

# Diurnal and nocturnal differences in hypothalamic–pituitary–adrenal axis function in Galápagos marine iguanas

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## Abstract

Temporal modulation of the stress response is a ubiquitous characteristic of animals. Here, we investigate possible mechanisms underlying daily changes in corticosterone release in an ectotherm model system. Earlier work indicated that free-living Galápagos marine iguanas (*Amblyrhynchus cristatus*) have lower corticosterone concentrations during the night than during the day. This could result from: (i) a lower circadian secretion of adrenocorticotrophic hormone (ACTH) as seen in mammals; (ii) from an increase in corticosterone negative feedback; or (iii) reflect lower metabolic activity during the night when core body temperature falls (from 35 °C during the day to as low as 21 °C during the night). To begin to distinguish between these three possibilities, exogenous ACTH was used to compare diel differences in adrenocortical tissue responsiveness, and dexamethasone was used to compare diel differences in the efficacy of corticosterone negative feedback. Low levels of exogenous ACTH (30 IU/kg body weight) potently stimulated both daytime and nighttime corticosterone release. Dexamethasone (1 mg/kg) inhibited only daytime, but not nighttime endogenous corticosterone release. Because the response to ACTH was similar between day and night we suggest that a simple lowering of core body temperature cannot explain the nighttime reduction in corticosterone release. However, the failure of negative feedback at night suggests that the response is not equivalent to the controlled downregulation seen in mammals.

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## 1. Introduction

The factors underlying the pervasive cyclic changes in glucocorticoid release in vertebrates are still poorly understood (Romero, 2002). Plasma concentrations of glucocorticoids change both on a seasonal and on a daily basis in all free-living animals investigated so far (Romero, 2001; Romero and Wingfield, 1998). To understand some of the mechanisms responsible for cyclic glucocorticoid release we invoked the approach of using non-traditional model systems (Schlinger et al., 2001), in our case a reptile that is exposed to changing body temperatures and daily changes

in foraging time (Wikelski and Hau, 1995). Such a system might allow us to distinguish between the various hypotheses that could explain changes in corticosterone levels (see below).

We recently showed that corticosterone concentrations in free-living Galápagos marine iguanas (*Amblyrhynchus cristatus*) vary in a diel manner (Woodley et al., 2003). Corticosterone was low at night and increased during the day. One question that arose from this work was whether the day/night differences were regulated, or simply a byproduct of body temperature differences. Since marine iguanas are ectotherms, their daytime core body temperatures of approximately 35 °C fall to approximately 26 °C at night (Butler et al., 2002). The reduced nocturnal corticosterone concentrations, therefore, may be a consequence of a lower metabolism at nighttime temperatures as seen with other

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species (Cree et al., 2003). Alternatively, the lower nocturnal corticosterone concentrations could reflect the circadian nadir of controlled regulation as seen in mammals (Dallman and Bhatnagar, 2001).

We began to test these two hypotheses by comparing the day/night functional sensitivities of two regulatory points of the hypothalamic–pituitary–adrenal (HPA) axis that are particularly amenable for study in free-living vertebrates (Romero, 2004). The first regulatory point is the adrenal cortical tissue's ability to respond to ACTH. Injecting exogenous ACTH during the day and night should result in equivalent stimulation of corticosterone release if there is a controlled downregulation of endogenous ACTH release (e.g., Romero, 2001; Romero et al., 1998; Romero and Wingfield, 1998). The second regulatory point is the termination of the corticosterone response via negative feedback (Dallman et al., 1992). A more effective negative feedback signal at night could result in lower corticosterone concentrations. The most accepted way to test the efficacy of negative feedback is with a dexamethasone (DEX) suppression test (Reul et al., 1990; Sapolsky and Altmann, 1991). DEX is a synthetic glucocorticoid that binds to corticosterone receptors, thereby artificially stimulating negative feedback. This results in a decrease in circulating endogenous corticosterone concentrations if feedback is functioning normally. This decrease can be monitored since DEX does not bind to the commonly used antibodies in the radioimmunoassays. In a DEX suppression test for free-living wild animals, DEX is injected immediately after capture and corticosterone levels are monitored over time (Astheimer et al., 1994; Sapolsky and Altmann, 1991). Comparing how these two regulatory points function in the day and at night is the first step to determining the mechanism controlling nocturnal corticosterone concentrations. Finally, changes in body temperature or associated changes in energy expenditure between night and day could also in itself be responsible for changes in corticosterone levels.

## 2. Materials and methods

Marine iguanas from Santa Fe Island (0°50' S, 90°5' W) were captured from January 12–13, 2001 and November 19–21, 2002. Animals were captured either by hand or with a noose attached to a long pole within 30 s of approach. We randomly selected animals from the adult population and they weighed between 0.7 and 2.6 kg. Although sex was determined for these animals, it was ignored in the analyses because corticosterone responses in marine iguanas do not differ by sex at these times of year (Romero and Wikelski, 2001). Immediately upon capture, we placed each iguana head-first into an opaque cloth bag and took a blood sample from the caudal vein on the underside of the tail using a 2 ml heparinized vacutainer collection vial (Becton Dickinson). The initial blood sample was taken usually within 1–2 min of capture, and never more than 3 min after capture. Corticosterone concentrations do not increase within the first 3 min of capture in this species (Romero and Reed,

2005). All initial samples were grouped together for statistical purposes.

After collecting the initial blood sample, one group of iguanas was injected intraperitoneally (i.p.) with porcine ACTH (Sigma Chemical). Porcine ACTH was chosen because it is the most common form previously administered to reptiles. ACTH was dissolved in dH<sub>2</sub>O, diluted with lactated Ringer's solution, and injected in doses of 30, 50, and 80 IU/kg, all doses injected in a volume of 0.2 ml. Powdered ACTH was reconstituted on site, maintained on ice, and used within 24 h. Controls for the ACTH injections consisted of 0.2 ml of lactated Ringer's solution injected i.p. Subsequent to ACTH injections, blood samples were collected into heparinized microhematocrit tubes from a hypodermic needle at 15, 30, and 60 min post injection.

A second group of iguanas was maintained in the cloth bags for 15 min, a second blood sample was collected, and the animals were injected i.p. with DEX (Sigma Chemical). DEX was dissolved in ethanol and injected at doses of 100 µg/kg (in 10 µl) or 1 mg/kg (in 100 µl). Controls for the DEX injections consisted of 100 µl ethanol injected i.p. Subsequent to DEX injection, blood samples were collected in microhematocrit tubes at 1, 2, 3, 4, 5, and 6 h post injection. This protocol reflects a standard clinical DEX suppression test (e.g., Carroll et al., 1981) as modified for use in free-living animals (Sapolsky and Altmann, 1991). Furthermore, for the DEX-injected animals during the day, groups of iguanas were injected with 50 IU/kg ACTH immediately after the 2, 3, and 4 h blood samples, with a final blood sample taken 30 min later. Unfortunately, at the time we did not feel it necessary to inject ACTH after DEX administration at night. Iguanas were kept in the opaque bags throughout the sampling period.

At the end of all blood collections, iguanas were marked with synthetic paint to avoid recaptures, weighed, measured for their snout-to-vent length, and released at the point of capture. All procedures were conducted in accordance with the guidelines from the American Society of Ichthyologists and Herpetologists and approved by the Tufts University Institutional Animal Care and Use Committee.

Blood samples were stored on ice for less than 12 h and then centrifuged at approximately 400g for 5 min. Plasma was then removed and stored on ice for transport to Tufts University. Samples were assayed for corticosterone using a previously described radioimmunoassay (Romero and Wikelski, 2001; Wingfield et al., 1992). Briefly, plasma was equilibrated with a small amount of tritiated corticosterone to measure subsequent recovery, and then steroids were extracted with redistilled dichloromethane. Each sample was then assayed in duplicate, with intraassay and interassay variations of 10 and 20%, respectively.

Data were analyzed using repeated measures ANOVA (with diel time and injection treatment as factors, and changes in corticosterone concentrations over time as the repeated measure) followed by Fisher's Protected Least Squares Difference (PLSD) post hoc tests that make adjustments for the repeated measure.

**3. Results**

ACTH was very effective at stimulating corticosterone release (Fig. 1). Corticosterone increased over the 60 min of restraint in all treatments (overall effect of sample time  $F_{3,165}=98.7, p<0.0001$ ). ACTH significantly augmented this corticosterone release (overall effect of treatment  $F_{3,55}=7.52, p<0.0005$ ). Post hoc analysis showed that all ACTH doses were different from controls ( $p<0.005$  for each comparison), but no ACTH dose was statistically different from any other. Furthermore, there was an overall difference between iguanas injected during the day and night ( $F_{1,55}=17.0, p<0.0001$ ), as well as diel differences between how the iguanas responded to restraint (interaction between sample time and diel time,  $F_{3,165}=6.17, p<0.0005$ ) and the effect of ACTH injection (interaction between diel time and treatment,  $F_{3,55}=4.68, p<0.01$ ). Finally, ACTH significantly altered the corticosterone response to restraint (interaction between sample time and treatment,  $F_{9,165}=3.60, p<0.0005$ ).

DEX dramatically reduced endogenous corticosterone release during the day (Fig. 2A). The 6 h of restraint significantly changed corticosterone concentrations (overall effect of sampling time,  $F_{7,77}=9.67, p<0.0001$ ). DEX significantly

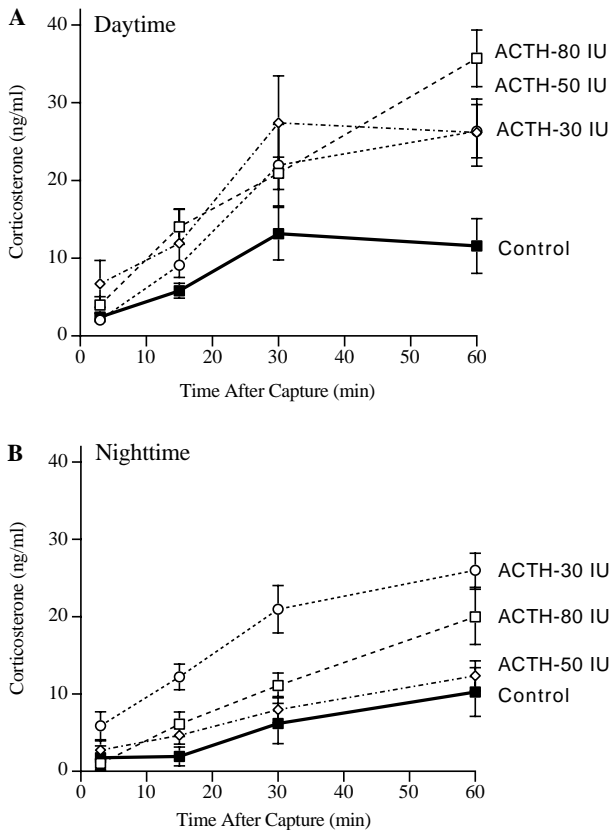


Fig. 1. Corticosterone responses to different doses of porcine ACTH injected immediately after capture in marine iguanas captured during the day (A) and at night (B). Each point represents the mean  $\pm$  SEM for  $n=7$  for controls and  $n=6,8,8$  for increasing ACTH doses during the day, and  $n=8$  for controls and  $n=8,10,8$  for increasing ACTH doses at night.

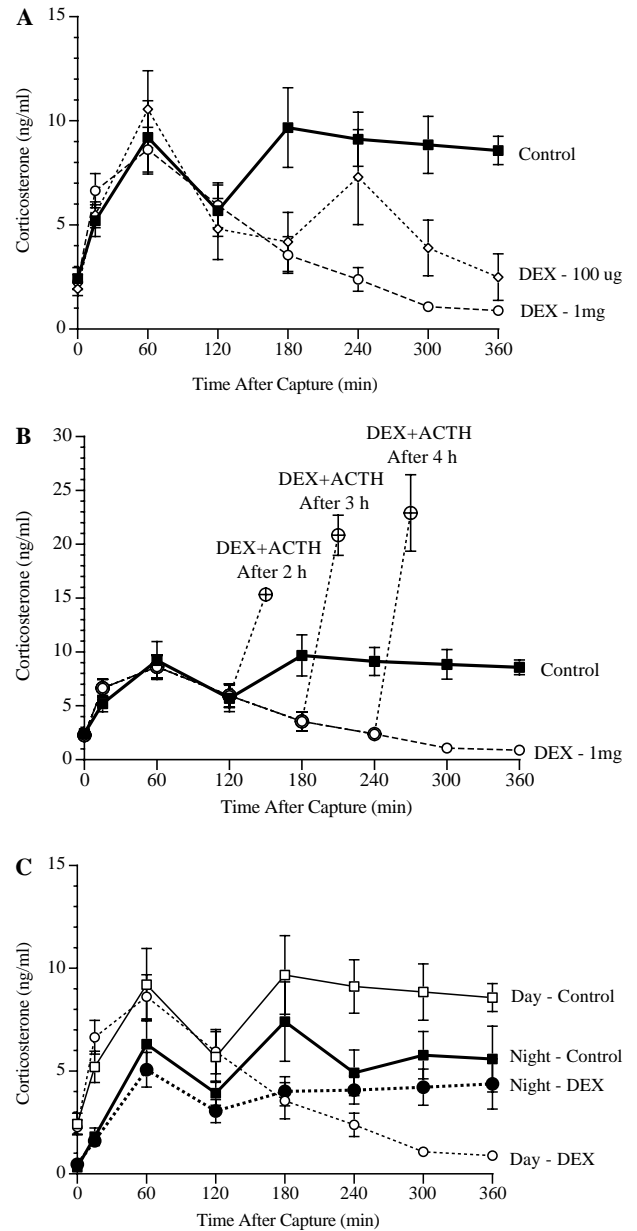


Fig. 2. Corticosterone responses to DEX administration immediately after capture in marine iguanas. (A) Response to different doses of DEX during the day.  $n=5$  for controls and  $n=5,4$  for increasing doses of DEX. (B) Response of 1 mg/kg DEX-injected iguanas to subsequent ACTH challenge. Controls are repeated from panel A.  $n=5$  for each ACTH injection. All DEX-injected groups were combined prior to ACTH injection, resulting in  $n=19,19,19,19,14,9,4,4$  for 0,15,60,120,180,240,300,360 min samples, respectively. (C) Differences in responsiveness to 1 mg/kg DEX during the day and night. Day values are repeated from panel A.  $n=5$  for both control and treatment at night.

altered this response (overall treatment effect,  $F_{2,11}=6.77, p<0.02$ ; interaction between sample time and treatment,  $F_{14,77}=4.49, p<0.0001$ ). Post hoc analysis indicated that the 1 mg/kg dose ( $p<0.005$ ) and nearly the 100  $\mu$ g/kg dose ( $p=0.051$ ) were statistically different from controls, but the two DEX doses did not differ from each other. DEX's inhibition of endogenous corticosterone release was reversible by injecting ACTH (Fig. 2B).

The response to 1 mg/kg DEX at night was very different (Fig. 2C). There was an overall treatment effect when comparing day and night ( $F_{1,15} = 12.64$ ,  $p < 0.003$ ), but post hoc analysis indicated that this was entirely driven by the response to DEX during the day. There was no difference between control and DEX injections at night. This led to an overall difference between day and night at the  $p = 0.069$  level ( $F_{1,15} = 3.85$ ), and a difference between the response to DEX during day and night at the  $p = 0.068$  level (interaction between diel time and treatment,  $F_{1,15} = 3.87$ ).

#### 4. Discussion

Several studies have shown that corticosterone increases in response to capture and handling in a variety of reptile species (e.g., Cash et al., 1997; Jessop et al., 2004; Knapp and Moore, 1995; Lance and Elsey, 1999; Lance et al., 2004; Manzo et al., 1994; Moore et al., 1991) and marine iguanas are no exception (Romero and Wikelski, 2001, 2002; Wikelski et al., 2001). Corticosterone concentrations are typically low when samples are collected within a few minutes of capture (reviewed by Tyrrell and Cree, 1998), and restraint in a cloth bag increases corticosterone concentrations, although stress-induced samples are often not taken for several hours (Guillette et al., 1995; Tyrrell and Cree, 1998). Several studies indicate that corticosterone responses in reptiles can change dramatically in different seasons (reviewed by Guillette et al., 1995; Romero, 2002), therefore all iguanas in this study were captured at the same time of year. Nevertheless, similar to our earlier study (Woodley et al., 2003) the corticosterone response is attenuated at night, which is consistent with studies in other reptile species showing a connection between corticosterone concentrations and body temperature (e.g., Jessop et al., 2000; Jones and Bell, 2004). The control treatments during the day are higher than the control treatments at night (Fig. 1).

The iguanas in this study responded robustly to an ACTH challenge. This is consistent with several other studies that showed a range of effective ACTH doses in several reptile species (Baverstock and Bradshaw, 1975; Dauphin-Villemant et al., 1990; Lance and Lauren, 1984; Mahmoud et al., 1996), including an iguanid (Daugherty and Callard, 1972). It was surprising, however, that all doses were equally potent (Fig. 1), although the i.p. route of injection may have masked any sensitive dose–response. Even though endogenous ACTH does not fully stimulate corticosterone secretion, these data indicate that 30 IU/kg, and perhaps less, saturates the response. Conclusions from the data at night, however, are not as strong since it is unclear why 50 IU/kg should be less effective than either 30 or 80 IU/kg. Regardless, the data indicate that iguanas maintain a reserve capacity to secrete corticosterone at night despite the nocturnal downregulation. This suggests that the mechanism is not simply a temperature-dependent lowering of metabolism resulting in reduced secretion of corticosterone. Instead, these data imply that there is less endogenous ACTH being secreted from the pituitary at

night. However, exogenous ACTH produces a slightly lower response at night, suggesting that there may be a modest decrease in secretory capacity at night that may be akin to seasonal alterations in steroidogenic function (Carasia and John-Alder, 2003).

Although the nocturnal differences in ACTH efficacy are slight, there is a large nocturnal change in the response to DEX. During the day, DEX potently inhibits endogenous corticosterone release with a delay of about 3 h (Fig. 2A). This is similar to the response seen for birds and mammals (e.g., Astheimer et al., 1994; Reul et al., 1990; Sapolsky and Altmann, 1991) and is thought to reflect a time delay resulting from DEX binding to receptors in the brain, altering gene transcription rates, and finally decreasing HPA axis activity (Dallman et al., 1992). A similar dose, however, was ineffective at decreasing corticosterone release in captive American alligators (Mahmoud et al., 1996). DEX-induced suppression presumably reflects a strong negative feedback signal throughout the HPA axis (Dallman et al., 1992; Jacobson and Sapolsky, 1991). The conclusion that DEX is inhibiting corticosterone secretion via negative feedback is strengthened when exogenous ACTH can short-circuit the response (Fig. 2B). However, DEX is essentially ineffective at night (Fig. 2C), implying that negative feedback is not functioning. These data are consistent with a lower metabolic function. Many different mechanisms, however, could account for the lack of a response at night, such as decreased corticosterone receptor numbers. Furthermore, there could also be a change in the ability of DEX to cross the blood–brain barrier, as is seen in mammals (Pariante et al., 2004). To our knowledge, DEX has not been previously administered to a free-living reptile.

In conclusion, nighttime corticosterone regulation appears more complex than either a temperature-driven decrease in overall metabolism or a controlled downregulation of the response. The exogenous ACTH data indicate that less endogenous ACTH is being secreted at night, because a larger ACTH signal could elicit a larger corticosterone response. Consequently, the metabolic capacity to produce corticosterone is only slightly reduced at night, if at all. On the other hand, the DEX data clearly suggest that HPA axis function is not simply downregulated, but is qualitatively different. Whether or not this is related to lower metabolism due to a lower core body temperature remains to be tested.

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