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## Are hotshots always hot? A longitudinal study of hormones, behavior, and reproductive success in male marine iguanas

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### ABSTRACT

Polygynous lek-mating systems are characterized by high reproductive skew, with a small number of males gaining a disproportionate share of copulations. In lekking species, where female choice drives male mating success and patterns of reproductive skew, female preferences for 'good genes' should lead to preferred males having high reproductive success in all years. Here we investigate whether these 'hot-shot' males have steroid hormone patterns that are consistent over time (between two mating seasons), and whether hormone levels consistently predict display behavior. We test these questions in the Galápagos marine iguana (*Amblyrhynchus cristatus*), a lekking vertebrate with high male reproductive skew. We found that male mating success and testosterone levels were not consistent across years. The most successful males showed an inverse relationship in copulation success between years. Similarly, territorial males that had high testosterone in one year had low levels in the next. Across years, testosterone was strongly associated with head-bob display, suggesting that this steroid plays a key role in mate attraction. These results suggest that female marine iguanas are not choosing the same 'hotshot' males in every year, but instead base their reproductive decisions on male behavioral traits that are hormonally mediated and variable across years. By using testosterone to regulate their costly display behaviors male marine iguanas appear to have a mechanism that allows them to adjust their reproductive effort depending on extrinsic and/or intrinsic factors.

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### 1. Introduction

When animals aggregate for reproductive purposes, such as in lek systems, there is frequently a high mating skew (Emlen and Oring, 1977; Höglund and Alatalo, 1995) indicating that some males are disproportionately selected by females (Hill, 1991; Lanctot et al., 1997, 1998). In lekking species, female preferences are often based on male display (Gibson and Bradbury, 1985; Andersson, 1989; Höglund and Robertson, 1990; Pruett-Jones and Pruett-Jones, 1990; McElligott et al., 1998), but it is not clear whether display is used as an indicator of genetic quality. If female preferences are based on 'good genes' then male mating success should be relatively consistent across years. For example, lekking great snipe (*Gallinago media*) show high consistency in reproductive success between years (Sæther et al., 2005), but it is not known whether this pattern is typical of lekking species. Male reproductive dis-

plays incur significant costs in many species (Magurran and Nowak, 1991; Magnhagen, 1991; Isvaran and Jhala, 2000; Basolo and Alcaraz, 2003; Woods et al., 2007) and in some cases are linked to steroid hormone levels (Marler and Ryan, 1996; Mayer et al., 2004; Day et al., 2006; Neal and Wade, 2007). Here we test the consistency of mating success in lekking male Galápagos marine iguanas (*Amblyrhynchus cristatus*) over a two-year period, and investigate whether the physiological and hormonal correlates of copulation success consistently predict individual performance.

In order to determine the behavioral and physiological trade-offs males face in deciding how much energy to devote to reproduction in a given season, we observed individual male marine iguanas during two successive mating periods. We were particularly interested in examining how the decision to reproduce in one year affects a male's likelihood of reproducing in the subsequent season (Beletsky et al., 1992). We attempted to determine which characteristics contribute to reproductive success in any given time period, and whether these traits are inherent to individual males. If a male gained a large number of copulations in one breeding season would he also be successful in subsequent years? Investigating these questions provides insight into patterns of male

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reproductive success in lekking species, the cues that females use to assess mates in these systems, and how males adjust their reproductive effort depending on intrinsic and extrinsic factors.

## 2. Materials and methods

### 2.1. Study system

Galápagos marine iguanas are an ideal system in which to study mating success and reproductive trade-offs because they exhibit strong male mating skew and several alternative male mating tactics with differing costs and benefits (Wikelski et al., 1996, 2001). Moreover, because females typically mate only once per season (Trillmich, 1983), copulation number is a reasonable proxy for male reproductive success. On Santa Fe Island, the breeding season of Galápagos marine iguanas commences in November when males begin establishing territories, and finishes in early January, by which time all receptive females have mated and males cease to defend territories. Successful territorial males maintain nearly constant presence on territories during the mate choice period, and may fast for up to 35 days during the reproductive period (Trillmich, 1979), losing up to 30% of their body mass (M. Vitousek, unpublished data). Marine iguanas experience strong selection pressure during periodic El Niño events that cause high mortality, particularly among larger animals (Wikelski and Trillmich, 1997; Wikelski et al., 1997; Laurie, 1990; Laurie and Brown, 1990a,b). Males that are territorial in the year prior to an El Niño event show increased mortality, likely due to a combination of their large body size and the high energetic demands of territorial defense (Wikelski and Trillmich, 1997). Male reproductive behavior is not affected by predation, as adult-sized males experience no native predators of sufficient size to capture them. Marine iguana reproduction occurs in the open and is highly visible, unlike the mating behavior of more cryptic reptile species.

### 2.2. Data collection

All data were collected at the 'Miedo' study site on Santa Fe Island, Galápagos (0°S 50'S; 09°S 5'W) from November 1999 to January 2001. Beginning in late November we weighed, measured, marked, and took blood samples from all large (potentially territorial) males on the two peninsulas in the area. Any males that subsequently established territories on the peninsulas were also captured, marked, measured, weighed, and bled. Animals captured in 1999–2000 (year 1) and in 2000–2001 (year 2) were sampled at night (starting 30 min after sunset, ~19:30, until approximately 22:00) to avoid disturbance of mating aggregations. At the end of the 1999–2000 breeding season we permanently branded animals on the ventral side of the tail. We were able to recapture 26 of these branded animals in the following (2000–2001) breeding season and track their body condition, behavior, hormone levels, and mating success. Of the 26 animals followed for two years, 11 were territorial for both years, two were territorial only in year 1, and five were territorial only in year 2. The rest were not territorial in either year.

### 2.3. Hormone sampling

Sampling occurred over the same time frame in each year of the study. In year 1, hormone samples were collected three times per individual between November 23 and January 10. In year 2, a mean of two samples per individual were collected between November 27 and January 12. Repeated samples were averaged for each individual. At each sampling period approximately 1.5 mL of blood was collected ventrally from the caudal vein into a heparinized vacutainer tube using a 21-gauge needle. Blood samples were taken within three minutes of approach and capture, after which time corticosterone levels may begin to rise (Romero and Reed, 2005). In year 1, samples were placed in heparinized centrifuge tubes and spun in a field centrifuge for 4 min; in year 2, samples were allowed to settle naturally before extraction. Plasma was extracted with a Hamilton syringe and stored in a vial containing 750 mL of ethanol. In the field, vials were stored in the dark at ambient temperature for up to 4 weeks. Upon returning to the lab the samples were stored at -20° C until assayed. The field technique of storing plasma samples in ethanol has been previously validated (Tarlow et al., 2001; Wikelski unpublished data), and does not change relative steroid hormone concentrations (see Goymann et al., 2007).

Samples were analyzed for testosterone (T) and corticosterone (CORT) using radioimmunoassay protocols described by Wingfield and Farner (1975). A volume of 100 mL of plasma was used for analyses of CORT and 15 mL for T. Plasma aliquots were equilibrated overnight with 2000 cpm of 3 H-labeled hormone at 4 °C, extracted with dichloromethane, dried in a 40 °C water bath under nitrogen gas, and re-dissolved in 550 mL of buffer. Samples were allowed to equilibrate with buffer overnight at 4 °C. Duplicates of 200 mL were used in the RIA as described previously (e.g., Wingfield and Farner 1975; T antibody, T3003, Wien Laboratories, Succasunna, NJ; CORT antibody, B3-163 Endocrine Sciences, Calabasas, CA). Fractions of 100 mL were directly counted for the determination of recovery. Mean recovery was 74.6 ± 2.2% for T and 78.4 ± 1.8% for CORT. Two 400 mL aliquots of distilled water (water blanks) and a total of four 400 mL aliquots containing 0.15, 0.25, or 0.50 ng of non-radioactive hormone standards were taken through the whole as-

say procedure to estimate nonspecific interference, assay accuracy, and intra-assay variation. Blanks were below the detection limit, and the accuracy of the hormone standards was 7% for T and 11% for CORT. The intra-assay variation between two RIAs per hormone type was 9% for T and 12% for CORT. The assay sensitivity was 0.14 ng/mL for T and 1.7 ng/mL for CORT. Protocols were approved by the University of Illinois Institutional Animal Care and Use Committee, Princeton University's Laboratory Animal Research, and by the local Ecuadorian authorities (the Galápagos National Park Service).

### 2.4. Behavioral observations

We gathered data on territoriality, head-bob rate, and copulation success using a combination of focal and incidental observations. During focal samples, each marked male was observed for 10 min per day: 5 min in the morning and 5 min in the afternoon. Animals were rotated arbitrarily through the list so that they were sampled at different times every day. Focal observations recorded whether the male was present on his territory, the number of head-bobbing displays performed, and any interactions between the territorial male and other iguanas. Incidental observations were used to record the total number of copulations. Our study site encompassed two peninsulas that jut into the ocean. Observers were stationed on elevated outcrops above the animals and conducted incidental observations between 08:00 and 18:00 every day. At the beginning of the study period observers were trained for 3–5 days to limit inter-observer variability. The lava peninsulas are completely bare of vegetation, making it possible to observe virtually all interactions occurring on male territories.

### 2.5. Statistical analysis

We compared the behavior, hormone levels, and mating success of all males that were territorial in at least one of the two years of study ( $n = 18$ ). Data were analyzed using JMP 7, with  $\alpha$  at 0.05. When necessary, data were transformed to meet the assumptions of normality and homogeneity of variances using log and square-root transformations. 'Copulation success' could not be transformed to satisfy a normal distribution, so comparisons involving this variable were tested using non-parametric statistics that did not enable us to control for individual when comparing values between years. The mating success of males that were territorial in one year was compared to that of males territorial in both years using a Wilcoxon signed-rank test. Female preferences, the physiological correlates of preferred behaviors, and the consistency of behavioral and physiological traits between years were investigated using generalized linear mixed models (GLMM) and Spearman's rank correlations. We added 'individual' as a random effect to all GLMMs to account for repeated samples taken over multiple years, included as a fixed effect when appropriate. Annual differences in the reproductive behavior and physiology of territorial males were compared using paired  $t$ -tests.

## 3. Results

### 3.1. Mating success

We compared the two-year reproductive success (total number of copulations) of males that were territorial during both years and those that were only territorial for one of the two study years. These groups showed no difference in copulation success (territorial in one year:  $9.14 \pm 4.11$ ,  $n = 7$ ; both years:  $11.00 \pm 2.47$ ,  $n = 11$ ;  $U = 1.82$ ,  $df = 1$ ,  $P = 0.18$ ); therefore, we combined data from all territorial males for analysis. When copulation success was examined

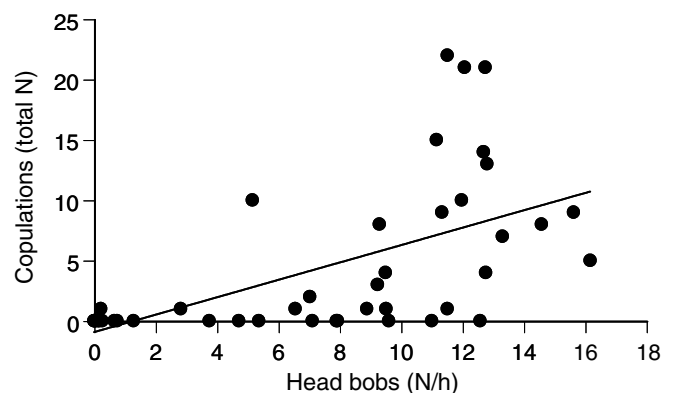


Fig. 1. The total number of copulations per year was highly correlated with head-bob rate. Each data point represents an individual male in one year.

over both years of study, the total number of copulations a male received was strongly associated with his head-bob rate (Fig. 1; head-bob:  $r_s = 0.56$ ,  $P = 0.0008$ ), but not with his body mass ( $r_s = 0.19$ ,  $P = 0.19$ ).

3.2. Physiological correlates of preferred traits

In order to determine whether females choose males based on a physiologically controlled trait, we investigated the relationship between hormones and head-bobbing. Testosterone was strongly associated with head-bob rate (Fig. 2a;  $F_{1,28.66} = 10.43$ ,  $P = 0.0031$ ). Although testosterone levels differed between years ( $F_{1,14.31} = 19.23$ ,  $P = 0.0006$ ), there was no interaction between head-bob rate and year ( $F_{1,27.08} = 0.34$ ,  $P = 0.56$ ). Circulating corticosterone was not associated with head-bob rate (Fig. 2b;  $F_{1,19.66} = 0.02$ ,  $P = 0.90$ ). Corticosterone differed between years ( $F_{1,10.59} = 27.82$ ,  $P = 0.0003$ ), but there was no interaction between head-bob and year ( $F_{1,24.22} = 2.19$ ,  $P = 0.15$ ).

Across years, head-bobbing was positively correlated with body mass, indicating that only larger males were able to sustain high head bob rates (Fig. 3;  $F_{1,28.85} = 8.76$ ,  $P = 0.0061$ ). Males with higher head-bob rates in year 1 also lost more mass between years than those that displayed less frequently ( $F_{1,14} = 8.11$ ,  $P = 0.013$ ,  $r = 0.606$ ).

3.3. Individual consistency

Mating success was not correlated between years when all territorial males were included ( $r_s = -0.11$ ,  $n = 18$ ,  $P = 0.67$ ); however, relatively successful and non-successful males showed different patterns. When all males that gained an average of one or fewer copulations per year were excluded from the analysis (leaving only

males that had high reproductive success), the remaining males showed an inverse correlation in mating success (Fig. 4;  $r_s = -0.66$ ,  $n = 11$ ,  $P = 0.028$ ), indicating that males with high copulatory success in one year had lower success the following year. Similarly, testosterone showed a tendency towards being negatively correlated between years (Fig. 5a;  $F_{1,14} = 4.49$ ,  $P = 0.052$ ,  $r = 0.492$ ).

Despite this apparent reproductive trade-off in reproductive success, males showed no relationship in head-bob rate ( $F_{1,13} = 0.47$ ,  $P = 0.50$ ,  $r = -0.187$ ), body mass ( $F_{1,14} = 2.43$ ,  $P = 0.14$ ,  $r = 0.384$ ) or corticosterone levels between years (Fig. 5b;  $F_{1,12} = 0.84$ ,  $P = 0.38$ ,  $r = 0.256$ ). However, individuals with higher testosterone did show a trend towards having lower corticosterone across both years ( $F_{1,20.61} = 3.74$ ,  $P = 0.067$ ).

3.4. Annual differences in behavior and physiology

We also examined differences in population means of head-bobbing behavior, hormone levels, and reproductive success across

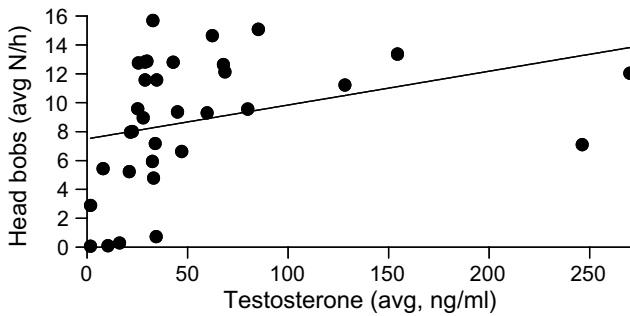


Fig. 2. Across years, head bob rate was strongly associated with circulating testosterone. The solid line indicates a significant linear regression.

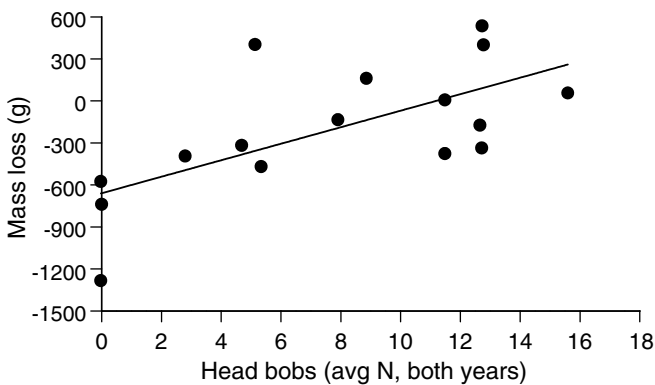


Fig. 3. Males that maintained higher head bob rates lost more body mass between years, suggesting that head bobbing is energetically costly. The solid line indicates a significant linear regression.

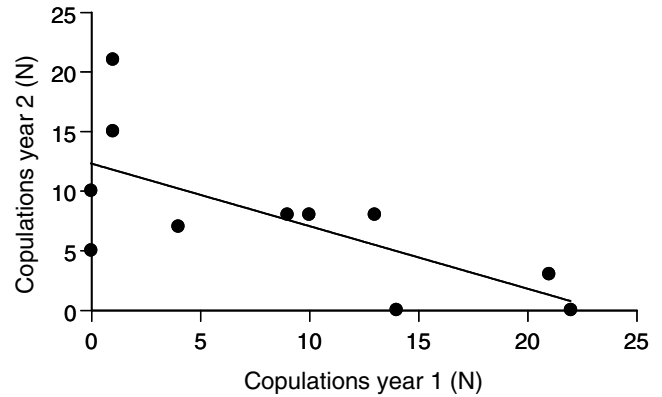


Fig. 4. Individual males that had high reproductive success in year 1 (measured by total number copulations) showed a decrease in mating success in year 2, and vice versa. The line indicates a significant linear regression.

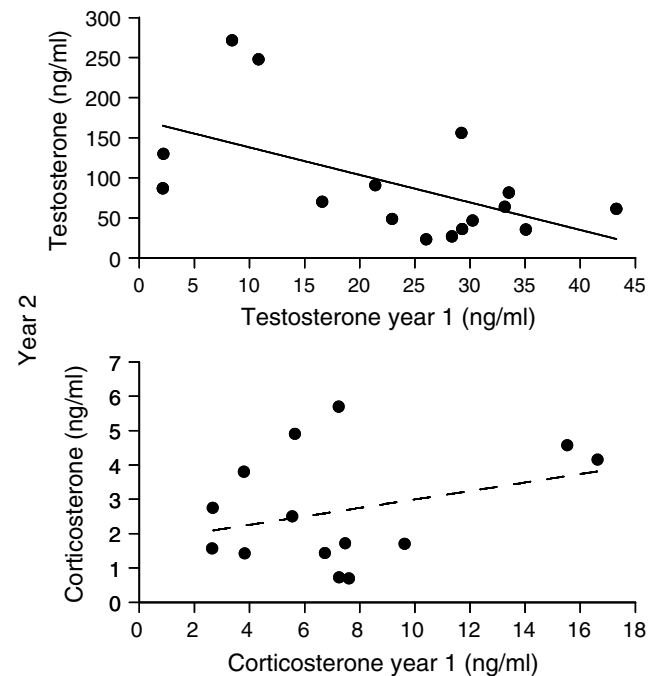


Fig. 5. Testosterone levels (top panel) were negatively correlated between years. Circulating corticosterone (lower panel) was not individually consistent.

years. The total number of copulations garnered by males did not differ between years ( $U = -0.021$ ,  $df = 1$ ,  $P = 0.88$ ). Likewise, neither body mass (paired  $t = 1.66$ ,  $df = 15$ ,  $P = 0.12$ ) nor head-bob rate (paired  $t = 0.93$ ,  $df = 14$ ,  $P = 0.37$ ) differed significantly over the study period.

Males did, however, show substantial differences in hormone levels. Circulating testosterone was lower in year 1 than in year 2 (Fig. 6a; paired  $t = 3.77$ ,  $df = 15$ ,  $P = 0.0019$ ). In contrast, males showed significantly higher baseline corticosterone in year 1 than in year 2 (Fig. 6b; paired  $t = 5.04$ ,  $df = 13$ ,  $P = 0.0002$ ).

#### 4. Discussion

Our results indicate that female marine iguanas appear to be choosing between territorial males based on their ability to sustain high rates of a costly display behavior: head-bobbing. Males with higher head-bob rates lost more mass between seasons than those with lower display rates. Similarly, only large males were able to sustain high head-bob rates. Head-bobbing behavior has previously been shown to be associated with both territorial defense and mate attraction in this species (Wikelski et al., 2001, 2005; Vitousek et al., 2007). The dependence of male mating success on display behavior is consistent with findings in many other lekking species (Gibson and Bradbury, 1985; Andersson, 1989; Höglund and Robertson, 1990; Pruett-Jones and Pruett-Jones, 1990; McElligott et al., 1998).

##### 4.1. Hormones and display behavior

The strong relationship between head-bobbing and testosterone suggests that this hormone plays a role in modulating costly mating displays. This hypothesis is consistent with previous find-

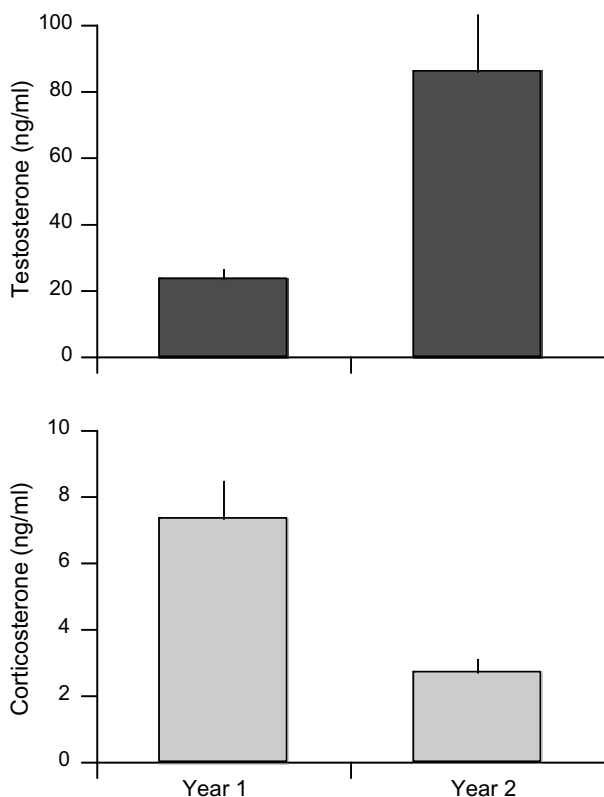


Fig. 6. Mean population-level testosterone and corticosterone differed between years. Testosterone (top panel) was higher during year 2; corticosterone (lower panel) was higher in year 1. Data show means  $\pm$  1 SE.

ings that male marine iguanas administered exogenous testosterone immediately increased both head-bobbing rate and territory size (Wikelski et al., 2005). Experimentally elevated testosterone has been shown to increase territorial behavior at the expense of condition and survival in a variety of lizards (Marler and Moore, 1991; Marler et al., 1995; Klukowski et al., 1998; Sinervo et al., 2000), but it is not known whether females in lekking species typically choose mates based on testosterone-dependent traits. Circulating levels of corticosterone were not related to head-bob display rates.

##### 4.2. Reproductive trade-offs

Successful territorial male marine iguanas showed an inverse correlation in reproductive success between years: males that gained a large number of copulations in one year were less likely to be successful in the following year. This apparent trade-off was observed only among the most successful males, and not those that gained relatively few copulations in both years. Over a two-year period, males that were territorial in both years did not have higher total copulation success than males that were territorial in only one season. This indicates that males may face a choice between following an intermediate strategy of being territorial in consecutive years and alternating highly costly years of greater reproductive success with much less successful non-territorial years.

Testosterone showed a negative relationship between years, further suggesting its role in modulating reproductive investment. Although males with low testosterone tended to have higher corticosterone, individuals showed little consistency in corticosterone between years (Romero and Reed, 2008). Interestingly, an inverse correlation was not present in head-bob rate or body mass. It is unclear why we did not see the expected correlation here, but it is possible that other factors related to the health or vigor of individual 'loser' males render them unlikely mating partners to females.

If female marine iguanas were choosing males based on 'good genes' alone (Fisher, 1915; Williams, 1966; Trivers, 1972; Zahavi, 1975, 1977; Hamilton and Zuk, 1982; Folstad and Karter, 1992; Andersson, 1992), then individual male mating success should be highly correlated between years. In contrast, we found that successful males showed a negative correlation in mating success between years. Thus, despite the high male mating skew within years (Wikelski et al., 1996, 2001), female marine iguanas are not simply selecting the same males (or those with the 'best genes') in every year. Between-year trade-offs in reproductive success, however, were only seen among the small group of highly successful males. Male marine iguanas exhibit four distinct strategies during the reproductive period: bachelor, sneaker, satellite, and territorial. Bachelor males are formerly territorial individuals taking the year off from breeding. Sneaker and satellite males are smaller, and typically have much lower reproductive success than territorial males (Wikelski et al., 1996). Even among territorial males, trade-offs were only apparent among males that gained an average of  $\geq 1$  copulation per season. Thus, although reproductive success is not consistent among this group of high-quality males, the ability to be territorial and maintain high levels of a costly, condition-dependent trait may indicate that males have considerably higher-than-average genetic quality. This latter hypothesis would argue for a minimum threshold of quality necessary for a male to be perceived as attractive.

By using hormones to regulate their costly display behaviors, male marine iguanas have a mechanism that allows them to adjust their reproductive effort depending on extrinsic and/or intrinsic factors (e.g. resource availability, social environment, body condition; Beletsky et al., 1992). Previous experiments indicated that

males almost immediately adjusted their territorial and reproductive behavior in response to experimental elevation or blocking of testosterone (Wikelski et al., 2005). The ability to rapidly modulate reproductive effort may help male marine iguanas to maximize their lifetime reproductive success while simultaneously minimizing their chances of mortality in an unpredictable environment (Laurie and Brown, 1990a,b).

#### 4.3. Annual differences in steroid hormones

Although neither mean copulation success nor the frequency of territorial and reproductive behaviors differed between years, males showed significant differences in hormone levels across the study period. Mean corticosterone levels were much higher during the first year of study (1999–2000), whereas testosterone was significantly higher during the second year (2000–2001). Both years were characterized by similar climatic conditions in the region, in which an abundance of food was available to marine iguanas. The occurrence of two consecutive resource-rich years is somewhat unusual for marine iguanas whose primary food source, macrophytic marine algae, is highly influenced by the El Niño Southern Oscillation (Laurie, 1987, 1990; Wikelski and Trillmich, 1997). During strong El Niño events marine iguanas experience high mortality, and typically exhibit almost complete reproductive failure in the following season (Laurie and Brown, 1990a; Wikelski et al., 1996). In the 1998–1999 reproductive period it is likely that almost no individuals reproduced due to the unusually strong El Niño that occurred during the previous season. Thus, because nearly all individuals gained a significant amount of weight between the first and second season of our study, it appears as though marine iguanas initiated the 1999–2000 mating season at lower than normal weights. During this season marine iguanas may still have been suffering from stress due to the prior El Niño event, as evidenced by their significantly higher levels of corticosterone and lower testosterone.

## 5. Conclusion

Our results indicate that the mating success of male marine iguanas is associated with their rate of head-bobbing, a costly display behavior that appears to be modulated by testosterone. Marine iguanas show a strong male mating skew within years. Among the small number of highly successful males, individuals showed a negative correlation in mating success between years, indicating that males that are 'hotshots' in one year suffer a cost in terms of copulation success in the following year. This trade-off in reproductive success suggests that among territorial males, head-bob rate is not a reliable indicator of 'good genes'. However, because only a small proportion of males are able to sustain the high head-bob rates necessary to obtain copulations, it is possible that females could still be using the ability to maintain costly mating displays as an indicator of relative fitness. This scenario would help to explain the maintenance of genetic variation in lekking species despite high male mating skew (Rowe and Houle, 1996; Kotiaho et al., 2001).

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