, one might predict them to have an important role in regulating the timing of hatching, as well as other life history transitions such as metamorphosis and birth (Crespi and Denver, 2005). Indeed, an increase in endogenous glucocorticoids is found among embryonic birds and crocodiles near hatching (Marie, 1981; Scott et al., 1981; Shepherdley et al., 2002), larval amphibians near metamorphosis (Glennemeier and Denver, 2002), and fetal mammals near parturition (Mulay et al., 1973; Fencil et al., 1980; Whittle et al., 2001). In addition, environmental stressors are known to increase both corticosteroids and development rates in near-metamorphic tadpoles and late gestation fetuses (Crespi and Denver, 2005). However, little work has directly manipulated corticosteroid levels to examine their regulatory role in the timing of hatching.

Here, we provide direct evidence that embryonic corticosterone accelerates the hatching event in oviparous tree lizards, *Urosaurus ornatus*. In this species, yolk corticosterone levels increase during incubation and peak near the time of hatching (Jennings et al., 2000). The corticosterone is produced by embryonic adrenal glands (Jennings et al., 2004) and gets sequestered into the yolk during embryogenesis. The functional significance of elevated yolk corticosterone in near-term embryos is not known, but is suspected to have a role in the regulation of development and of hatching.

Herein, we begin by describing an unexpected outcome from a study aimed at addressing the role of progesterone on early development using application of the synthetic progesterone- and corticosterone-antagonist RU-486 to nearly full-term embryos. Based on the results from this study, we hypothesized that elevated corticosterone is important for successful hatching of viable tree lizard embryos. We present two studies that experimentally test this hypothesis.

MATERIALS AND METHODS

Animal and egg collection

Female tree lizards were collected June – August 2000, July – August 2003, and May 2004 in Maricopa County, Arizona within a 10 km radius of the intersection of Arizona Highway 87 and Sycamore Creek-Sugarloaf Mountain Road at an elevation of 400–700 m. Females were held in captivity in 37 × 42 × 45 cm wire cages until they were determined by palpation to be ready to oviposit. Each cage was supplied with water, food (crickets), shelter and perches, and had a 25W incandescent bulb in a reflector suspended 2 cm over the top of the cage to provide

additional heat. These heat lamps, as well as black lights and 40 W full spectrum fluorescent lights, were on a 14:10 h light:dark cycle.

In 2000 and 2004, we collected only gravid females and housed them in all-females groups. On average, these females were in captivity for 8.2 ± 0.5 (SE) days prior to oviposition. In 2003, we collected vitellogenic females and housed them in groups of two females and one male per cage. On average, these females were in captivity for 34.1 ± 2.4 days prior to oviposition. Oviposition was induced via an intraperitoneal injection of oxytocin (2 USP units in 0.1 ml aqueous solution).

On the day of oviposition, egg mass was determined (to 0.001 g) using an analytical balance, and in 2003 and 2004 egg length and width also were measured (to 0.01 mm) using digital calipers. Eggs were incubated in an environmental chamber maintained at $30 \pm 1^\circ \text{C}$, individually buried in cups of moist vermiculite that were sealed with parafilm. On the day of hatch, we measured hatchling mass and snout-vent length (2003 and 2004 only) using an analytical balance and digital calipers.

Egg treatments

On Day 30 (approximately 85%) of incubation, we uncovered eggs using a gentle vacuum to remove vermiculite without disturbing the egg. We treated the egg as described below, recovered it with freshly prepared moist vermiculite, and returned it to the environmental chamber. We checked egg containers for hatchlings beginning on Day 31 of incubation.

Experiment 1: Effect of RU-486 on hatching success and survival During a study designed to test the effect of early progesterone levels on the development of male secondary sexual characteristics (Weiss and Moore, unpublished data), we unexpectedly found that RU-486 treatment of eggs prevented hatching. In response, we modified the RU-486 dosage throughout the study. Herein, we present the percent of eggs that successfully hatched and the survival of resulting hatchlings from each of the following treatment groups. On Day 30 of incubation, we treated near-hatching eggs with 5 μl of an ethanol solution containing either: ethanol only (V; vehicle), 15 μg progesterone only (P), 322 μg RU-486 + 15 μg progesterone (20R:1P), 16 μg RU-486 + 15 μg progesterone (1R:1P), or 8 μg RU-486 only (0.5R). The solution was deposited via a Hamilton syringe onto the surface of the eggshell. We treated a total of 299 fertilized eggs from 63 mothers (V: $n = 95$, P: $n = 93$, three RU-486 doses combined: $n = 111$).

Also in response to reduced hatching success of RU-486 treated eggs, we examined the viability of some 1R:1P and 0.5R treated embryos by uncovering eggs on Day 35 or 36 of incubation and surgically removing the embryo from the egg. To estimate hatching success of eggs in these treatment groups, we used three methods that differ based on the assumed success of surgically opened eggs. In Method 1, we assume that all surgically opened eggs containing living embryos would have successfully hatched had they not been opened. In Method 2, we use the percent of all V treated eggs that hatched after Day 35 (46%) as the assumed hatching success of surgically opened eggs. In Method 3, we assume all embryos from surgically opened eggs would have otherwise died within the egg.

Experiment 2: Effect of corticosterone on hatch order In 2003, we randomly selected pairs of eggs from each of 13 clutches to treat with either 5 μl of ethanol (V, vehicle) or 5 μl of a corticosterone solution (CORT; 350 μg corticosterone/5 μl ethanol) on Day 30 of incubation. Egg containers were checked for hatchlings multiple times each day beginning on Day 31 of incubation, and we recorded which member of each pair of eggs hatched first.

Experiment 3: Effect of corticosterone on hatch order and hatch time In 2004, we treated eggs from 14 clutches similar to 2003 with the following exceptions. First, a third group of eggs was included in the study; these “untreated” eggs were uncovered on Day 30 of incubation, re-covered, returned to the incubator, and observed for hatch time. Second, we determined exact hatch time by constantly monitoring egg cups via a video camera. Video tapes were observed and the time at which the first movement could be detected on the surface of the egg cup was recorded as “hatch time”. We determined relative hatch time by standardizing the time that the first egg within each clutch hatched as 0 min, and then calculated mean ($\pm$ SE) relative hatch time for each treatment group.

RESULTS

Experiment 1: Effect of RU-486 on hatching success and survival

Average incubation time of viable V and P treated eggs was 35.5 ± 0.1 and 35.6 ± 0.1 days, respectively (Student t-test: $t = 0.91$, $df = 168$, $p > 0.30$). Hatching success of V and P treated eggs was 86.3% and 94.6%, respectively (Fisher’s Exact Test: $p = 0.08$). In contrast, hatching success of RU-486 treated eggs was much reduced (Table 1).

On 11 – 18 July 2000 we treated 11 eggs with 20R:1P. None of these eggs had hatched by Day 37 of incubation. In contrast, 72 and 100% of the V and P eggs treated during the same time period had hatched by this time. The 20R:1P eggs were uncovered and found to contain fully formed but dead embryos that were apparently unable to escape the eggshell. The egg shells appeared shriveled and stuck to the sides of the embryos, and in many cases, mold had begun to grow. In one instance, the embryo apparently died during the hatching process, as the tip of its nose was out of the eggshell at the time of death. Due to the toxicity of the 20R:1P dose, on 21 July 2000 we reduced the RU-486 dose to 1R:1P. In addition, on 15 August 2000 we began to use a lower dose of RU-486 with no progesterone (0.5R).

Of the first 15 1R:1P treated eggs, 11 died and four (27%) hatched with an average incubation time of 36.5 ± 0.3 days. At this time, we decided to examine the viability of 23 1R:1P treated embryos by uncovering eggs on Day 35 or 36 of incubation and surgically removing the embryo from the egg. Six of nine (67%) Day 35 embryos were removed from the eggshell alive, one of 13 (8%) Day 36 embryos were removed alive, and one Day 36 embryo hatched on its own. Thus, from that point on, we began opening all unhatched, viable 1R:1P treated eggs on Day 35. Of the remaining 36 1R:1P treated eggs, two hatched (6%; both on Day 35), 21 (58%) contained dead embryos, and 13 (36%) contained living embryos on Day 35.

The hatching success of 1R:1P treated eggs estimated using Method 1, 2, and 3 (described above) is 36.5% (27 of 74), 21.9% (16.2 of 74), and 9.5% (7 of 74), respectively (Table 1). We expect that Method 1 grossly overestimates hatching success, and suggest that true hatching success of 1R:1P treated eggs is somewhere between 9.5 and 21.9%.

Of the initial 8 0.5R treated eggs, four hatched (average incubation time: 36.0 ± 0.7 days) and four died. We decided to surgically open the remaining 0.5R eggs on Day 35. Of the remaining 18 eggs, one hatched (6%; on Day 35), four (22%) contained dead embryos, and 13 (72%) contained living embryos on Day 35. Using Method 1, 2, and 3, the estimated hatching success of 0.5R treated eggs is 69.2% (18 of 26), 42.2% (11 of 26), and 19.2% (5 of 26), respectively (Table 1). We expect true hatching success of 0.5R treated eggs to be between 19.2 and 42.2%.

The mass of eggs treated with vehicle, progesterone, and RU-486 (all doses combined) did not differ from each other ($F_{2,295} = 0.13$, $P > 0.80$), nor did the mass of the resulting hatchlings on the day of escape from the egg ($F_{2,210} = 1.13$, $P > 0.30$), at 30 days old ($F_{2,163} = 0.09$, $P > 0.90$).

or at 180 days old ($F_{2,108} = 1.52$, $P > 0.20$). However, egg treatment did appear to affect survivorship of hatchlings. For vehicle, progesterone, and RU-486 (1R:1P and 0.5R combined) treated animals, percent survival to 30 days old was 85.4, 83.0 and 51.1%, respectively (Pearson Chi-square test: $\chi^2 = 22.2$, $df = 2$, $p < 0.001$) and to 180 days old was 61.0, 58.0, and 22.0%, respectively (Pearson Chi-square test: $\chi^2 = 19.9$, $df = 2$, $p < 0.001$). Thus, RU-486 treated animals that were pulled from the egg alive may have had developmental defects that resulted in reduced survival.

Experiment 2: Effect of corticosterone on hatch order

Of the 13 clutches treated with V and CORT in 2003, we were unable to determine hatch order for two clutches; both eggs from these clutches were assigned Hatch Order 1 for analyses. Overall, CORT eggs were significantly more likely to hatch first than were V eggs (Fisher's Exact Test: $p < 0.02$; Table 2A).

V and CORT treated eggs did not differ in mass ($F_{1,24} = 0.23$, $p > 0.60$), length ($F_{1,24} = 0.10$, $p > 0.70$), or width ($F_{1,24} = 0.52$, $p > 0.40$). Similarly, the hatchlings resulting from these eggs did not differ in mass ($F_{1,24} = 0.09$, $p > 0.70$) or snout-vent length ($F_{1,24} = 0.38$, $p > 0.50$).

Experiment 3: Effect of corticosterone on hatch order and hatch time

Of the 14 clutches treated in 2004, three clutches had only two viable eggs available for treatment on Day 30 of incubation; in these cases, the untreated egg was excluded. Two clutches included a treated egg that did not successfully hatch (one V and one CORT). Thus, sample sizes for untreated, V, and CORT eggs are 11, 13, and 13, respectively. Due to a videotape malfunction, the first egg to hatch could not be determined for one clutch. In this case, the untreated and CORT treated eggs were discovered at the same time and were both assigned to Hatch Order 1 for analyses.

CORT eggs were significantly more likely to hatch first than were V and untreated eggs (Likelihood Ratio $\chi^2 = 14.5$, $p < 0.01$; Table 2B). CORT eggs hatched first in 9 of 12 clutches (excludes the clutch with a tie for Hatch Order 1), hatched before V eggs in 10 of 12 clutches (Fisher Exact Test: $p < 0.01$), and hatched before untreated eggs in 7 of 9 clutches (Fisher Exact Test: $p = 0.06$). Vehicle treatment alone does not stimulate hatching, as vehicle-treated eggs hatched before untreated eggs in only 3 of 10 clutches. Relative hatch times of untreated, V, and CORT eggs were 741.9 ± 209.0 , 1135.5 ± 240.8 , and 46.1 ± 38.0 min, respectively (Repeated measures ANOVA: $F_{2,16} = 7.21$, $p < 0.01$; Figure 1).

Untreated, V, and CORT eggs did not differ in mass ($F_{2,34} = 0.46$, $p > 0.60$), length ($F_{2,32} = 0.80$, $p > 0.40$), or width ($F_{2,34} = 0.55$, $p > 0.50$). Similarly, the hatchlings resulting from these eggs did not differ in mass ($F_{2,34} = 0.43$, $p > 0.60$) or snout-vent length ($F_{2,34} = 0.69$, $p > 0.50$).

DISCUSSION

We show that hatching of near full-term tree lizard embryos is prevented in a dose-dependent manner by RU-486, a synthetic progesterone- and corticosterone-antagonist. Progesterone does not appear to affect hatch time or success, therefore we focused our experimental efforts on the potential role of corticosterone in the regulation of the hatching event. We found that corticosterone promotes hatching of late-term embryos such that CORT treated eggs hatched, on average, 12 to 18 hours sooner than control eggs. The stimulatory effect of corticosteroids on hatching has previously been documented in teleosts, where corticosteroids are thought to play an indirect role in regulating hatching (Cloud, 1981; Schoots et al., 1982). The data

presented herein suggest that embryonic corticosteroids may not only be permissive, but may be required for successful hatching of tree lizards.

Rises in embryonic corticosteroid levels near hatching have been found in a number of species. Such rises are unlikely to be simple byproducts of adrenal development, but rather are likely to be functionally significant. Given that the hatching process is expected to involve strenuous movements and to represent an energetic challenge for embryos (Soffe, 1991; Speer-Blank and Martin, 2004; but see Ninness et al., 2006), the role of glucocorticoids in hatching may be associated with the mobilization of lipid substrates to provide fuel for the process. Glucocorticoids are known to regulate locomotion and to mobilize energy stores via gluconeogenesis in juvenile and adult animals (e.g., Sheridan, 1986; Sheridan, 1994; Breuner et al., 1998), and are expected to have similar effects on near-term embryos.

Glucocorticoids also are known to accelerate lung development in fetal mammals (Kotas et al., 1971; Torday et al., 1975) and, in combination with thyroid hormone, stimulate surfactant production in embryonic green sea turtles (Sullivan et al., 2001) and bearded dragons (Sullivan et al., 2002). Sullivan and colleagues (2002) suggest that species with precocial hatchlings/neonates, such as tree lizards and most reptiles, may be selected for a rapid and late embryonic development of the lungs, including absorption of the embryonic lung fluid, and production and secretion of surfactant. Thus, CORT treated eggs may have hatched sooner because those embryos more rapidly completed lung development and other preparations for the transition to the free-living hatchling life stage. Consistent with this idea, the observed reduction in survival, but not body mass, of RU-486 treated hatchlings may be a symptom of a corticosteroid-blocking effect of RU-486 on embryonic lung development.

Regardless of the underlying mechanisms, the regulation of hatching via corticosteroids may be adaptive if embryonic corticosteroids are sensitive to environmental conditions, such as oxygen availability and desiccation rate. Respiratory stress of the embryo inside the egg may result in an increase in corticosteroid production by the embryonic adrenal glands, and initiate a chain of events that results in the individual escaping from the eggshell. Indeed, the role of glucocorticoids in inducing escape from one life stage to the next has been found in association with deteriorating fetal and larval environments in mammals and amphibians, respectively (Denver, 1997; Crespi and Denver, 2005). For example, in response to pond drying or adverse social environments (e.g., extreme competition or predation pressure), larval amphibians experience an activation of the hypothalamic-pituitary-adrenal axis that results in an increase in development rate (Denver, 1997). This response is adaptive as it speeds up the tadpoles' escape from deadly conditions. Interestingly, however, elevated glucocorticoid levels that stimulate metamorphosis during late development may inhibit metamorphosis during early development (Hayes, 1997).

The effect of corticosteroids on embryonic development and hatching success of tree lizards also may depend on the time of delivery. Preliminary work suggests that super-physiological levels of corticosterone in egg yolk at the time of oviposition can greatly reduce hatching success of tree lizards (Johnston & Moore, unpublished data; L. D. Lucas & Weiss, unpublished data). We expect that this is due to gross developmental effects of corticosterone on early embryos rather than an effect on the hatching event itself. In other words, tree lizard embryos may be unlikely to develop to full-term in extremely high corticosterone environments. Interestingly, hatching success and hatch time are unaffected by corticosteroid manipulations during early embryonic development in damselfish (McCormick and Nechaev, 2002) and soho salmon (Stratholt et al., 1997). Generally, however, elevated corticosteroids in maternal circulation or egg yolk have widespread effects on embryonic development, as well as neonatal

behavior, growth and survival (e.g., Hayward and Wingfield, 2004; Crespi and Denver, 2005; Meylan and Clobert, 2005).

In summary, we found that treating late-term embryos with a corticosterone antagonist, RU-486, prevented hatching of viable embryos while treatment with corticosterone induced earlier hatching. Thus, corticosterone appears to play an important role in the success and timing of hatching in tree lizards. Further research should address if corticosterone regulates hatching in other reptiles, and if environmental conditions can alter embryonic corticosterone production and thus hatch time.

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Fig 1.

Effect of egg treatment on mean (+SE) relative hatch time. Eggs were either left untreated, were treated with 5 μ l ethanol (vehicle) or were treated with 350 μ g corticosterone in 5 μ l ethanol (CORT) on Day 30 of incubation. Relative Hatch Time indicates the time when a given egg hatched relative to the first hatching egg from the same clutch. CORT treated eggs hatched significantly earlier than untreated and vehicle eggs ($F_{2,16} = 7.21$, $p < 0.01$). Different letters over bars indicate significant differences between groups at a $p < 0.05$ level.

Table 1

Hatching success of eggs treated with ethanol (vehicle, V), progesterone (P), 20:1 RU-486:P (20R:1P), 1:1 RU-486:P (1R:1P), or a low dose of RU-486 only (0.5R) in 2000.

Day 30 treatment	Total treated	# dead	# hatched	# opened	True hatching success (%)	Estimated hatching success (%) ¹		
						Method1	Method2	Method3
V	95	13	82	.	86.3	.	.	.
P	93	5	88	.	94.6	.	.	.
20R:1P	11	11	0	.	0.0	.	.	.
1R:1P	74	47	7	20	.	36.5	21.9	9.5
0.5R	26	8	5	13	.	69.2	42.2	19.2

¹ See text for methods of estimating hatching success of surgically opened eggs. True hatching success of RU-486 treated eggs likely lies between the Method 2 and Method 3 estimates.

Table 2

Hatch order of eggs from (A) 2003 and (B) 2004.

(A)	Day 30 treatment ¹	Hatch Order 1 ²	Hatch Order 2	
	V	4	9	
	CORT	11	2	
(B)	Day 30 treatment ¹	Hatch Order 1 ²	Hatch Order 2	Hatch Order 3
	Untreated	4	4	3
	V	1	7	5
	CORT	10	2	1

¹ V: vehicle (ethanol); CORT: corticosterone.² Ties in order were assigned to Hatch Order 1.