Testosterone and Immune Function in Primates: A Brief Summary with Methodological Considerations

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Abstract The endocrine system serves as a mediator by which the body integrates environmental cues to organize physiological alterations, including changes in immunocompetence. Hormones are central mechanisms that contribute to the onset and timing of key life history events, the allocation of time and energy between competing functions, and in general modulate phenotypic development and variation. Here we provide a very brief review of testosterone and immunity, which highlights the physiological costs that elevated testosterone levels can incur as a result of reproductive investments. We focus primarily on nonhuman primates where possible. Although there is substantial evidence that testosterone exerts some influences on immune responses, results from *in vivo* studies involving human and nonhuman primates have yielded equivocal results regarding such immunomodulatory actions. There may be several reasons for this, including variation in study design, immunological measures used, levels of other hormones present, host energy status, and even social conditions. We therefore review some of these potential methodological issues, concluding that increased care must be taken to analyze seasonal variability in energy budgets, to collect an adequate number of samples from known individuals, to account for status in the dominance hierarchy when applicable, and to use multiple measures of immunity. We must also seek to understand the collaborative effects of multiple hormones (particularly dehydroepiandrosterone and estradiol) with relation to their downstream immunological effects, assessing both individual and multiplicative actions in both males and females. Such efforts would benefit from the development of additional noninvasive immune measures for primates.

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Introduction

Immunocompetence, or the ability to effectively mount an immunological response to a foreign antigen, is an integral part of organismal life histories because it is crucial for maximizing evolutionary fitness. It represents a phenotypically plastic response to stochastic ecological stressors, such as the presence of pathogens, allergens, and injury, producing a range of complex adaptations through natural and sexual selection. As such, it is modulated by various physiological (including hormonal), ecological, and even social factors in many species, including human and nonhuman primates. The endocrine system serves, in part, as a primary mediator from which the body integrates environmental cues to organize physiological alterations (Bribiescas and Ellison 2008; Bribiescas and Muehlenbein 2010; Muehlenbein and Flinn 2011), including specific changes in immunocompetence. Hormones are central mechanisms that contribute to the onset and timing of key life history events, the allocation of time and energy between competing functions, and in general modulate phenotypic development and variation. This is particularly true for steroids, lipid-soluble molecules derived from cholesterol and shared by all vertebrates, including androgens.

It can be predicted that the endocrine system modulates immunocompetence in an adaptive fashion to alter energetic investment in expensive life history functions, such as survivorship through immune activation. Immunity is energetically expensive to develop, maintain, and activate, and is therefore predicted to be involved in trade-offs with other physiological functions and life history traits, including growth and reproduction (Barnard and Behnke 2001; Lochmiller and Deerenberg 2000; Muehlenbein and Bribiescas 2005; Muehlenbein *et al.* 2010; Norris and Evans 2000; Schmid-Hempel 2003; Sheldon and Verhulst 1996;). Such trade-offs are inherently difficult to qualify and quantify in human and nonhuman primates, in part because of our protracted life histories, but also due to methodological limitations and logistical/ethical constraints when working with these species (particularly in obtaining repeated useful biological samples from wild and sometimes endangered primates). Yet there remains much communication between the reproductive endocrine and immune systems to investigate. Here we provide a brief review of testosterone and immunity, focusing primarily on nonhuman primates where possible. Because testosterone can modulate both reproductive investments and trade-offs with immune function, it is considered one of the principal physiological mediators of the costs of reproduction in males. Given the dearth of studies assessing these relationships in these species, we must also draw from the extensive medical literature (and growing evolutionary anthropology literature) in humans. As much of this has been reviewed earlier (see Muehlenbein and Bribiescas 2005), we focus primarily on methodological issues that may explain the tremendous variation in study results on this topic. We refer readers unfamiliar with some of the immune or endocrine topics discussed here to the many high quality publications on these fields for more background (Demas *et al.* 2011; Griffin and Ojeda 2004; Janeway *et al.* 2005; Muehlenbein and Lewis 2013).

Why Testosterone?

Testosterone, a steroid hormone important to male reproductive physiology, can facilitate male reproductive success by modifying behaviors like competition and sexual

motivation, in addition to physical attributes such as gametogenesis and musculoskel-etal function (Bribiescas 2001; Muehlenbein and Bribiescas 2010). Muscle anabolism can augment work capacity, intrasexual competition, intersexual coercion, and mate choice. However, high testosterone levels could also compromise survivorship by increasing energetic costs (Muehlenbein and Bribiescas 2005). This problem would become exacerbated in resource-limited environments. Because testosterone also ex-hibits effects on immunity, testosterone can be regarded as a mechanism to modulate energetic investment between the competing demands of reproduction and survival (Muehlenbein and Bribiescas 2005).

In a complementary perspective, testosterone is predicted to modulate mate choice via the expression of condition-dependent traits (Folstad and Karter 1992; Wedekind and Folstad 1994). That is, the expression of some secondary sexual characteristics, which are energetically expensive to develop and maintain and are influenced by endocrine signals, may indicate the ability of the individual to both thrive in his environment and to express quality signals, despite the immunological and energetic costs of androgens. Lower quality males may not be able to tolerate the immunosup-pressive effects or increased energetic costs of high testosterone levels. The antagonist pleiotropic effects of androgens may thus both limit trait exaggeration and have important influences on social behavior. Of course all of this is dependent on the assumption that testosterone causes suppression of immune functions.

Immunomodulation

There is tremendous evidence that testosterone exerts some influence on immune responses, and correlated changes in immunity and gonadal function reflect the man-ifold interactions between these two systems. Unfortunately, there are few studies that examine such effects in nonhuman primates. In an experimental study of captive male long-tailed macaques (*Macaca fasicularis*) infected with Venezuelan equine encepha-litis virus, testosterone levels were significantly reduced after infection as compared to baseline (Muehlenbein et al. 2006). Depressed testosterone levels during immune activation would function to limit energetic investment in energetically expensive anabolic functions, as well as prevent immunosuppression by otherwise higher testos-terone levels (Muehlenbein 2008). It may also be the case that individuals inhabiting high pathogen risk environments could benefit from decreased testosterone levels. Environmental conditions, including exposure to infectious agents, during development may ultimately play an important role in altering baseline testosterone (and other hormone) levels as well as amount of variation experienced in adulthood (Muehlenbein 2008). Evidence from laboratory animal models is somewhat consistent with these predictions, where changes in maternal nutrition, maternal infection before pregnancy, or maternal exposure to dexamethasone or endotoxin all alter offspring testosterone profiles (Curno et al. 2011; Nilsson et al. 2001; Page et al. 2001; Zambrano et al. 2005).

Other lines of evidence that testosterone is involved in immunomodulation come from comparisons of infection prevalence and immunological outcomes between males and females. For example, Poulin (1996), in a meta-analysis of vertebrate helminth infections, found males to be significantly more parasitized and to have larger parasites

than females. A more recent analysis of mammals found similar results, that males exhibited higher parasitism than females, polygynous species had a significantly higher degree of parasitism biased toward males, and that there was a significant association between male-biased mortality and male-biased parasitism (Moore and Wilson 2002). Several studies specifically looking at populations of nonhuman primates have found similar results in multiple species, including prevalence of helminths in *Papio ursinus* (Pettifer 1984), and intensity of infection of *Schistosoma* in *Papio anubis* (Muller-Graf *et al.* 1997), although other studies find no sex-bias in measures of parasitism (Muller-Graf *et al.* 1996; Stoner 1996). In humans 81% of studies examined indicated a lower prevalence of filariasis in females than males (Brabin 1990), with similar findings for hantavirus (Ferrer *et al.* 1998) and several helminthes (Kazura *et al.* 1984; Webster *et al.* 1997), among others. Some of these differences in the prevalence of infection between males and females may be due to differences in life history strategies independent of the role of testosterone (Nunn *et al.* 2009; Rolff 2002), behavioral differences leading to increased pathogen exposure (Klein 2000), as well as differences in nutritional requirements, diet, and social considerations (Nunn and Altizer 2006). However, a recent meta-analysis of sex bias in human disease suggests that sex differences in behavior are largely secondary to physiological differences mediating disease susceptibilities (Guerra-Silveira and Abad-Franch 2013). Overall, men appear to have poorer adaptive immunity, where women exhibit higher B cell number and activation, higher Th2 antibody response, higher concentration of antibodies, and a lower age-adjusted death rate (Abrams and Miller 2011; Muehlenbein and Bribiescas 2005).

Testosterone is a potent manipulator of some immunological markers. Testosterone can modify T-cell development and subset ratios (Olsen and Kovacs 2001; Olsen *et al.* 1991), and suppress lymphocyte function by increasing suppressor cell and decreasing helper cell counts (Grossman *et al.* 1991; Weinstein and Berkovich 1981). Testosterone can decrease nitric oxide and pro-inflammatory cytokine production in macrophages (Chao *et al.* 1994, 1995), impair natural killer cell and macrophage activity (Straub and Cutolo 2001), and possibly favor the development of Th1 cytokines (Daynes *et al.* 1991; Giltay *et al.* 2000).

In vivo studies of testosterone and immunity are much less clear. Baseline testosterone levels were positively associated with viremia (measured quantity of virus in biological samples) after experimental Venezuelan equine encephalitis virus infection in captive male long-tailed macaques (*Macaca fasicularis*) (Muehlenbein *et al.* 2006). Testosterone was also positively associated with helminth and protozoan parasite richness (number of unique intestinal parasite species recovered from hosts' fecal samples) in a large population of wild, habituated male chimpanzees in Uganda (Muehlenbein 2006). In this case, elevated testosterone levels may alter the ability of the primate host to mount an effective response against concomitant infection with multiple parasitic species, or possibly increase the likelihood of acquiring multiple infections.

In longitudinal human studies, testosterone levels typically decrease in response to illness, and often correspond to the severity of perturbation (Spratt *et al.* 1993). In a study of endocrine responses to *Plasmodium vivax* in Honduran males, subjects exhibited lower testosterone and higher cortisol during infection when compared to samples taken after recovery as well as age-matched healthy controls (Muehlenbein

et al. 2005). Similarly, in a sample of 25 nonfebrile college-aged adult males who were naturally infected with respiratory viruses, testosterone concentrations were reduced by 10% during infection, and a subset of males exhibited a 30% decrease in testosterone levels when ill as compared to after recovery (Muehlenbein *et al.* 2010). In addition, testosterone concentrations were found to be negatively associated with immunoglobulin A (sIgA; Granger *et al.* 2000) and C-reactive protein (Lassek and Gaulin 2009; Tang *et al.* 2007), and positively associated with malaria parasitemia in Hondurans (Muehlenbein *et al.* 2005), among others. Conversely, testosterone was negatively associated with malaria parasitemia in one age group of Kenyan men (Kurtis *et al.* 2001). In a sample of college-aged men, testosterone was positively associated with the antibody response to hepatitis vaccination (Rantala *et al.* 2012). Likewise, changes in testosterone and sIgA were positively associated in a study of men from the Philippines (Gettler *et al.* 2014). No association between testosterone and sIgA was identified in a smaller study of young adults (van Anders 2010). In a study of healthy male athletes, there were no associations found between testosterone and the cytokines interleukin-6 (IL-6) or IL-1β (FitzGerald *et al.* 2012). In addition, in a tightly controlled sample (i.e., absence of any known infectious or chronic disease, history of drug or alcohol abuse, recent weight change, injury or surgery, mental disorder, use of prescription or non-prescription medications, etc.) of healthy college-aged adults (37 men and 57 women), testosterone (assessed through multiple samples across several days) was positively associated with a salivary measure of functional innate immunity (bacteria killing assay: Muehlenbein *et al.* 2011; Prall *et al.* 2011).

In total, results from *in vivo* studies involving human and nonhuman primates have been equivocal regarding the immunomodulatory actions of testosterone, and some studies that do find significant relationships have a relatively low effect size (Granger *et al.* 2000), suggesting that the association may not be biologically meaningful. There may be several reasons for these discrepancies, including host energy status, social conditions, variation in study design, immunological measures used, and levels of other hormones present.

Methodological Considerations

Few studies have focused on androgen-mediated immunity in nonhuman primates. This is likely due to the methodological complications in measuring endocrine and immune functions in wild primates, but there are many other practical hurdles. Here we outline some of the most important host factors and methods to consider.

Energetic Status

Because life history theory predicts trade-offs in energetic expenditure to occur during resource restriction, it is very likely that energy availability may play a role in mediating both endocrine markers and immunological outcomes. For example, there is evidence that fasting can temporarily reduce testosterone concentrations (Röjdmark 1987; Trumble *et al.* 2010). Unlike females, mammalian male reproductive physiology is energetically inexpensive, and evidence suggests that modest energy deficits do little to alter testosterone concentrations in humans (Bentley *et al.* 1993; Ellison and Panter-

Brick 1996). However, though short-term modifications in energy availability do not appear to modulate androgen concentrations directly, modulation of energy budgets is predicted to alter life history strategies. Muehlenbein and Bribiescas (2005) argue that testosterone is the driving mechanism mediating the trade-off between reproductive investment and survival, where it is predicted to redistribute energetic resources toward reproductive facets at the cost of immunocompetence. Even so, when energy is plentiful and the population is in relatively good health, there is little reason to suspect a negative relationship to appear. This is supported by the finding that testosterone is largely found to be unassociated or positively associated with measurements of immune function in humans in samples from wealthy developed countries (Prall *et al.* 2011; Rantala *et al.* 2012), while it is negatively related to a variety of measures from populations in developing countries (Campbell *et al.* 2001; Muehlenbein *et al.* 2005).

Variation in energy intake can have impacts on immune function directly. For example, military trainees who experienced weight loss exhibited decreased *in vitro* lymphocyte proliferation to immune activation, which were partially mitigated by increased food intake (Kramer *et al.* 1997). Similar results were found in red colobus (*Piliocolobus tephrosceles*), where a decline in food availability was linked to an increase in parasite prevalence in fragmented habitats (Chapman *et al.* 2006). When monitoring hormone-mediated immune function in wild primates, care must be taken to consider the role of food availability on immunological measures. This may be particularly true in populations that experience seasonal variations in food availability. Future studies should use seasonal variability in energy budgets to examine whether androgen mediated trade-offs appear only during times of energy deficit.

Social Factors

Social status is related to both endocrine function and health outcomes in complex ways. Testosterone can co-vary with dominance status, but not in all species under all conditions. For example, testosterone correlated positively with rank in chimpanzees using urinary steroid concentrations at Kanyawara, Kibale National Park in Uganda (Muller and Wrangham 2004). Similarly, fecal steroid concentrations correlated positively with rank in the much larger chimpanzee group at Ngogo, Kibale National Park (Muehlenbein *et al.* 2004). Using urinary steroid concentrations at a much later time, Sobolewski *et al.* (2013) found no such relationship at Ngogo. Testosterone may be a positive predictor of dominance rank in some species only during periods of social instability, such as during challenges by conspecific males for territory or access to mates, the establishment of territorial boundaries, or the presence of receptive females (Beehner *et al.* 2006; Bergman *et al.* 2006; Rose *et al.* 1975; Wingfield *et al.* 1990).

Independent of the role of androgens, status in the dominance hierarchy can directly and indirectly affect immune function through several mechanisms. Higher ranking individuals may experience altered probability of illness and infection via increases in sexual activity, differences in social interaction between group members, greater access to food resources, and differential grooming opportunities and social support (Nunn and Altizer 2006; Sapolsky 2005). Changes in rank even alter expression of many genes related to immune function in female macaques (Tung *et al.* 2012).

Assuming that testosterone is associated with both dominance-related behaviors in addition to immunocompetence, then it is possible that testosterone-associated

immunosuppression may be a significant cost to maintaining dominance and interacting in a dynamic social environment. Individual males that are more resistant to the costs of maintaining high testosterone may be able to maintain physiological and behavioral investment in reproduction, and be more likely to maintain a high dominance rank (Muehlenbein and Watts 2010). In this way, status in the dominance hierarchy is predicted to be an honest indicator of individual male quality. Of course, studies have yielded mixed results.

Muller-Graf and others (1996) found no association between helminth infection and dominance rank in olive baboons, whereas Hausfater and Watson (1976) did demonstrate higher intestinal helminth infection in high-ranking yellow baboons. Lower rank is associated with increased louse prevalence, lower insulin-like growth factor I, and fewer circulating lymphocytes in olive baboons (Eley *et al.* 1989; Sapolsky 2005; Sapolsky and Spencer 1997). In a longitudinal study of baboon males (*Papio cynocephalus*), high-ranking males had faster healing rates to external injuries (Archie *et al.* 2012), despite the fact that there is substantial evidence that testosterone acts in an immunosuppressive fashion, and has been found to inhibit wound healing rates in some contexts (Engeland *et al.* 2009; Gilliver *et al.* 2007). In captive macaques, the results are as mixed. Dominant male longtailed macaques (*Macaca fascicularis*) exhibit lower primary antibody responses to tetanus toxoid (Cunnick *et al.* 1991) but lower risk of adenovirus infection compared with subordinate animals (Cohen *et al.* 1997). High-ranking female rhesus macaques (*Macaca mulatta*) had higher CD4+ and CD8+ lymphocyte counts than lower ranking females (Schapiro *et al.* 1989).

Muehlenbein and Watts (2010) examined the associations between fecal testosterone, intestinal parasitism, and dominance rank in a sample of 22 wild male chimpanzees (*Pan troglodytes schweinfurthii*) from Ngogo, Kibale National Park in Uganda. In this study, dominance hierarchy rank was assessed via longitudinal observations of social behavior, while multiple fecal samples were collected for hormone and parasite analyses. Dominance rank and testosterone significantly positively correlated to helminth parasite richness in this study, so that higher ranked individuals had higher number of unique helminth species present alongside higher testosterone levels. Variation in the results of these aforementioned studies may be the result of differences in immunological methods employed and perhaps even variability in animal social systems. Regardless, such variation highlights the importance of accounting for social status when assessing immune-endocrine relationships in primates.

Sampling Strategies

Outcomes are certainly dependent on the methods employed to answer the research question. Many recent papers on primate ecoimmunology and behavioral endocrinology present arguably questionable results as a direct effect of their compromised sampling strategies. A majority of these studies are opportunistic, retrospectively analyzing samples to evaluate post hoc hypotheses relevant to immune-endocrine questions. Examples include studies that randomly sample fecal samples from unknown individuals within a population; such sampling will produce an inaccurate measure of prevalence because the unit of analysis is fecal samples instead of infected/susceptible hosts. Fecal samples are not randomly distributed in the environment, as ill individuals *may* defecate more frequently than healthy hosts. This is particularly

problematic if your pathogen of interest is not evenly distributed throughout the host population, which is usually the case. Furthermore, it is critical that multiple samples be collected from the same animal across time. Hormones fluctuate hourly (and often times more frequently) in response to a multitude of factors. Intestinal parasites are shed only intermittently. Muehlenbein (2005) demonstrated that not one of the 12 parasitic species recovered from the chimpanzee Ngogo group in Kibale National Park was found in all samples from any one individual, and the most commonly occurring parasites were found in all of the serial samples of only a fraction of the chimpanzees sampled. Cumulative parasitic species richness for the chimpanzee hosts significantly increased for every sequential sample (up to four samples) taken per individual during this study. Estimates of worm burdens are highly vulnerable to variability in fiber content, consistency, parasite factors, and host characteristics (Stear *et al.* 1995). Though a single fecal sample may be adequate for determining antibody levels indicative of past infection, current parasitic infection usually requires multiple samples.

Timing of sample collection is also important. In seasonally breeding species, care must be taken to measure differences between reproductive and nonreproductive periods, as males may have altered endocrine profiles and changes in disease load and immunological measures. Some species, including many primates, have elevated testosterone during the breeding season or in the presence of receptive females (Arlet *et al.* 2011; Mehlman *et al.* 1997), and this is consistent with theoretical predictions and avian models (Wingfield *et al.* 1990). There are few studies that assess the role of changing androgens during breeding season as it relates to immune function. In an analysis of the role of testosterone on parasitism increases during the breeding season in brown mouse lemurs (*Microcebus rufus*), testosterone was found to be unassociated with both endoparasite and ectoparasite loads (Zohdy *et al.* 2012). Nevertheless, the impact of elevated androgens during seasonally focused reproductive effort is a useful natural experiment to determine the outcome of increased testosterone concentrations, and one that should be expanded to include other primates with other methods of measurement.

Assessing hormone-mediated immune function in wild primates can be accomplished through several different approaches. The most widely used method is to compare cross-sectional measures of fecal or urinary hormones with some measure of immune function. However, while technically considered cross-sectional, all sample collection cannot be completed at the same time. As multiple samples from each individual are required, alongside the difficulty of collecting samples from wild primates, sample collection for a single "cross-sectional" study can take weeks or even months. In this time, investigators need to be aware of changing host factors that can modify endocrine or immune measures, and exclude individuals or samples accordingly. For example, chimpanzee fecal samples collected after aggressive encounters with other groups will likely indicate elevated testosterone, and integrating these samples for a mean baseline may be inappropriate.

A second approach to measuring androgens and immune function in wild primates is a longitudinal pre-/posttreatment, comparing the influence of some changing environmental or host factor such as which may influence one or more outcome variables. For example, sample collection before and after breeding season, to assess the influence of differences in testosterone concentrations is a useful but underutilized approach. Here investigators need to consider changes in activity patterns, aggressive interactions, and

nutritional intake, all which are likely to change during the breeding season and could impact host immune function. Nevertheless, increased androgen concentrations surrounding periods of intensified reproductive investment or competition provide a useful natural experiment to assess these relationships. In a similar vein, measuring the response of androgens to periods of infection, or the relationship between androgens at baseline as compared to infection, can provide a great deal of information on both the influence of androgens in modulating the physiological impact of infection, as well as the adaptive endocrine response to infection. Using this approach, investigation could include treatment of anthelmintic reagents to individuals previously identified as infected with parasites or other pathogens, or measuring endocrine concentrations before and after an experimentally induced infection.

Outcome Measures

The measurement of health outcomes is determined by the research questions, which are constrained by the natural history and habitat of the primate species under investigation. The most important thing to know is: Do not reinvent the wheel. There are experts in immunology who should be consulted. Measurement error is caused by both inappropriate methods and untrained individuals. Much variation that occurs in published (and unpublished) studies surely results in variation in these factors relating to reliability, validity, sensitivity, specificity, and choice of methods.

Methods of assessing hormone concentration via fecal or urinary samples have been well developed, and there are several good texts outlining such methodologies (Muehlenbein 2009; Ziegler and Wittwer 2005). Measures of immune function in wild primates are comparatively very limited (*cf.* Muehlenbein and Lewis 2013 for advice on primate epidemiology and health assessment). Table I lists several of the most common methods available. Nearly all measures of primate health have been limited to determination of exposure, i.e., baseline parasite levels, primarily owing to the difficulty in collecting invasive samples from wild, and sometimes endangered, primates. However, using measures of parasitism as indexes of health or disease resistance are problematic, as many wild primates maintain low levels of chronic infection with little consequence. Caution must be taken as not all reports of parasitism are equal, and poor methodology can produce extremely inconsistent results. In particular, using fecal egg counts as a proxy of infection intensity is problematic, as egg counts are dependent upon parasite age, fiber content, and various host factors (Stear *et al.* 1995), making egg counts unpredictably related to adult parasite infection. Similarly, immunophenotyping, i.e., measurement of leukocyte counts and ratios, can be difficult to interpret because higher levels may not represent better immune capabilities, but rather subclinical infections. It is critical to remember that baseline immune measures in wild animals do not necessarily represent a "disease absent" state, as some up-regulation of immunity due to the ubiquitous presence of parasites is to be assumed.

It is recommended that researchers rely on multiple measures of immunity in their studies, as it is not possible to adequately represent the functioning of an entire coordinated system through a singular measure (Norris and Evans 2000; Sheldon and Verhulst 1996; Westneat and Birkhead 1998). There is no reason to believe that results from multiple studies on a singular host system, e.g., macaques, should yield similar results when assessing relationships between testosterone and white blood cell count

Table I Selection of immune measures available for some sample types from primates

Sample type	Immune measures	Recommendations
Urine	Cytokine profiles[1]; C-reactive protein; neopterin; β2-microglobulin; secretory IgA and other antibody levels; *ex vivo* bacteria killing ability; nitric oxide; some pathogen diagnostics.	It may be important to control for sample water content (usually via creatinine or specific gravity analyses) and diurnal variation.
Feces	Intestinal parasite richness (number of unique pathogens infecting a host at any given time)[2]; some pathogen diagnostics; C-reactive protein	Multiple samples should be taken from each individual to account for variation in parasite shedding. If you are untrained in parasitology, establish a collaboration with an experienced investigator. Collect samples from known individuals (not randomly throughout the habitat). Consider sampling across seasons. Do not rely on fecal egg counts.
Whole blood	Lymphocyte proliferation followed by cytokine quantification in cell culture supernatant,[3] cell counts and differentials; most pathogen diagnostics; *ex vivo* bacteria killing activity[4, 5]; gene expression of immune effector pathways.	Samples can be fixed and stained for later hematological analyses, but cannot be frozen for use in flow cytometry. Keep in mind that elevated white blood cell count may indicate subclinical infection (and not better immunity in that animal).
Isolated peripheral blood mononuclear cells	Lymphocyte proliferation followed by cytokine quantification in cell culture supernatant[6–8]; macrophage phagocytic ability; natural killer cell cytotoxicity[9]; gene expression of immune effector pathways	Lymphocyte separation requires careful centrifugation. Freezing lymphocytes for later proliferation requires liquid nitrogen and cryopreservatives.
Serum or plasma	Complement proteins; hemolytic complement activity[10, 11]; *ex vivo* bacteria killing activity[4]; C-reactive protein; cytokine profiles[1]; antibodies; most pathogen diagnostics; antioxidant activity	Serum separation requires centrifugation. Collect samples across multiple days. Most importantly (and for all sample types): use multiple measures of immunity when possible.

[1] Sachdeva and Asthana (2007); [2] Muehlenbein (2005); [3] Coe *et al.* (2002); [4] Liebl and Martin (2009); [5] Millet *et al.* (2007); [6] Weinberg *et al.* (1998); [7] Froebel *et al.* (1999); [8] Hearing *et al.* (1999); [9] Claus *et al.* (2009); [10] Mayer (1948); [11] Sinclair and Lochmiller (2000).

versus testosterone and sIgA levels, particularly when not accounting for reproductive season, social status and a number of samples per individual.

Functional measures are those that allow for the direct assessment of a component of the immune system to respond to an immune challenge, taking into account dozens of individual factors and pathways in some cases, and delivering a relative measure of immunocompetence independent of previous exposure. Examples include lymphocyte proliferation (ability of T and B cells to undergo mitosis *in vitro* after exposure to a

mitogen); hemolytic complement activity of serum to lyse antigens; and *ex vivo* bacteria killing abilities of blood, saliva, and urine (bacteria killing assay). Outside of functional measures, concentrations of cytokines, antibodies, lysozyme, and various antimicrobials can be assessed through enzyme immunoassay, multiplexing, Western blot, immunofluorescence, or hemagglutination. Gene expression of immune effector pathways can be determined using quantitative polymerase chain reaction (PCR), sequencing or microarrays. In brief, measuring multiple branches of immune function is required to adequately understand immune-endocrine relationships, and there are many such assays available (Demas *et al.* 2011), although few can be used for noninvasively collected samples.

Other Hormones

Though testosterone appears to have interactions with multiple facets of immune function, it is clear that testosterone does not work in isolation with respect to its physiological effects, and understanding hormone-mediated immunity by analyzing only testosterone is inadequate and inappropriate. Instead, measuring the complete hormonal milieu in relation to its downstream immunological associations will yield a more complete picture, and there is a great deal of evidence to suggest that additional hormones have equally potent interactions. For example, glucocorticoids are known to have potent effects on aspects of immune function, the impact dependent on the length and severity of stimulation (McEwen *et al.* 1997). Alberts *et al.* (1992) observed an inverse association between cortisol and total lymphocyte levels in wild female baboons, and Sapolsky and Spencer (1997) found an inverse association between cortisol and insulin-like growth factor I in wild male baboons. Glucocorticoids can also co-vary with social and reproductive status in primates (Bergman *et al.* 2005; Ostner *et al.* 2008; Sapolsky 2005; Setchell *et al.* 2010). Depending on social conditions, glucocorticoids can exhibit potent interactions with androgens in determining immunological outcomes. For example, in chimpanzees, both cortisol and testosterone were positively associated with parasite richness when placed in a mixed model (Muehlenbein 2006). Similarly in humans, cortisol was found to have a moderating influence on the relationship between testosterone and immune function (Rantala *et al.* 2012).

The adrenal androgen dehydroepiandrosterone (DHEA, and its sulfated form DHEA-S) also has numerous associations with immunity responses both *in vivo* and *in vitro*. In various experimental treatments, DHEA can increase T-cell and natural killer cell cytotoxicity (Casson *et al.* 1993; Suzuki *et al.* 1991), secretion of some cytokines (Daynes *et al.* 1990), and proliferation of some immune cells to an antigenic challenge (Sakakura *et al.* 2006). It is possible that DHEA also facilitates the production of Th2 over Th1 cytokines (Powell and Sonnenfeld 2006). DHEA has also been implicated in increasing Treg cytokine production (Auci *et al.* 2007; Coles *et al.* 2005). DHEA was found to exert protective effects against schistosomiasis and malaria infections in several studies (Abebe *et al.* 2003; Kurtis *et al.* 2006; Leenstra *et al.* 2003). And because of its potential protective effects, DHEA may be elevated during illness relative to testosterone levels (Prall and Muehlenbein 2011). However, DHEA may also increase the expression of several inhibitory proteins, which regulate the complement cascade (Falus *et al.* 1990; McLachlan *et al.* 1996). Though this has not been investigated in a larger physiological context, initial results suggest that DHEA-S is negatively related to complement action in humans (Prall and Muehlenbein, *unpubl. data*).

DHEA may play some role in sexual dimorphism in primate immune function. In one study of captive chimpanzees, DHEA-S was higher in females compared with males (Nadler *et al.* 1987). DHEA and DHEA-S are also higher in female baboons (*Papio cynocephalus* and *Papio hamadryas anubis*) as compared to males, but no sex differences were found in sooty mangabeys (*Cercocebus atys*) (Bernstein *et al.* 2008; Castracane *et al.* 1981). It is possible that variation in DHEA/S in specific age groups of some species could influence immune function. In addition, variability in DHEA may be more important in males in determining immune outcomes, as DHEA supplementation in mice resulted in significantly lower *Trypanosoma* parasitemia in males only (dos Santos *et al.* 2005). The importance of DHEA in ameliorating the immunological costs of corticosteroids has been explored in laboratory mice (Ben-Nathan *et al.* 1992), but has yet to be expanded to nonhuman primates. And, given that hypothalamic–pituitary–adrenal activation can increase both DHEA and cortisol simultaneously (Lennartsson *et al.* 2012), and such action can go on to modify other endocrine signals, it is clear that these hormones will act in concert in relation to their effects on the immune system.

Estradiol and many other steroid and peptide hormones exhibit potential effects on some immune components. 17β-Estradiol may bias T-cell fractions toward CD8+ cells (Athreya *et al.* 1993), modulate lymphocyte development and differentiation (Lang 2004), increase the production of several immunoglobulins (Kanda and Tamaki 1999) and cytokines (Cutolo *et al.* 2006; Olsen and Kovacs 1996), up-regulate the production of antioxidant enzymes (Vina *et al.* 2006), and protect against the oxygen radicals produced by inflammatory stress (Asaba *et al.* 2004).

Clearly, hormones are not universally immunosuppressive, and a more nuanced understanding of the specific effects of particular endocrine signals to particular branches of immunity will yield a greater understanding of these relationships. Hormone-immune relationships cannot be described simply in broad strokes. Future studies must seek to understand the collaborative effects of multiple hormones with relation to their downstream immunological effects, assessing both individual and multiplicative actions. Equally critical is the appropriate choice of functional immune measures and increased sampling effort, both of which would benefit from the additional development of noninvasive immune measures in primates.

Glossary

Androgen	Group of steroid hormones, including testosterone, utilizing specific receptors whose primary role includes the maintenance and development of male reproductive characteristics.
Antibody	Also known as immunogloublins, antibodies are crucial proteins in the immune system, which act by binding onto foreign molecules and instigating a cascade of immunological effects. There are several types, including

	IgA, which is primarily secreted into the oral cavity and other mucus membranes and binds to pathogens to prevent their attachment to the cell surface.
B cells	A specialized lymphocyte involved in the humoral branch of the immune system. When activated, B cells produce antibodies specific to a pathogen.
Bacterial killing assay	An *in vitro* assay of innate immune function, primarily related to complement proteins and various antimicrobials, where serum or saliva is cultured with a known quantity of bacteria.
Complement system	The complement system, a facet of innate immunity, is a system of plasma proteins that, when activated via several different pathways, result in immunological responses. These responses include phagocyte recruitment, increasing pathogen phagocytosis, and formation of a protein complex (membrane attack complex) that causes lysis of pathogens.
C-reactive protein	An inflammatory protein synthesized by the liver whose primary role is to signal for the removal of dead cells or pathogens.
Cortisol	A glucocorticoid produced by the adrenal gland whose primary role involves increasing plasma glucose, but also has anti-inflammatory properties and regulates immune function at several levels. Is a key component of normal stress responses.
Cytokines	Specialized protein messengers that act as signaling molecules for the immune system separated into various groups depending on function. Th-1 cytokines (pro-inflammatory) include tumor necrosis factor α (TNF-α), interferon γ (IFNγ), interleukin-1β (IL-1β), IL-2, IL-12, and others, and play a role in regulating cellular immunity. Th-2 cytokines (anti-inflammatory) are primarily involved in humoral immunity and include IL-4, IL-5, IL-6, IL-10, and others.
Dehydroepiandrosterone (DHEA)	An adrenal androgen (along with the sulfated version dehydroepiandrosterone-sulfate, or DHEA-S) whose primary role is as a precursor for the synthesis of other hormones.
Estradiol	More specifically, 17β-estradiol, is the most potent estrogen synthesized from the granulosa cells of the ovaries, whose primary role includes the development and maintenance of female reproductive physiology.
Hemolytic complement assay	An *in vitro* assay measuring the ability of the complement system (specifically the antibody-dependent pathway) to lyse sheep red blood cells.
Insulin-like growth factor 1 (IGF1)	A hormone produced by the liver stimulated by growth hormone, whose primary role is related to growth and development.
Lymphocyte	Specific white blood cells, including T and B cells, that play important roles in adaptive immune responses, including the

	release of cytokines and antibodies, and lysis of foreign cells or pathogens.
Lymphocyte proliferation assay	An assay measuring the functional ability of T and B cells to undergo mitosis *in vitro* in response to an exposure to a mitogen.
Lysozyme	An enzyme found in large quantities in mucous membranes that acts to rupture the cell walls of bacteria.
T cells	A specialized lymphocyte involved in cell-mediated immunity, with several different subtypes each with a different function. CD8 or cytotoxic T cells attach and destroy pathogens, CD4 or helper T cells secrete cytokines to aid in the maturation of other immunological cells, and regulatory T cells (Treg) act to suppress cell-mediated immunity after infection.
Testosterone	An androgen synthesized in the Leydig cells of the testes (as well as the adrenal glands), important in the development and maturation of secondary sexual characteristics, as well as in other various physiological systems including muscle anabolism and spermatogenesis.

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