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Corticosterone suppresses immune activity in territorial Galápagos marine iguanas during reproduction

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Abstract

Individuals that display elaborate sexually selected characters often show reduced immune function. According to the immunocompetence handicap hypothesis, testosterone (T) is responsible for this result as it drives the development and maintenance of sexual characters and causes immunosuppression. But glucocorticoids also have strong influences on immune function and may also be elevated in reproductively active males. Here, we compared immune activity using the phytohemagglutinin (PHA) skin test in three discrete groups of male marine iguanas (*Amblyrhynchus cristatus*): territorials, satellites, and bachelors. Males of these three reproductive phenotypes had indistinguishable T concentrations during the height of the breeding season, but their corticosterone (cort) concentrations, body condition and hematocrit were significantly different. Territorial males, the animals with the most elaborate sexual ornaments and behaviors, had lower immune responses and body condition but higher cort concentrations and hematocrit than satellites or bachelors. To test directly cort's immunosuppressive role, we elevated cort by either restraining animals or additionally injecting cort and compared their PHA swelling response with the response of free-roaming animals. Such experimental elevation of cort significantly decreased immune activity in both restrained and cort-injected animals. Our data show that cort can induce immunosuppression, but they do not support the immunocompetence handicap hypothesis in its narrow sense because T concentrations were not related to immunosuppression.

Keywords: Immunocompetence; Trade-off; Resource allocation; Testosterone; Corticosterone; Iguana; Phytohemagglutinin

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Introduction

Immune function is often compromised in males with the most elaborate secondary sexual traits (Verhulst et al., 1999; Zuk and Johnsen, 2000; Zuk et al., 1995), but the proximate causes for this relationship are not well understood. It is often suggested that the androgen testosterone (T) is responsible for both exaggerated sexual traits and immunosuppression, as formalized by the immunocompetence handicap hypothesis (Folstad and Karter, 1992). Trade-offs between T-related secondary sexual characters and immune activity exist because both are costly (Demas et al., 1997; Gustafsson et al., 1995; Hanssen et al., 2004; Lochmiller and Deerenberg, 2000, Martin et al., 2003; Råberg et al., 2000; Sheldon and Verhulst, 1996). Only high quality males can display elaborate traits and simultaneously fend off ubiquitous attacks by parasites and pathogens (Hamilton and Zuk, 1982).

In support of the T-immunity trade-off, experimental studies in various vertebrates have found elevated T concentrations at the time when immune activity is reduced (Hillgarth and Wingfield, 1997; Nelson and Klein, 2000; Verhulst et al., 1999; Zuk et al., 1995). Experiments with T-implanted males have shown that T can directly cause

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immunosuppression as measured by a reduction in serum antibody titers to antigenic challenge (Duffy et al., 2002; Saad et al., 1990) and by a suppressed cell-mediated immune response (Duffy et al., 2002). In several experiments, ectoparasite loads were associated with immunocompetence (Olsson et al., 2000; Poiani et al., 2000; Salvador et al., 1996). T-implanted animals harbored relatively higher ectoparasite loads—a fact that was explained as a result of compromised immune function. Other studies, however, have been unable to confirm immunosuppression by elevated concentrations of plasma T. Saino et al. (1995) have shown that T-implantation resulted in higher levels of parasite infection, but could not find an obligatory immunosuppressive effect of T as measured by leucocyte counts and immunoglobulin concentrations. Further studies also found that neither antibody responses (Hasselquist et al., 1999; Lindström et al., 2001) nor cell-mediated immune response (Greenman et al., in press) were suppressed by above-normal concentrations of plasma T. The contradictory results of these T-implantation experiments suggest that the immune system is a very complex system and there may be no consistency in the effects of T on specific immune responses. Furthermore, the influence of T on immune function may be condition-dependent (Peters, 2000). Peters (2000) found T-induced immunosuppression in superb fairy-wrens only under laboratory conditions; under natural conditions, individuals with the highest T concentrations had the strongest immune response. She suggested that T-induced immunosuppression is highly condition-dependent and only immunosuppressive when resource availability is standardized, such as in the lab. In the field, where differences in resource acquisition ability and hence in body condition exist, the relationship between T and immunocompetence is not consistent (Peters, 2000).

While these results are exciting, other hormones may be important, and elevations in glucocorticoids may explain the apparent condition-dependent effects of T.

For instance, Casto et al. (2001) showed that male dark-eyed juncos (*Junco hyemalis*) implanted with T had increased concentrations of corticosterone (cort), a steroid hormone whose circulating blood concentrations are increased during times of stress. Thus, whether the observed immunosuppression in his and other birds is actually due to T's direct effects on immunity or to T's influence on cort production remains unclear. Indeed, Evans et al. (2000) also found elevated cort concentrations after experimental T augmentation in house sparrows, which in turn were related to the degree of immunosuppression. After statistically controlling for the effect of cort, T apparently enhanced the birds' ability to produce antibodies. A causal role for glucocorticoids in immunosuppression is conceivable as many studies have documented that circulating concentrations of corticosteroids are inversely correlated with immune function (Munoz et al., 2000; Nelson and Demas, 1996; Saad and El Ridi, 1988; Sapolsky, 1992). Similarly, the administration of exogenous cort provided strong evidence for cort-induced immunosuppression in lizards (*Chalcides ocellatus*) and alligators (*Alligator mississippiensis*) (Morici et al., 1997; Saad et al., 1986). However, it is still unclear whether wild animals show immunosuppression when exposed to naturally occurring elevations of cort in free-living animals.

In the present study, we used wild male marine iguanas (*Amblyrhynchus cristatus*) as a model system to investigate the interaction between reproductive behavior, body condition, immune function and steroid hormones. Because male marine iguanas differ in reproductive activity, which is probably associated with hormone concentrations, they are most suitable for these kinds of studies. Furthermore, endocrinological methods are already validated and well established in this species (Romero and Wikelski, 2001), and ease of capture and their calmness minimizes handling times. The marine iguana is a polygynous reptile species where males display in mating aggregations. Three male types can be classified that differ in morphology and behavior: territorials, satellites, and bachelors (Carpenter, 1966; Eibl-Eibesfeldt, 1966; Moore et al., 2000a; Wikelski and Baeurle, 1996; Wikelski et al., 1996). Territorial males are large and the most ornamented group. During the reproductive season, they have distinct skin coloration and elongated dorsal spines. They frequently show ritualized head-bobbing, a typical reproductive behavior to attract females and threaten off rivals (Carpenter, 1966; Wikelski et al., 2001). Territorial males, which defend clustered mating territories, are often surrounded by numerous females, and receive 95% of all copulations (Partecke et al., 2002). Satellite males are smaller and less ornamented than territorial males. They try to force-copulate with females leaving male territories, a strategy that results in low reproductive success (Wikelski et al., 1996). In general, male marine iguanas do not participate in reproduction each consecutive year probably to replenish their energy reserves after reproductive efforts. Males that were territorial in previous reproductive seasons, but are now reproductively quiescent during the current year, are called bachelor males. They do not exhibit reproductive behavior or ornamentation, which is probably associated with low T concentrations and regressed gonads. They spend most of their time grouped with other bachelors outside clusters of reproducing males (Dellinger, 1991; Laurie, 1989; Wikelski et al., 1996).

At the beginning of the reproductive season when territories are being established, territorial males have the highest T concentrations, followed by satellites and bachelors. However, at the peak of reproduction, T concentrations decrease in territorial and satellite males and do not differ between reproductive phenotypes (Nelson, 2003; Wikelski et al., 2005). We took advantage of this seasonal decrease in T concentrations and compared T-lymphocyte-mediated immunity in territorial, satellite, and bachelor males when they had similar T concentrations. The aim of our study was to test whether reproductively active males show immunosuppression in the absence of T differences and if other factors such as cort play a more important role in compromising the immune system in these males. In addition, we measured the influence of experimentally elevated cort concentrations on immune function in individual bachelor males. This allowed us to directly test whether cort alone can suppress the immune system of marine iguanas.

Methods

Field site and animals

Our field site, ‘Miedo’ (0°50′S, 90°02′W), is located on the southeastern coastline of Santa Fé island. Marine iguanas at Miedo live in large, dense aggregations. The coastline consists of lava rocks and extended inter-tidal zones with macrophytic marine algae exposed during low tide, on which marine iguana feed exclusively (Trillmich and Trillmich, 1984; Wikelski and Hau, 1995; Wikelski and Trillmich, 1994; Wikelski et al., 1993). Lava rocks above the inter-tidal zone offer territories for males. This study was performed from December 11, 2002 to January 3, 2003 during the peak of the reproductive season. Individuals were caught with a noose and marked with synthetic paint to ease observation and recapture. Paint markings did not change iguana behavior or interactions in previous studies (Audet and Wikelski, unpublished data).

Phenotypic comparisons

Male status

Males were classified as territorial, satellite, or bachelor by repeatedly observing their behavior for 10 min at different times of different days. A male was categorized as territorial if it exhibited frequent head-bobbing against other males and was found at least three times at the same location, successfully defending that location against rivals (Partecke et al., 2002; Trillmich, 1983; Wikelski et al., 1996). Satellite males generally head-bob less and are not consistently found at the same site. Males were classified as bachelors if they did not head-bob or engage in other obvious reproductive behaviors. We marked (a number with synthetic paint) and investigated a total of 49 individual males (24 territorials, 16 satellites, 9 bachelors).

Immune function

We measured immune activity using the phytohemagglutinin swelling response (PHA-test). PHA causes local swelling and edema, driven by mitogenesis and infiltration of immune cells into injected tissue (Goto et al., 1978). The PHA-test has been used increasingly in ecological studies of free-ranging birds and is supposed to be harmless to animals. Because to the best of our knowledge, the PHA-test had never been used in iguanids and only once in lizards (Svensson et al., 2001), we tested our planned methodology in a pilot study in the lab conducted on green iguanas (*Iguana iguana*), caught in the wild in Florida. We determined the lowest doses of PHA which induces a measurable swelling and confirmed that no obvious behavioral effects or indications of sickness were occurring. 5 mg PHA/ml induced a significant swelling peak 18 h after injection. To ensure there was no species bias in our calibration, we re-validated our technique in 23 male marine iguanas using exclusively non-territorial males. In these animals, we injected 0.1 ml of 5 mg/ml PHA solution (PHA-P, Sigma L-8754) into the toe-web between the fourth and fifth toe of the right back foot. As a control, we injected 0.1 ml of pure saline solution into the toe-web of the left back foot. To determine peak swelling, we measured the thickness of the toe-web immediately before injection and 6, 12, 18, 24, and 48 h after injection at the injection site to the nearest 0.05 mm using a pressure sensitive spessimeter (Teclock, model SI-510).

After each measurement, animals were released and recaptured just prior to the next measurement period to allow us to obtain repeated PHA responses of free-living animals under natural conditions. We found that peak swelling in marine iguanas occurred 12 h after PHA injection (Fig. 1). Handling during all measurements was kept at a minimum to prevent prolonged cort increase, which may have reduced the immune response. For the ensuing experiments, we applied the validated PHA-test in three male reproductive phenotypes by measuring the PHA swelling response 18 h after injection to avoid swelling measurements during the night.

Hormone concentrations

We used the standard capture and restraint stress protocol (Romero and Wikelski, 2001; Wingfield, 1994) to quantify plasma cort concentrations. Males were caught with a noose and an initial blood sample (within 3 min of capture) of about 1 ml was collected from the tail vein into sodium heparinized Vacutainer tubes (Becton Dickinson, Franklin

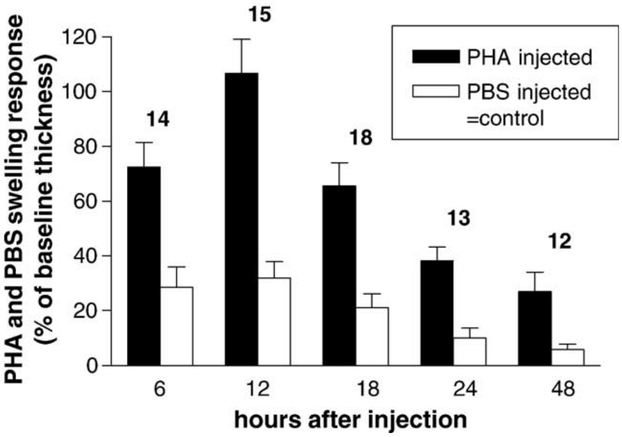

Fig. 1. PHA and PBS (control) swelling response in male marine iguanas 6, 12, 18, 24, and 48 h after injection. Bars represent means ± 1 SE. Numbers above bars indicate sample sizes.

Lakes, NJ). Sampling was only done from noon to late afternoon over 4 consecutive days to control for variation due to circadian and tidal cycles in circulating T and cort (Nelson, 2003; Woodley et al., 2003).

Body temperature was measured in the cloaca with a digital thermometer (accuracy 0.1°C) immediately after initial blood collection to control for its potential influence on cort, and we excluded animals with body temperatures lower than 30°C. Iguanas were subsequently held in an opaque cloth bag for 30 min when a second blood sample was collected. Blood samples were stored cool for up to 6 h in a thermos bottle on cooling gel packets and then centrifuged at 2000 × *g* for 10 min. Plasma was removed from each sample and stored at 4°C for 7 days in a gas-fueled cooling box until it could be frozen at −20°C. A subset of the plasma of initial blood samples was separated for T analysis and its volume was measured with a Hamilton syringe. This aliquot of plasma was stored in a tight vial containing 750 µl of 99.5% ethanol.

Body condition

We determined hematocrit by centrifuging two blood-filled capillary tubes from initial blood samples in a Mini Centrifuge (Compur, Bayer Diagnostika, Germany) at 1400 × *g* for 3 min. We used the mean of the two samples as our estimate. Also, each iguana was weighed (to the nearest 10 g) and the snout-vent length (SVL) was determined to calculate a body condition index $((\text{body mass}/\text{SVL}^3)*10^6)$ as a measure of the physical condition (Laurie, 1989). Iguanas die at indices of less than ≈25 and are maximally fat at indices of 60 (Romero and Wikelski, 2001).

Stress-induced immunosuppression—an experimental approach

To test whether elevated cort concentrations directly induce immunosuppression, we compared the PHA swelling response 6, 12, and 18 h after immune challenge between three groups of bachelor marine iguanas: (i) animals caught and held for 12 h under restraint stress in an opaque bag (n = 9), (ii) cort-injected animals under the same restraint stress (n = 8), and (iii) free-living animals, which were caught and released immediately after the treatment (n = 23). For groups (i) and (ii), we caught males in the late afternoon to ensure that animals did not miss a foraging period. Furthermore, we ran the experiment during the night to avoid holding animals for 12 h in high ambient air temperatures.

After catching each animal, we used the standard stress protocol (see above) to obtain plasma baseline cort concentrations and cort responses. From this pool of animals, we randomly chose 9 animals for cort injections (treatment) and 8 animals for pure peanut oil injections (control). After the 60-min blood sample of the stress protocol, we injected each treatment animal with cort diluted in peanut oil (200 µl solution/kg body weight for a dose of 1 mg cort/kg body weight). After injection, we took additional blood samples for cort analysis at intervals of 120, 240, 360, and 720 min after initial capture from both cort and control injected animals; the PHA swelling response was also measured at 360, 720, and 1080 min after PHA injection without knowledge of the treatment group. All procedures on iguanas were conducted in accordance with the guidelines set forth by the American Society of Ichthyologists and Herpetologists and were approved by the Princeton and Tufts University Institutional Animal Care and Use Committees (accordant to NIH standards for the use of animals in research) and accomplished with permission of the Parque Nacional Galápagos.

Steroid assays

Cort and T were extracted with dichloromethane and analyzed by indirect radioimmunoassay (described in detail in Romero and Wikelski, 2001; Wingfield and Farner, 1975; Wingfield et al., 1992). We used T antibody, T 3003, from Wien Laboratories, Succasunna, NJ, and cort antibody, B3-163, from Esoterix Endocrinology, Austin, TX. Each sample was assayed in duplicate with a small amount of radio-labeled steroid added to determine recovery. Intra-assay variation of the hormone assays was on average 3.8% for cort and 12% for T. The detection limit was 0.13 ng/ml for cort and 0.17 ng/ml for T. The alcohol-preserved samples were prepared for dichloromethane extraction as described in Tarlow et al. (2003).

Statistical analysis

We used univariate AN(C)OVA (Analysis of (Co)Variance) models and Tukey HSD post hoc pairwise comparisons to compare PHA swelling response, T, baseline cort, cort response, body condition, and hematocrit among male phenotypes. Linear regression models were used to investigate possible correlations among measured variables. Baseline cort values were $\log_{10}$ transformed and hematocrit percentages were arcsine square-root transformed to achieve normality and homogeneity of variances of our data.

For analysis of data from the stress-induced immunosuppression experiment, we used unpaired Student's t tests to compare cort concentrations between groups and paired t tests to compare cort concentrations within groups between different time points. Differences in PHA swelling response between free-living animals, restrained, and cort injected animals were tested with univariate ANOVA and Tukey HSD post hoc pairwise comparisons. 18 h PHA swelling responses were $\log_{10}$ transformed to achieve homogeneity of variances. To test the PHA method, we compared the PHA swelling response with the PBS response (control) with paired t tests to determine if controls had significantly different (lower) responses than the PHA-injected animals. Sample sizes for hormone analysis, body condition variables, and PHA swelling responses differed among groups because not all of the males could be recaptured, bled, and measured at all times. Data were analyzed with SPSS for Windows 10.0 and α was set to $P = 0.05$.

Results

Validating the PHA swelling response

The PHA swelling response was strongest at 12 h, compared to 6, 18, 24, and 48 h ($N = 23$). At each time point, the PHA swelling response was significantly larger than the PBS swelling response (6 h: $t = 5.22$, $P < 0.001$; 12 h: $t = 8.81$, $P < 0.001$; 18 h: $t = 6.11$, $P < 0.001$; 24 h: $t = 5.04$, $P < 0.001$; 48 h: $t = 3.26$, $P = 0.008$; Fig. 1).

Comparison between reproductive phenotypes

The PHA swelling response was significantly different between male types ($F_{2,37} = 4.4$, $P = 0.02$). Territorial males exhibited a lower swelling response than satellites (Fig. 2a). However, T concentrations were not significantly different between types (Fig. 2b). Body temperature (ranged from 31.3 to 39.2°C) had a significant influence on baseline cort ($F_{1,43} = 11.15$, $P = 0.002$) and on cort response ($F_{1,42} = 8.26$, $P = 0.006$). We ignored any possible influence of body temperature on swelling response in our study because we predicted similar mean body temperatures during the PHA induced immune response over 18 h. We found a difference between male types in baseline cort ($F_{2,43} = 14.72$, $P < 0.001$), but not in cort response. Territorial males exhibited higher baseline cort concentrations than satellites and bachelors (Fig. 2c). Furthermore, the male types differed significantly in hematocrit ($F_{2,44} = 3.8$, $P = 0.03$) and body condition ($F_{2,46} = 9.4$, $P < 0.001$). Body condition was lower in territorials compared to satellites or bachelor males (Fig. 2e). Territorial males had a significantly higher hematocrit than bachelors (Fig. 2f). We found no significant differences between satellites and bachelors in baseline cort, body condition index, hematocrit, or swelling response (Fig. 2). In sum, territorial males exhibited the lowest immune response, the highest baseline cort, the lowest body condition index, and the highest hematocrit in comparison with the two other reproductive phenotypes.

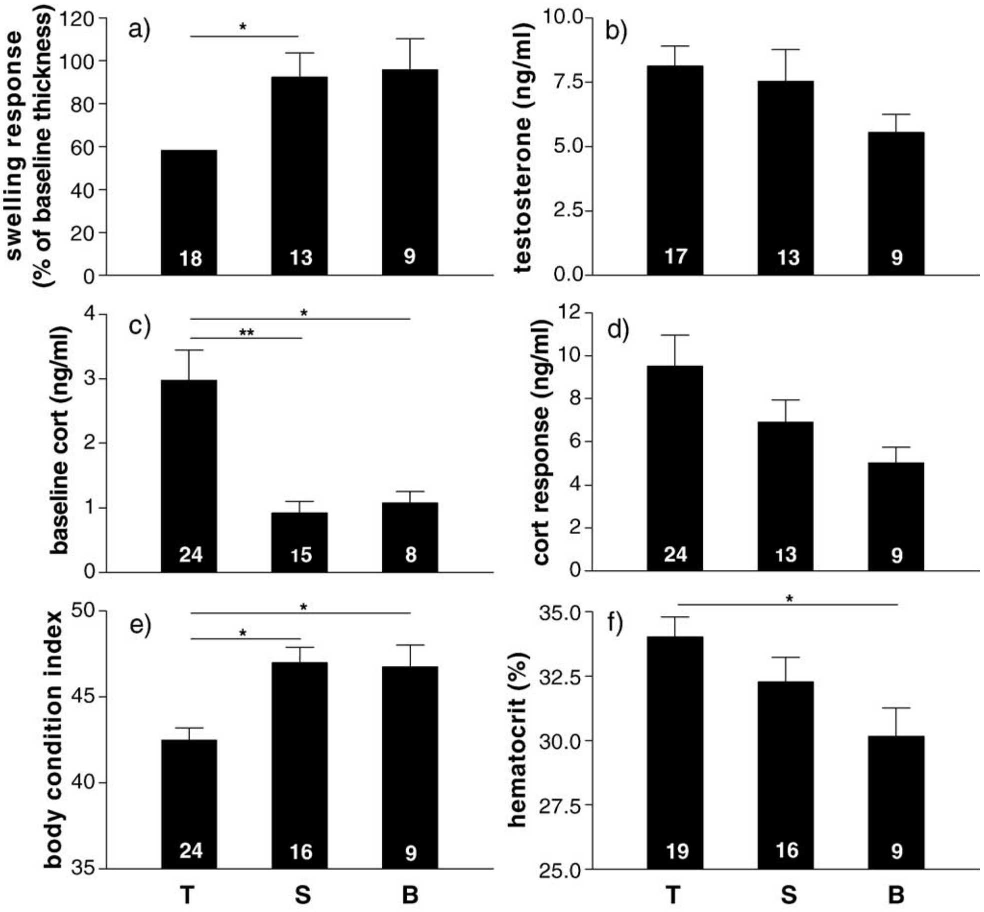

Fig. 2. (a) Swelling response, (b) T, (c) baseline cort, (d) induced cort response, (e) body condition index ((body mass/snout-vent length^3)* 10^6), and (f) hematocrit in male marine iguanas with different reproductive phenotypes: territorials (T), satellites (S), and bachelors (B). Bars represent means ± 1 SE. Numbers in bars indicate sample sizes. Horizontal lines above bars indicate statistical significances for Tukey post hoc pair-wise comparisons: $^{**}P < 0.01$; $^{*}P < 0.05$; other comparisons are not significant.

Baseline cort was negatively correlated with body condition index (F = 9.019, P = 0.004; Fig. 3b). Furthermore, we found a negative linear regression between PHA swelling response and baseline cort (F = 6.12, P = 0.018; Fig. 3a) and between PHA swelling response and hematocrit (F = 13.25, P = 0.001; Fig. 3c).

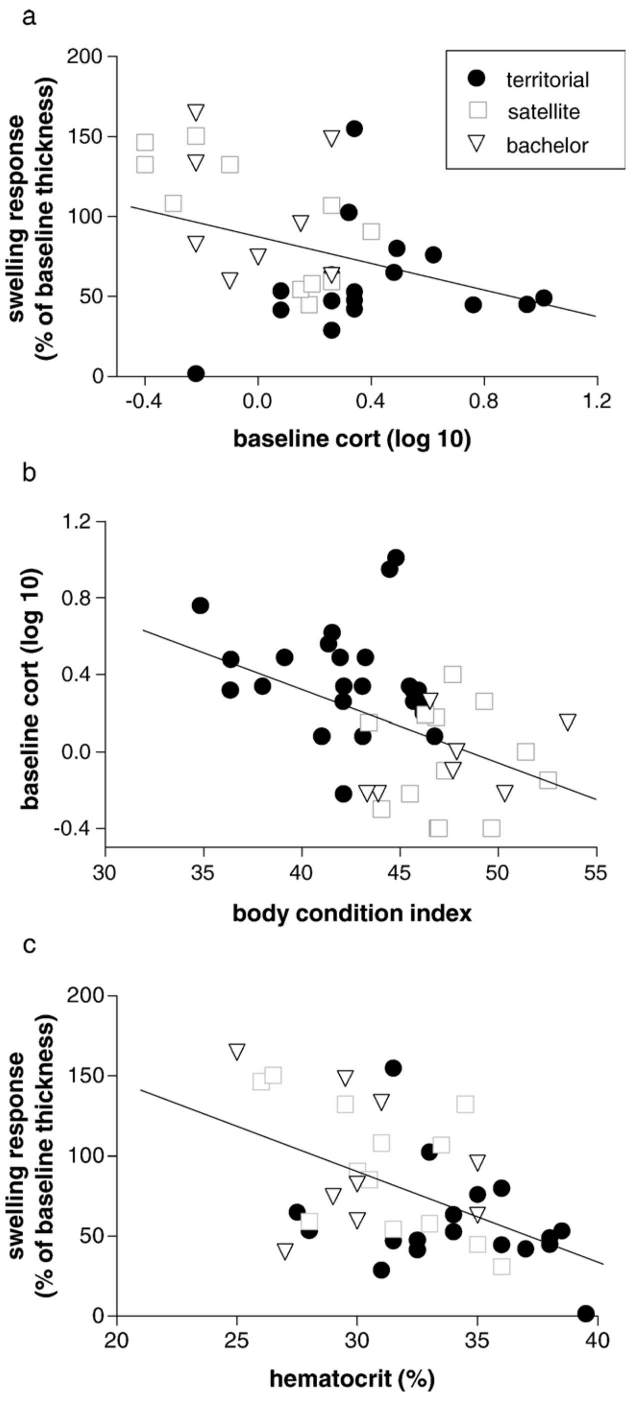

Fig. 3. (a) Swelling response correlates with baseline cort concentrations (P = 0.018, N = 38), (b) baseline cort concentrations correlates with body condition (P = 0.004, N = 47), and (c) swelling response correlates with hematocrit (P = 0.001, N = 40) in territorials, satellites, and bachelors. The linear regression models include males of all three reproductive phenotypes.

Stress-induced immunosuppression—an experimental approach

After 30 and 60 min restraint stress, cort concentrations were significantly higher as compared to baseline concentrations (t = −6.87, P < 0.001; Fig. 4). Animals kept under 120 min restraint stress and injected with peanut oil (control) exhibited lower cort concentrations than captive animals injected with cort (t = −7.57, P < 0.001; Fig. 4). There was no significant change between the 60- and 120-min samples in the control group, but cort increased significantly after cort injection (t = −7.26, P < 0.001; Fig. 4). During the 240 and 360 min of restraint stress, the cort injected and oil injected group differed significant in cort concentrations (240 min: t = −8.74, P < 0.001, 360 min: t = −7.36, P < 0.001; Fig. 4), but there was no longer a significant difference after 720 min. Both groups of males under restraint stress (with cort or with oil injection alone) had a lower PHA swelling response 12 and 18 h after PHA injection compared to free-roaming males (12 h: $F_{2,25}$ = 5.10, P = 0.01; 18 h: $F_{2,28}$ = 10.20, P < 0.001; Fig. 5). There was no significant difference in the swelling response between male types 6 h after PHA injection. Thus, male marine iguanas with elevated cort concentrations, either caused by restraint stress or cort injection, exhibited a reduced immune responsiveness as compared to free-roaming animals.

Discussion

Territorial male marine iguanas showed significantly lower PHA-induced immune activity than satellite or bachelor males. At the same time, all males had indistinguishable T concentrations, but differed strongly in their baseline cort concentrations. Linear regression models showed negative correlations between baseline cort concentrations and immune activity and between baseline cort and body condition. We confirmed this apparent role of cort in immunosuppression by experimentally elevating cort in males and detecting a decrease in immune activity. Our result is overall consistent with the idea of a resource-based trade-off between the immune system and reproduction (Rigby and Moret, 2000; Sheldon and Verhulst, 1996), but we could not find an involvement of T in this trade-off as suggested by Folstad and Karter (1992).

Body condition and cort

Glucocorticoids are often elevated during starvation (Kitaysky et al., 1999; Romero and Wikelski, 2001). One of the general physiological roles of glucocorticoids is to help supply adequate amounts of energy during strenuous times (McEwen and Wingfield, 2003; Moore et al., 2000b; Romero, 2002; Wingfield et al., 1998). In our study, territorial males had low body condition but high baseline

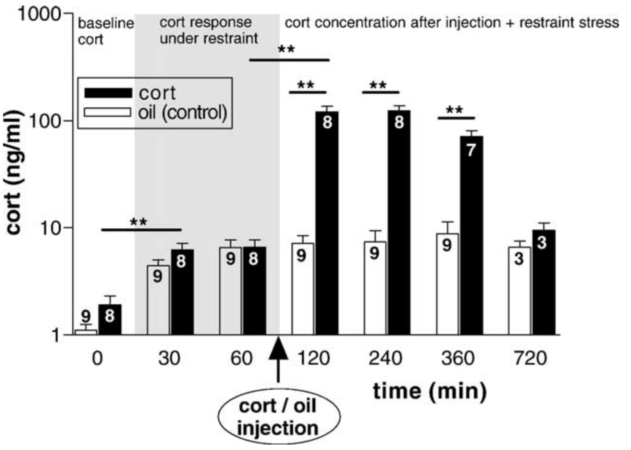

Fig. 4. Plasma cort concentrations in male marine iguanas (bachelor males) before restraint stress (baseline cort), under restraint stress (oil injected = control), and under restraint stress + cort injection. Bars represent means ± 1 SE. Numbers in bars indicate sample sizes. The symbols between bars indicate statistical significances: $^{**}P < 0.01$; $^{*}P < 0.05$; other comparisons are not significant. For analysis, paired *t* test and unpaired *t* test were used.

cort, suggesting that these males were faced with strong energy demands during their vigorous reproductive activities. In fact, Trillmich (1983) found that territorial male marine iguanas shorten their foraging time and even fast for some time (median of fasting period: 12 days) to ensure that they keep their territories. They lose up to 26% body weight during this period, which correlates strongly with the length of their fasting period (Trillmich, 1983). Wikelski et al. (1996) found one territorial male that fasted for 35 days, that is, did not feed during the entire reproductive period.

Hematocrit is often positively correlated with body condition (Amand, 1986; Cucco et al., 2002; Potti et al., 1999). Surprisingly however, territorial iguanas showed the opposite pattern: high hematocrit but low body condition, as well as a negative correlation between immune activity and hematocrit. Two explanations for elevated hematocrit in territorials are possible. First, high hematocrit may be a prerequisite for high aerobic capacity to satisfy the higher oxygen requirements of reproductive behavior. For example, jungle fowls with higher hematocrit had higher aerobic capacity (Chappell et al., 1997). Second, an elevated hematocrit might indicate dehydration (Fitzsimons and Kaufman, 1977). During hibernation, desert reptiles (*Varanus griseus*) increase their hematocrit because they do not eat or drink, resulting in a state of dehydration (Haggag et al., 1974).

T and immune reactivity

Contrary to numerous studies in vertebrates, which have found elevated T concentrations at the time when immune activity is reduced (Hillgarth and Wingfield, 1997; Nelson and Klein, 2000; Verhulst et al., 1999; Zuk et al., 1995), T was not elevated when the PHA response was suppressed in our territorial males. This suggests that T plays a less important role as immunosuppressive agent in reproductive male marine iguanas than postulated in the immunocompetence handicap hypothesis. We did not test, however, whether the immune system was suppressed as a consequence of elevated T at an earlier point during reproduction, such as when T concentrations were elevated during territory establishment. Our results could also be expected if the immune system of territorials is more sensitive against circulating T than in other male groups. Thus, T could have caused immunosuppression in territorial males, although T concentrations were statistically equivalent in all male groups. Furthermore, it might be possible that steroid binding protein concentrations decreased in territorial males while measured T concentrations were not different between male groups. This could result in territorial males having higher levels of free T than the other male groups as shown for cort in tree lizards (*Urosaurus ornatus*) (Jennings et al., 2000). Overall, we could not demonstrate a direct effect of T on the immune system in male marine iguanas, but an experimental verification of this result remains to be done.

Cort and immune reactivity

Previous studies have shown that glucocorticoids have bi-directional effects on immune function, such that temporarily elevated plasma concentrations of glucocorticoids may be immune enhancing, whereas chronically elevated concentrations may be immunosuppressive (Dhabhar and McEwen, 1997). We assume, based on previous findings (Nelson, 2003), that our territorial males had elevated baseline cort concentrations over several weeks to satisfy the high-energy demands associated with continuous vigorous reproductive behavior (cf. Greenberg and Wingfield, 1987; Guillete et al., 1995). Therefore, chronically elevated cort concentrations can have a suppressing

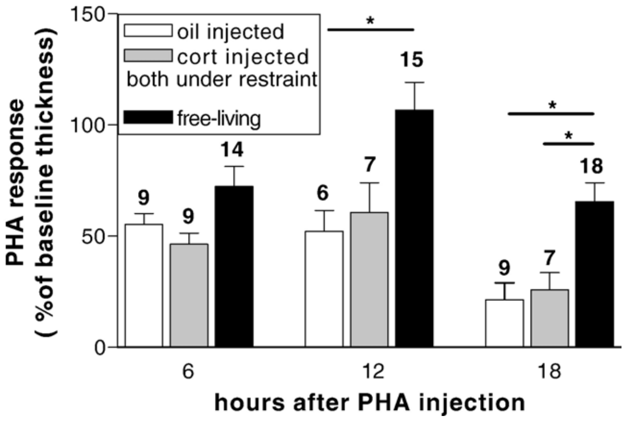

Fig. 5. PHA swelling response 6, 12, and 18 h after PHA injection in male marine iguanas (bachelor) after oil or cort injection (both under restraint) and in free-living male marine iguanas (bachelor). Error bars represent means ± 1 SE. Numbers above bars indicate sample sizes. The symbols between bars indicate statistical significance for Tukey post hoc pair-wise comparisons between oil injected (under restraint) and free-living animals and between cort injected and free-living animals: $^{*}P < 0.05$; other comparisons are not significant.

effect on immune activity in territorials as we found in our study. Such immunosuppression could be adaptive because cort may reallocate scarce nutritional reserves away from costly immune responses during situations in which an animal is under stress (Apanius, 1998).

In our experiment, the PHA response was reduced in both cort injected animals and restrained animals as compared to free-ranging animals. This confirms our result of cort-induced immunosuppression in territorial males and indicates that both exogenous and endogenous cort concentrations can cause immunosuppression in marine iguanas. Interestingly, the PHA response was similar when cort was elevated by injection or by restraint, although cort concentrations were significantly higher in cort injected than in restraint animals. We therefore hypothesize that cort concentrations surpassed a threshold that caused immunosuppression, while above the threshold the amount of cort did not suppress immune response any further.

Cort and T interactions

Territorial males had the highest circulating baseline cort concentrations in our study, possibly to provide sufficient energy for their intense aggressive behavior (Goymann and Wingfield, 2004; Moore and Jessop, 2003; Wingfield et al., 1998). However, cort is typically highest during breeding in many species for reasons that are currently unknown (Romero, 2002). In contrast, all males had similar circulating T concentrations as we had expected based on previous findings. This suggests that T in territorial marine iguanas is high only during the establishment of territories and the early reproductive period; during the peak of reproduction, it does no longer differ from other marine iguana phenotypes (Nelson, 2003).

Such a variability in T concentrations during reproduction is somewhat expected from the challenge hypothesis (Wingfield et al., 1990) which states that T is highest during times of social instability, for example, during territory establishment. Similarly, Manzo et al. (1994) have shown in lizards (*Podarcis sicula sicula*) that T concentrations are elevated during the territorial establishment and defense period, but low during the mating phase. Cort on the other hand followed opposite trends. Manzo et al. (1994) suggested that cort, which is released by the adrenals after territories are established, acts on T synthesis to reduce aggressive behavior and thereby allows breeding. In fact, cort and T have been found to be antagonistic in many reptiles (Grassman and Hess, 1992; Moore et al., 1991; Tokarz, 1987). A variation of the Energetics-Hormone Vocalization (EHV) Model proposed for anuran calling behavior by Emerson (2001) may also explain the negative association between T and cort concentrations in male marine iguanas: T increases reproductive behavior, but due to high energetic demands of reproductive behavior, plasma cort concentrations also increase. At some point, cort concentrations surpass a threshold and create a negative feedback for T concentrations, which then decline.

So far, it is unclear whether reproductive behavior, glucocorticoids and T interact in the same way in reproductive males of different vertebrate species. Glucocorticoid concentrations have been found to differ in males depending on their reproductive state or rank in a social hierarchy. Beletsky et al. (1989) found in red-winged blackbirds (*Agelaius phoeniceus*) that territory owners had higher cort concentrations than adult floaters presumably because of greater energetic demands. Greenberg et al. (1984) measured lower cort concentrations in dominant lizard males (*Anolis carolinensis*) than in subordinates, contrary to what is often shown in other taxa.

Such variability in glucocorticoid concentrations may be explained by the amount of energetic costs needed to acquire and maintain territorial or dominance status (Goymann and Wingfield, 2004). Accordingly, we hypothesize that energetic costs and concomitantly plasma cort are high in territorial male marine iguanas because their territoriality has to be defended and can only be maintained with constant physical aggression toward rivals. On the other hand, in tree lizard males (*Urosaurus ornatus*), territorials and non-territorials had similar basal concentrations of total plasma cort, but territorial males showed a greater androgen-glucocorticoid-binding globulin capacity than non-territorial males (Jennings et al., 2000). This could have resulted in higher concentrations of free cort in non-territorial versus territorial males (Jennings et al., 2000). It remains unclear whether cort binding globulins (CBGs) might also differ among male marine iguana types in addition to the differences we found in circulating plasma concentrations of cort (Breuner and Orchinik, 2001).

In general, our data support the expected trade-off between reproductive activity and immune responses. This trade-off is most likely mediated by the fact that both the expression of sexual characters and mounting an immune defense require significant amounts of energy or nutrients (Demas et al., 1997; Gustafsson et al., 1995; Hamilton and Zuk, 1982; Hanssen et al., 2004; Lochmiller and Deerenberg, 2000; Martin et al., 2003; Råberg et al., 2000; Sheldon and Verhulst, 1996). It is also possible, however, that hormones such as T and cort may play a role in the avoidance of auto-immune disease (Råberg et al., 1998) or help to influence how immune resources are allocated, as formalized by the immunoredistribution hypothesis (Bilbo et al., 2002; Braude et al., 1999; Dhabhar et al., 1995). Further, risk of infection, as mediated by steroid hormones, may affect immune-endocrine trade-offs, but little empirical work has been done to test this hypothesis.

Despite this multitude of factors that can theoretically be involved, our data demonstrate a strong pattern suggesting that cort concentration is an important factor driving differences in immune function among groups of male marine iguanas. We hypothesize that other factors, such as hematocrit and body condition, probably influence immune function as well, but most likely by indirect routes, which are ultimately mediated by cort. Indeed, cort and body condition are linked to each other and, in turn, influence reproductive behavior. We propose that cort has an important integrative role in modulating marine iguana behavior and physiology during the strenuous reproductive period. Most likely, elevated cort concentrations in reproductively active males coordinate the immune system and reproductive activities in order to utilize the available energy as efficiently as possible.

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