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# Food supplementation and testosterone interact to influence reproductive behavior and immune function in *Sceloporous graciosus*

**Mayté Ruiz**[1,*], **Susannah S. French**[2], **Gregory E. Demas**[1], and **Emília P. Martins**[1]

[1]Department of Biology and Center for the Integrative Study of Animal Behavior, Indiana University, Bloomington, Indiana 47405, USA

[2]Department of Biology, Utah State University, Logan, Utah 84322, USA

## Abstract

The energetic resources in an organism's environment are essential for executing a wide range of life history functions, including immunity and reproduction. Most energetic budgets, however, are limited, which can lead to trade-offs among competing functions. Increasing reproductive effort tends to decrease immunity in many cases; and increasing total energy via supplemental feedings can eliminate this effect. Testosterone (T), an important regulator of reproduction, and food availability are thus both potential factors regulating life-history processes, yet they are often tested in isolation of each other. In this study, we considered the effect of both food availability and elevated T on immune function and reproductive behavior in sagebrush lizards, *Sceloporus graciosus*, to assess how T and energy availability affect these trade-offs. We experimentally manipulated diet (via supplemental feedings) and T (via dermal patches) in males from a natural population. We determined innate immune response by calculating the bacterial killing capability of collected plasma exposed to *E. coli* ex vivo. We measured reproductive behavior by counting the number of courtship displays produced in a 20-min sampling period. We observed an interactive effect of food availability and T-patch on immune function, with food supplementation increasing immunity in T-patch lizards. Additionally, T increased courtship displays in control food lizards. Lizards with supplemental food had higher circulating T than controls. Collectively, this study shows that the energetic state of the animal plays a critical role in modulating the interactions among T, behavior and immunity in sagebrush lizards and likely other species.

### Keywords

Context-dependent; Energy allocation; Innate immunity; Life history; Resources; *Sceloporus*; Trade-offs

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*Corresponding Author: mayruiz@indiana.edu, Phone: 812-855-5652.

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## Introduction

Animals often use behavioral and hormonal modifications to moderate the impact of variation in food availability in their natural environment. When food is limited, animals may experience trade-offs between survival and fecundity (e.g., Therrien et al., 2008; Waelti and Reyer, 2007). These effects, however, are sometimes modulated by behavioral or hormonal shifts that buffer the animal from sudden shifts in food supply (Guo et al., 2008, Mattila and Otis, 2007). For example, hormones have been implicated in producing the alternative reproductive strategies exhibited by different male side-blotched lizard morphs (Mills et al., 2008). Here, we begin to tease apart the effects of food resources and hormones by manipulating breeding male sagebrush lizards (*Sceloporus graciosus*) in the field and simultaneously assessing the effects of our manipulations on both immune function and reproductive condition.

The nutritional resources available to organisms in their natural environment determine the energy available at a given time (Owen et al., 1992). This energy is essential for an organism to carry out the cellular processes required for survival or reproduction (e.g., Hill et al., 2008). However, energy is not limitless; finite energy reserves are required to maintain a wide range of physiological and behavioral functions, often resulting in trade-offs among competing functions (Klein and Nelson, 1999; Marler and Moore, 1989). Thus, changes in food availability in an organism's environment could lead to effects on survival and fecundity of these organisms (Houston et al., 2007, Macdonald and Bayne, 1993). Fecundity is reduced in organisms with low food supply (Crawford et al., 2006; Nagy and Holmes, 2005), and this could possibly be due to behavioral or hormonal modifications produced by food limitation. Survival is also impacted by food availability (Le Galliard et al., 2005; Marler and Moore, 1988; Marler and Moore, 1991), likely due to effects on immunity and/or energy stores.

Trade-offs between reproduction and immunity are not always observed (e.g., Greenman et al., 2005; Nakazawa et al., 1997). This is most likely due to the large differences in the amount of resources available to individuals within the population (McDade 2003). For example, when energy is not limiting (e.g., when animals are fed *ad libitum*), trade-offs between reproduction and immune function may not occur because individuals can simply increase their energy expenditure to accommodate increased energetic demands. Trade-offs may therefore be a plastic response to limited diet (Owen-Ashley et al., 2004). In support of this idea, limited resources decrease immune function in reproductively active female tree lizards (*Urosaurus ornatus*), but this trade-off is not apparent in lizards provided with ample food resources (French et al., 2007a). We thus expect individuals to increase investment into both reproductive and immune functions when resources are plentiful, as trade-offs are expected to be relaxed when energy availability is high.

The gonadal steroid hormone testosterone (T) serves as the predominant hormone regulating reproduction in males in a large variety of vertebrate taxa and is an important factor regulating life-history processes. High circulating T levels during the breeding season are correlated with an increase in testis size (Dixson and Anderson, 2004), induce male copulatory behavior (James and Nyby, 2002), activate elaboration of male ornamental traits or reproductive coloration (Cooper et al., 1987; Eens et al., 2000), and increase courtship song rate (Ketterson et al., 2001; Sartor et al., 2005). Individuals with high T are thus expected to be allocating large amounts of energetic output towards reproductive physiology and behavior. Additionally, T can increase metabolism and energy expenditure (Marler et al., 1995; Tobler et al., 2007). Consequently, maintenance of high T levels could significantly reduce the energy available for immune function (Muehlenbein and Bribiescas, 2005). Testosterone is indeed immunosuppressive in some cases. (Cox and John-Alder, 2007; Derting and Virk, 2005; Greives et al., 2006; Opplinger et al., 2004). Thus under high levels of T, we expect elevated reproductive investment to come at a cost to immune function. Many species exhibit a trade-

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off between reproductive effort and immunity by decreasing one or more functions when another is enhanced (see Ardia, 2005; Ahtiainen et al., 2005; Tomas et al., 2007; Uller et al., 2006 for examples).

Resource availability and T may both act separately to determine how much an individual should invest into reproductive versus immune processes, or they may interact. In this study, we examined the effects of both T and food supplementation on trade-offs between reproduction and immunity. Specifically, we predicted that increasing reproduction and courtship behavior via T treatment will impair immune function in sagebrush lizards. Furthermore, if this effect is due to an energetic trade-off, then food supplementation should attenuate T-induced immunosuppression by increasing energy availability. Considering these factors simultaneously allows us to better understand how life-history processes are mediated through both environmental and endocrine factors.

## Methods

### Overview

We conducted a field study on a natural population of male *Sceloporus graciosus* in southern California USA (33°N, 116°W). *S. graciosus* are small (50–70mm snout-to-vent length; 5–11g mass), territorial lizards with overlapping male and female territories (Martins, 1993). Their robust site fidelity makes this a particularly useful species for conducting field studies and for manipulating resources. We studied these animals in May and June 2008, at the peak of their breeding season (Martins, 1993). We captured and paint-marked individual lizards with a unique 3-color code, and gave each lizard a dermal patch (see testosterone manipulation below) before releasing them at the site of capture.

Seven to ten days after initial capture, we recaptured lizards in the afternoon hours (12:30 – 17:20 PST, except for one individual recaptured at 10:53 PST) immediately following behavioral observations. We immediately took blood samples from each lizard via rupturing the post-orbital sinus with a heparin-coated microcapillary tube. We measured mass upon initial capture and recapture to confirm the effectiveness of food manipulation, and then released the lizards at the site of capture. Within 8 h of collection, we centrifuged the blood at 4000 rpm for 10 min, and stored plasma samples at −20C for subsequent endocrine and immune assays. We treated all animals in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and as approved by the Bloomington Institutional Animal Care and Use Committee (BIACUC) and by the California Department of Fish and Game.

### Testosterone manipulation

During the initial capture, we manipulated hormone levels by placing an external dermal patch on the lower back of each lizard. This is a non-invasive technique that has been shown to effectively elevate hormone levels over a 24-h period (Knapp and Moore 1997). Patches contained either 5 ul of 4ug testosterone (Sigma-Aldrich, Saint Louis, MO) suspended in sesame oil (T-manipulated group, N=21) or sesame oil vehicle alone (control group, N=19). We assembled patches from small pieces (approximately 0.3 $cm^2$) of adhesive bandages (Johnson and Johnson©) and electrical tape (approximately 1.0 $cm^2$), as described in Knapp and Moore (1997). We attached patches to the backs of lizards with SC Nexaband glue.

### Resource manipulation

During the week between captures, we placed a petri-dish on the territories of each marked individual. We fed 3–6 vitamin-dusted (Herptivite® Multivitamins, Rep-Cal) mealworms and/or 2–4 vitamin-dusted crickets to individuals in the food supplemented group (N= 20, vs. N=20

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without food supplementation) every day until the day of recapture. On days in which marked lizards in the food-supplemented group were spotted on their territories, we noted that these lizards readily consumed the prey. We additionally used mass increases as an indirect measure of food intake. Lizards in the non-supplemented group remained with empty petri-dishes in their home ranges until recapture.

## Behavioral observations

Seven to ten days after initial capture, we located marked individuals in the afternoon hours (noon – 16:30 PST, except for one individual re-spotted at 10:30 PST) and conducted behavioral observations in the field prior to recapture. Elevated T is expected to increase reproductive behaviors, such as courtship (Woolley et al. 2004). We thus conducted behavioral observations in order to confirm the effect of the T-patch. We recorded behaviors produced by the target individual for 20 min, paying particular attention to headbob and shudder displays (Martins 1994). During the breeding season, *S. graciosus* males engage in frequent pushup (stereotyped single and double headbobs) and shudder (short, rapid headbobs with little fine-scale structure) displays (Kelso and Martins, 2008). These displays require considerable energy expenditure to perform (Brandt 2003). In other lizards, display frequency is also T-dependent (Tokarz et al., 2002; Watt et al., 2003).

We also recorded foraging behavior performed during this time, specifically the number of times a focal individual was spotted consuming prey. We considered foraging behavior in addition to changes in mass as factors for validating food manipulation in the field.

## Bacteria killing assay

We determined innate immune response by calculating the bacterial killing capability of collected plasma exposed to *E. coli* (ATCC#8739, Microbiologics, St. Cloud, MN) *ex vivo*. This assay assesses the ability of complement in the blood to kill a bacterial colony. To do this, we diluted plasma in a 1:5 dilution with glutamine enriched $CO_2$-independent media under laminar-flow conditions (Sigma-Aldrich). We used a 1:5 dilution due to preliminary dilution-response curve results for sagebrush lizard plasma. We added a working bacteria solution of *E. coli* in PBS to our diluted plasma samples (ratio 1:10 bacteria:plasma). We then incubated the plasma/bacteria cocktails for 30 min at 37°C and subsequently plated each sample on agar, in duplicate, including a positive control (of only bacteria) and a negative control (with no bacteria). Plates were incubated overnight at 37°C to allow colony growth. We quantified percent bacteria killed with the equation 100% - [(# of colonies on sample plated / # of colonies on positive control plate)*100]. No colonies formed on negative control plates.

## Hormone analysis

We analyzed circulating levels of testosterone (T) and corticosterone (CORT) for each lizard using radioimmunoassay (RIA). We measured CORT since T manipulation has been shown to elevate CORT in other species (Ketterson et al., 2001, Zysling et al., 2006); we therefore considered CORT as a possible mediator of T effects. Samples were assayed as described under a previously published protocol (Moore, 1986) with slight modifications, and performed in the Ketterson laboratory at Indiana University. Briefly, we extracted samples with diethyl ether, separating the ether phase by snap freezing in a bath of dry ice in methanol, and subsequently resuspended samples in 10% ethyl acetate in isooctane. We separated individual hormones using column chromatography (T and CORT), with short-columns composed of one layer celite (Celite 521, Sigma-Aldrich) evenly mixed with water and a second layer of celite evenly mixed with glycol. We separated T from the column with an elution of 20% ethyl acetate: isooctane; and CORT with 52% ethyl acetate: isooctane. Separated hormones were collected, dried, resuspended in PBS, and assayed in duplicate. For each sample, we used an aliquot of the resuspended fractions to measure individual recoveries following extraction and

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chromatography. These recoveries were used to adjust the final sample concentration values to account for any losses during these procedures. Intra-assay coefficients of variation were 6% for T and 13% for CORT.

### Statistical analysis

We performed multiple regression analyses using SAS (2002) to test whether lizard mass and food consumption during behavioral observations were predictors of food supplementation and whether headbob and shudder displays were predictors of patch group. Once manipulations were confirmed, we conducted two-way analysis of variance (ANOVA) with an interaction effect to test the ability of food supplementation, dermal patch group, and the interaction between food and patch group to predict each of our response measures (separately). Specifically, these response measures were immunity (percent of bacteria killed by the lizard's plasma), and circulating hormone levels (T and CORT, separately).

## Results

### Mass and foraging behavior confirmed resource manipulation

We used lizards in which we did not manipulate T-levels to determine the effectiveness of our food manipulation for use in the field. Food-supplemented lizards gained an average of 0.3 gm (SE = 0.26) during the week of resource manipulation, whereas controls lost an average of 0.3 gm (SE = 0.17). ($F_{1,35} = 4.60$, $p < 0.05$; Fig. 1a). Foraging behavior also differed between groups. Food supplemented lizards did not eat during behavioral assays, whereas we usually observed control individuals eating at least once during the 20 min trial ($F_{1,36} = 10.99$, $p < 0.01$; Fig. 1b). Together, these observations suggest that the food manipulation was successful, and individuals in the food-supplemented group were in fact supplied with enhanced energy resources.

### Courtship behavior confirmed testosterone manipulation

Considering only lizards that did not receive supplemental food, males that were provided with a short-term burst of T (T-patch) produced over 4 times more courtship shudder bouts (mean = 8.3 shudder displays/20 min, SE = 3.1) than did males that received only a sesame oil patch (mean = 1.9 shudder displays/20 min, SE = 0.9). ($F_{1,36} = 5.60$, $p < 0.03$; Fig. 2). However, we did not observe a similar effect of dermal patch treatment on headbob displays ($F_{1,36} = 0.00$, $p = 0.98$).

### Food supplementation and T manipulation interact to affect immune function

Food supplementation and T manipulation interacted to affect bacteria killing capability (Table 1, Fig. 3a). Food supplemented lizards had 3 times greater immune activity than control lizards (Table 1, Fig. 3a). This effect was mainly due to a significant increase in immune function for food-supplemented lizards that also received a T-patch. T-patch lizards had elevated immune function with food-supplementation when compared to T-patch individuals that were not fed ($t = 2.12$, $df = 16$, $p < 0.001$). This effect of food supplementation on immunity was not pronounced in control-patch lizards, leading to a significant interaction effect between food and patch in the two-way ANOVAs (Table 1).

### Food supplementation increases circulating testosterone

Food, but not patch, manipulation predicted average circulating T levels between groups (Table 1). Lizards provided with additional food resources had 1.5 times higher circulating T than control lizards (Fig. 3b). This was true even when considering only control-patch individuals. Although there was a trend towards a weak correlation between T and CORT ($r = 0.277$, $df = 38$, $p = 0.08$), food supplementation was not a good predictor of CORT levels (Table 1, Fig.

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3c). Within each food treatment group, circulating T and immune function were not correlated (food-supplemented: r = 0.153, df = 14, p = 0.57; control: r = 0.025, df = 17, p = 0.92).

## Discussion

We found significant interactions between the reproductive system, the immune system, and food availability. Food supplementation and T manipulation interacted to influence immune function in male sagebrush lizards. Consistent with our predictions, food supplementation increased immune response in lizards and this response was more pronounced in T-treated compared with control animals. Furthermore, food supplementation increased plasma T levels in both T-treated and control lizards. In contrast to our predictions, however, T enhanced immunity in food supplemented lizards. These results suggest that T can act differentially to alter immune function, depending on the energetic state of the animal. For example, T may enhance both reproduction and immunity when energy is abundant, but T may also mediate trade-offs between these two physiological systems when resources are limiting. These context-dependent effects of T may reconcile the conflicting evidence for both immunosuppression and immunoenhancement of T in the literature (Muehlenbein and Bribiescas, 2005). Variability in lab or field conditions or in species requirements of energetic resources may account for these variable effects of T. Trade-offs between reproduction (as measured by T) and immunity may also be due to the indirect effects of energy allocation (Owen-Ashley et al., 2004), explaining our lack of immunosuppression in food supplemented lizards. Nevertheless, we only considered one area of immunity, namely innate immune function, and may have attained different responses (ie., noted immunosuppression with T) if we had measured acquired or cell-mediated immunity.

Although T-treated individuals had increased courtship behavior when food-limited, T-patch did not produce long-term elevation of circulating levels of T, as plasma T was not significantly elevated at the time of blood sampling. The fact that T-patches increased courtship behavior in food-limited lizards one week after patch administration even without a measured effect on circulating T levels, suggests that some behavioral regulation may be in response to previous hormone levels. Similar to the way in which CORT-patches acted on circulating CORT levels (Knapp and Moore, 1997), T-patches likely produced a short-term elevation of T. This may have up-regulated androgen receptors, producing a prolonged behavioral and immune response.

One of the most pronounced effects of our study is that food supplementation increased levels of circulating testosterone in both T-patch and control individuals, as well as was involved in the enhancement of immune function. Mechanisms are now unclear but may involve differences in clearance rates or conversion from other precursors. An increase in energy resources provided by an increase in food may have allowed for greater energy distribution to multiple functions, including reproduction and immunity (French et al., 2007b). The fact that increasing food intake allowed for the elevation of both reproductive and immune processes suggests that these processes are not mutually exclusive and thus trade-offs may exist due to limitations in energy uptake. Female snow skinks in good condition may produce high levels of both reproductive investment and immune function, whereas costs arise in females that do not have the resources to produce high levels of both processes (Olsson et al., 2001). Tropical pythons with low body condition have decreased immunity (Ujvari and Madsen, 2006). Food availability may be one factor affecting individual quality. High levels of activity have immunosuppressive effects in female mice on a restricted diet (Schubert et al., 2008), suggesting energetic requirements are important for allocation towards multiple functions.

In addition to acting internally to increase energy stores, food supplementation may impose other externally-derived motives for increasing circulating testosterone. Increases in food

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availability in some territories may affect social interactions within those territories. Androgen levels can then be modulated by the social environment (Oliveira et al., 2002). This may occur due to an increase in density of, and thus behavioral encounters within, these now higher quality territories, promoting elevated levels of T (Beletsky et al., 1992). Increasing food resources in some territories may also elevate aggression to retain high quality territories. We witnessed one highly aggressive encounter between a focal food supplemented lizard and a conspecific male (pers. obs.). In this case, food supplementation may have elevated T due to the increased potential for aggressive interactions in defense of high food resources. Variation in food abundance causes changes in home ranges and associated social structures of mice (Schradin and Pillay, 2006) and red squirrels (Wauters et al., 2005). The role of food availability on social structures has yet to be studied, however, in sagebrush lizards.

Supplemental food items may have also provided a stimulus for increased testosterone. Lizards in food-supplemented sites were supplied with vitamin-dusted crickets and mealworms, which are notably larger than most of the natural prey of these lizards (personal observation). The size of prey items correlates with rump coloration (a signal of condition) in kestrel fledglings (Vergara and Fargallo, 2008). Similarly, the interaction with larger prey objects, most likely also indicative of greater food intake, may have allowed for an elevation of T due to enhancement of individual quality. Furthermore, providing lizards in supplemented group with vitamin-dusted crickets and mealworms may not only increase the amount of available food but also the quality of the food by providing essential vitamins and both a protein source (crickets) and a fat source (mealworms). In this way, lizards were able to intake their required nutrients without much effort. Diet quality has the potential to influence the direction of trade-offs (Naya et al., 2007). This may have allowed for the increase of T in these higher quality individuals. Nevertheless, calorie restriction, not diet quality, is associated with suppression of reproduction (including T levels) in other studies (Govic et al., 2008, Santos et al., 2004).

In conclusion, the present findings demonstrate that several key factors may interact to influence important life history functions, such as immunity and reproduction. Here we considered testosterone, a reproductive mediator, in the context of energetic resources. Testosterone was found to influence both immune function and courtship behavior differently depending on the energetic state of the animal. Thus, T is not necessarily immunosuppressive in all species and across all conditions. Rather, the effects of T on immunity appear depend on the environmental condition of the animal. For example, T can be immunosuppressive when food is limited; however, our results also support T-induced immunoenhancement when food availability is high. These findings support the idea of an energetic trade-off between reproduction and immune function, at least when environmental resources are limiting. Furthermore, this study suggests a role for food availability in enhancing immune function (in presence of T) and on reproduction (by elevating circulating T). Further research is needed to determine the mechanism regulating hormone levels by energetic state.

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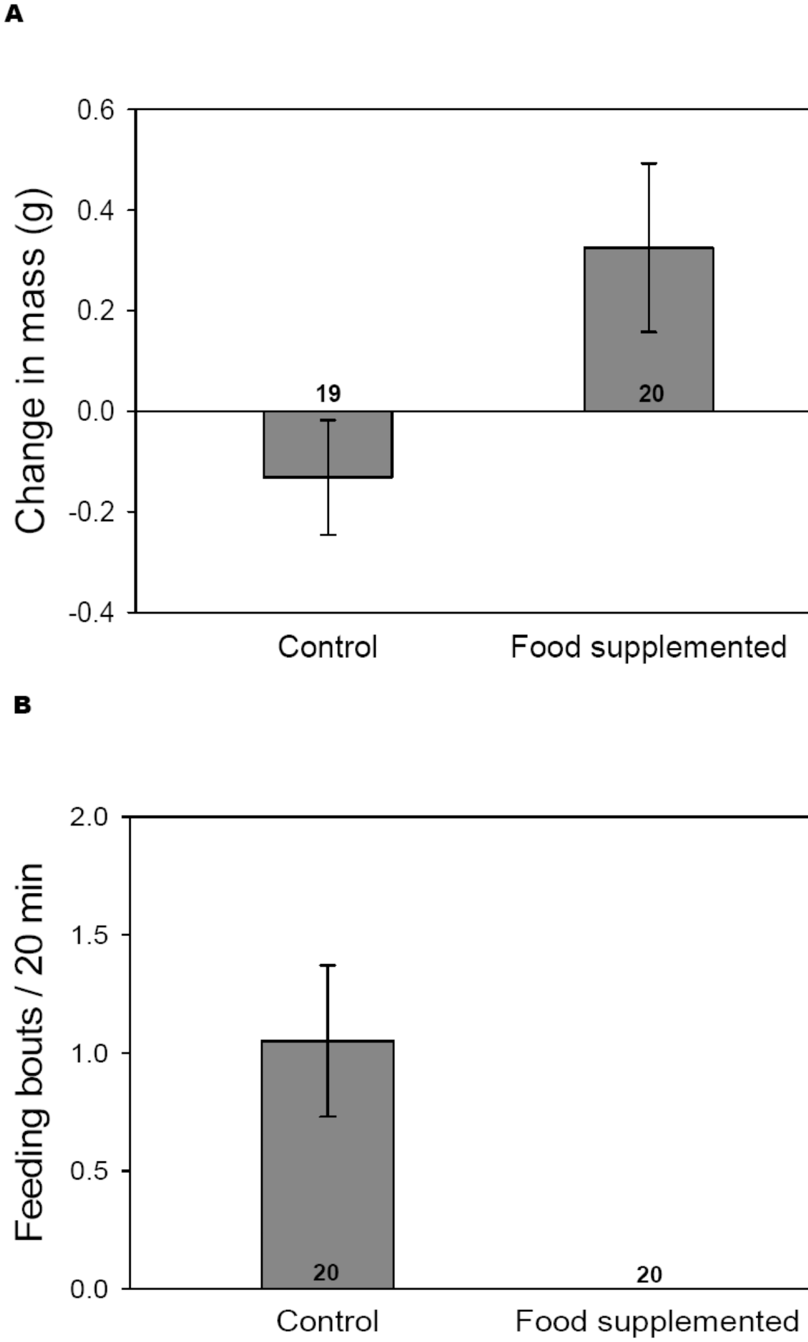

**Figure 1.**

Mean (+/− SEM) weight change (a) and number of times observed eating (b) in control and food supplemented lizards

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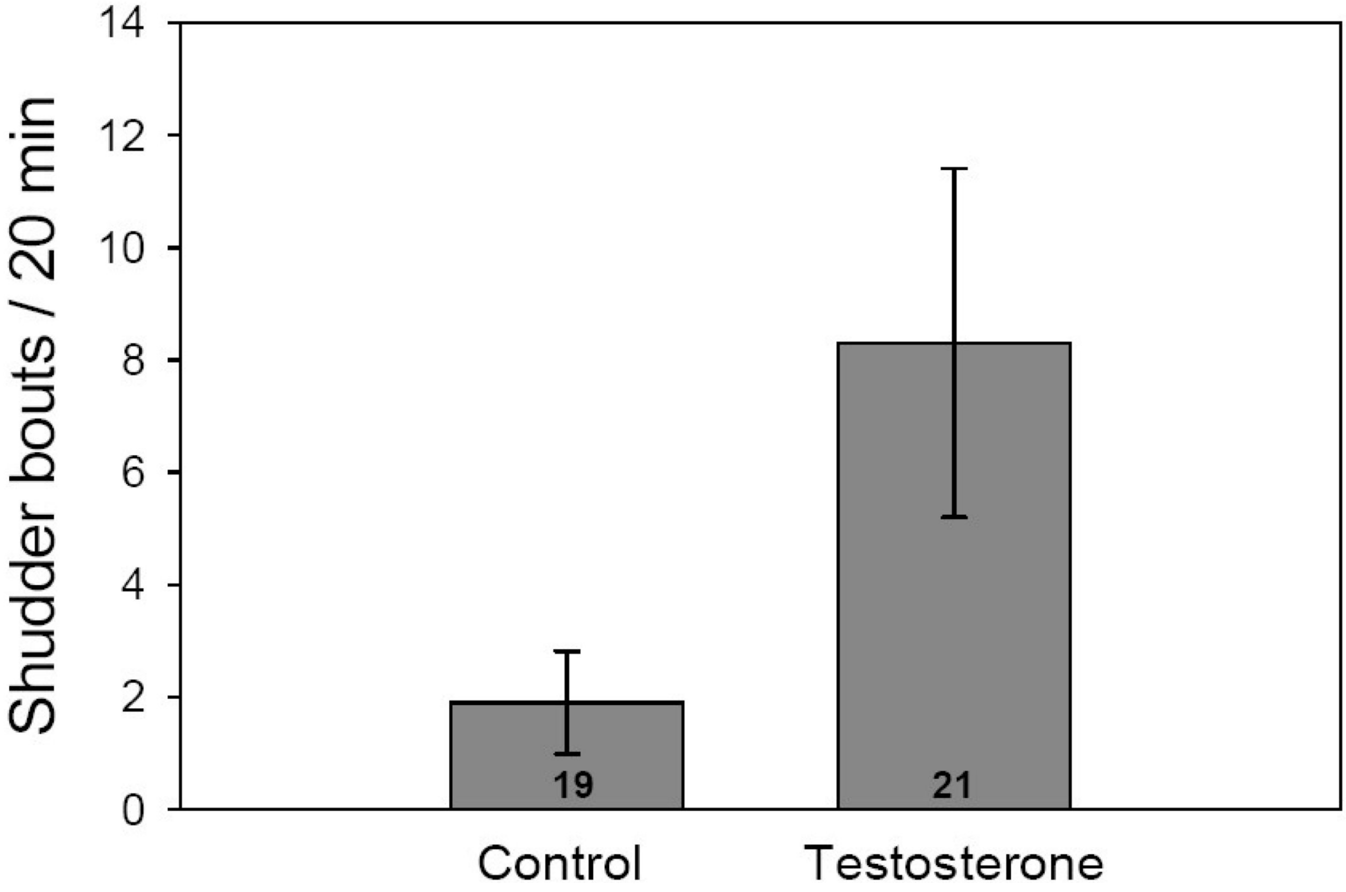

**Figure 2.**
Mean (+/− SEM) number of shudder bouts in vehicle or testosterone treated lizards without food supplementation

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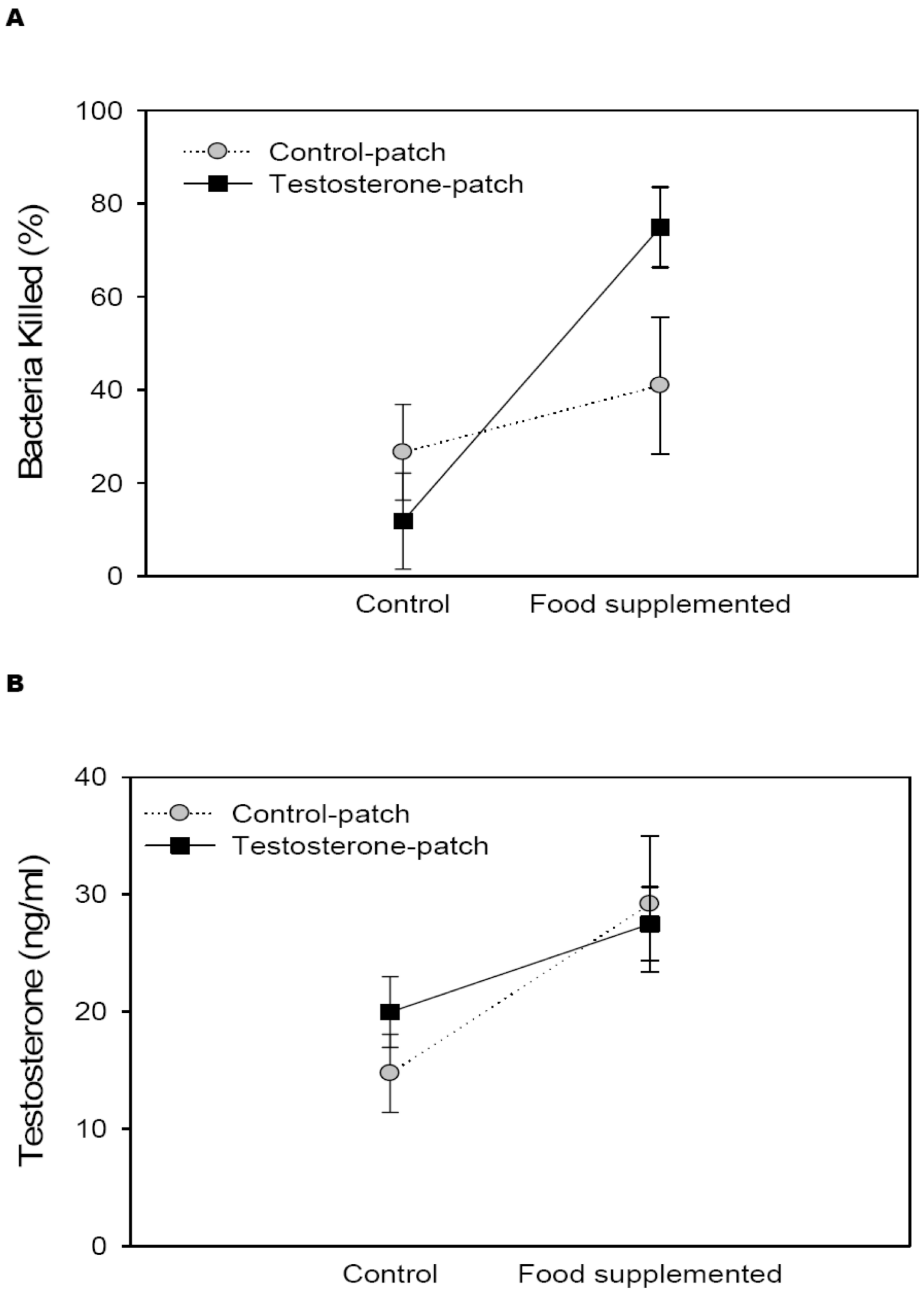

**Figure 3.**
Mean (+/− SEM) percent bacteria killed (a), circulating T (b) and CORT (c) in food supplemented or control lizards treated with exogenous testosterone (black squares) or vehicle (gray circles)

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## Table 1

Mean squares and F-values from two-way ANOVAS of immune (df = 1,31) and hormonal (df = 1,36) responses to food and patch (testosterone) manipulations

| | Bacteria killed (%) | | Circulating T | | Circulating CORT | |
|---|---|---|---|---|---|---|
| **Effect** | **MS** | **F** | **MS** | **F** | **MS** | **F** |
| **Food** | 12909.05* | 12.67* | 1197.93* | 8.14* | 0.01 | 0.00 |
| **T-Patch** | 794.90 | 0.78 | 31.17 | 0.21 | 24.81 | 2.72 |
| **Interaction** | 5121.94* | 5.03* | 119.24 | 0.81 | 4.11 | 0.45 |
| **Full model** | 6608.08* | 6.49* | 445.50* | 3.03* | 9.62 | 1.05 |

[*] $p<0.05$