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RESEARCH ARTICLE

Noninvasive measurement of mucosal immunity in a free-ranging baboon population

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Abstract

Ecoimmunological patterns and processes remain understudied in wild primates, in part because of the lack of noninvasive methods to measure immunity. Secretory immunoglobulin A (sIgA) is the most abundant antibody present at mammalian mucosal surfaces and provides an important first line of defense against pathogens. Recent studies show that sIgA can be measured noninvasively in feces and is a good marker of mucosal immunity. Here we validated a commercial ELISA kit to measure fecal IgA in baboons, tested the robustness of its results to variation in collection and storage conditions, and developed a cost-effective in-house ELISA for baboon fecal IgA. Using data from the custom ELISA, we assessed the relationship between fecal IgA concentrations and gastrointestinal parasite burden, and tested how sex, age, and reproductive effort predict fecal IgA in wild baboons. We find that IgA concentrations can be measured in baboon feces using an in-house ELISA and are highly correlated to the values obtained with a commercial kit. Fecal IgA concentrations are stable when extracts are stored for up to 22 months at −20°C. Fecal IgA concentrations were negatively correlated with parasite egg counts (*Trichuris trichiura*), but not parasite richness. Fecal IgA did not vary between the sexes, but for males, concentrations were higher in adults versus adolescents. Lactating females had significantly lower fecal IgA than pregnant females, but neither pregnant nor lactating female concentrations differed significantly from cycling females. Males who engaged in more mate-guarding exhibited similar IgA concentrations to those who engaged in little mate-guarding. These patterns may reflect the low energetic costs of mucosal immunity, or the complex dependence of IgA excretion on individual condition. Adding a noninvasive measure of mucosal immunity will promote a better understanding of how ecology modulates possible tradeoffs between the immune system and other energetically costly processes in the wild.

KEYWORDS

fecal immunoglobulin A, mucosal immunity, noninvasive, wild baboons

1 | INTRODUCTION

In the past two decades, there has been a growing interest in understanding how variation in individual and species ecology modulates tradeoffs between the immune system and other energetically costly processes (see reviews: Lazzaro & Little, 2009; Lee, 2006; Martin, Weil, & Nelson, 2006; McDade, Georgiev, & Kuzawa, 2016; Roberts, Buchanan, & Evans, 2004). Experimental studies on captive animals have been essential in demonstrating the existence of tradeoffs between immunity, growth, and reproduction (Lazzaro & Little, 2009; Lochmiller & Deerenberg, 2000; Rauw, 2012). However, while these systems offer exceptional experimental control, they often cannot replicate conditions in natural populations, where subjects experience considerable heterogeneity in access to resources, exposure to parasites, coinfections, and host genetic diversity (Lazzaro & Little, 2009; Pedersen & Babayan, 2011; Sheldon & Verhulst, 1996). As such, research in both captive and wild populations is necessary to gain a complete understanding of immune tradeoffs. However, researchers working in natural populations are often challenged to measure immune function because the assays are usually invasive, requiring researchers to inject antigens under the skin or measure immune response in blood samples (reviewed in Demas, Zysling, Beechler, Muehlenbein, & French, 2011). Such assays present especially large logistical and ethical challenges for large mammals and endangered species, including wild primates; hence Noninvasive assays of immune response are essential to understand ecoimmunological patterns and processes across the tree of life.

Recent studies have suggested that immunoglobulin A (IgA) can be measured noninvasively in feces, providing a measure of mucosal immunity in the intestine (in rats: Hau, Andersson, & Carlsson, 2001; in dogs: Peters, Calvert, Hall, & Day, 2004; in reindeer: Rehbinder & Hau, 2006; Yin et al., 2015; in Soay sheep: Watt, Nussey, Maclellan, Pilkington, & McNeilly, 2016; in humans: Dion, Montagne, Bene, & Faure, 2004; Vetvik, Grewal, Haugen, Ahren, & Haneberg, 1998; in chimpanzees: Lantz et al., 2018). Importantly, because immune responses are compartmentalized in different tissues, mucosal responses (including IgA) are often not correlated with other immune responses and cannot be used as a measure of immune response in the whole organism (Mestecky, 1987; Sinski et al., 1995; Watt et al., 2016). However, mucosal immunity, even in the absence of a strong link to systemic immunity, is highly relevant to animal health in wild populations. Wild animals are exposed daily to disease-causing agents that use mucosal tissues (e.g., respiratory, urinal, gastrointestinal, and genital tracts) as their primary point of entry, including viruses, bacteria, and parasites (Hart, 2011). Mucosal tissues also encounter diverse antigens from the environment (e.g., commensal bacteria, food, and pollen), and strong inflammatory responses to these nonpathogenic antigens would be detrimental (reviewed in Savage, 2005). Therefore, mucosal tissues have evolved a chronic but noninflammatory immune response that does not kill microorganisms, but prevents them from crossing epithelial barriers (Russell & Kilian, 2005).

A key component of the mucosal immune defense is the production of secretory immunoglobulin A (SIgA). SIgA is the most abundant antibody at mammalian mucosal surfaces. It provides an important first line of defense against infectious agents including viruses (e.g., influenza, rotavirus), bacteria (e.g., *Streptococcus*, *Salmonella*, *Chlamydia*), and parasites (e.g., Giardia, Helminths), and it prevents commensal bacteria from penetrating the mucosal epithelium (reviewed in Russell & Kilian, 2005). SIgA acts by entrapping pathogens in mucus, which facilitates their removal, often via excretion (reviewed in Grencis, 2015; Kaetzel, 2014; McNeilly, Devaney, & Matthews, 2009; Russell & Kilian, 2005). SIgA can also neutralize bacterial toxins and enzymes (Lycke, Eriksen, & Holmgren, 1987, see also review by Russell & Kilian, 2005). In addition to its role in defense against infections, sIgA also minimizes damage to the mucosal epithelium by regulating inflammatory responses. SIgA is less inflammatory than other immunoglobulins, as it does not bind to complement well (reviewed in Russell & Kilian, 2005; Williams, 2012). SIgA also downregulates the release of proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin 6 (IL6), and interferon gamma (IFNγ), while inducing the production of the immunomodulatory cytokine interleukin 10 (IL10; reviewed in Mantis, Rol, & Corthesy, 2011; Williams, 2012). SIgA is produced in mucosal-associated lymphoid tissues upon activation of lymphocyte B cells. This activation is mediated through the secretion of cytokines that are produced by endothelial cells as well as by cells of the innate immune system (e.g., macrophages, neutrophils, dendritic cells; reviewed in Strober, Fagarasan, & Lycke, 2005).

In this study, we validated the determination of fecal IgA assays in baboons and tested how fecal IgA varies in wild baboons as a function of parasite burdens, sex, age, and reproductive effort. Our wild subjects were members of the Amboseli baboon population in Kenya, which has been under continuous, individual-based monitoring by the Amboseli Baboon Research Project (ABRP) for more than 45 years. Considerable data on these baboons' ecology, demography, behavior, life-history, and physiology are available (e.g., Alberts & Altmann, 2012; Gesquiere, Wango, Alberts, & Altmann, 2007). The baboons live in a harsh environment, including a 5-month long dry season (June through October) during which virtually no rain falls, and they experience low food availability.

Because of energy limitation, members of this population face tradeoffs between investment in current offspring, future offspring, and their own maintenance and growth (Altmann & Alberts, 2003, 2005; Beehner, Onderdonk, Alberts, & Altmann, 2006; Gesquiere, Altmann, Archie, & Alberts, 2018). Tradeoffs between reproductive effort and immunity seem to be particularly complex. For instance, in Amboseli, female baboons exhibit a tradeoff between reproduction and wound healing, but not between reproduction and parasite infection risk. Specifically, lactating females, who bear high energetic costs of milk production and infant carrying, heal more slowly than pregnant and cycling females (Archie, Altmann, & Alberts, 2014). However, females do not differ in helminth infection risk as a function of reproductive state, perhaps because helminth infection is

predicted by exposure as well as individual traits or states (Akinyi et al., 2019). Male baboons in Amboseli exhibit little evidence for tradeoffs between reproductive effort and immunity: high-ranking males engage in higher reproductive effort than low-ranking males, yet they also heal faster from wounds (Archie, Altmann, & Alberts, 2012) and exhibit equivalent parasite loads to low-ranking males (Habig et al., 2019). The difference between male and female baboons may reflect differences in male and female life histories: females reproduce steadily throughout relatively long lives while males concentrate reproductive effort in their prime age, rapidly declining in social status and reproductive success at an age when females are still reproductively active. Therefore, males may benefit from investing simultaneously in immune function and reproduction in their prime, even at the cost of a more rapid decline with age.

The immune system is complex, comprising multiple arms, and individuals are likely to experience selection to up or down-regulate different immune components depending on the threats they face and the resources they can invest (Lee, 2006; McDade et al., 2016). For example, following a wound, an infection of the blood as well as the connective tissues is likely, because of the disruption of the skin protective barrier. In response, T-helper type 1 (Th1) cells and fibroblasts, among other cell types, will be activated and will result in the elimination of the pathogen and the repair of damaged tissues (Young & McNaught, 2011). In contrast, a helminth infection of the intestinal mucosae will generate a T-helper type 2 (Th2) and mucosal B cell response with the production of immunoglobulin such as sIgA and IgE (reviewed in Abd-Alla & Ravdin, 2005; Williams, 2012). Each arm of the immune system has its costs and benefits (reviewed in Lee, 2006; McDade et al., 2016), and life history traits are likely to influence which aspect of the immune system is favored. For example, high-ranking male baboons, who invest heavily in reproduction, seem to favor defense against infections (which involve a Th1 response) over defense against helminth parasites (which involve a Th2 response; Archie et al., 2012; Habig et al., 2019).

We had six specific research goals (see Table 1). First, we investigated whether a commercially available monkey IgA ELISA kit, designed to measure IgA in old world monkey serum or plasma, could be used to measure IgA in baboon feces. We validated the IgA assay by checking for parallelism, accuracy, and intra- and inter-assay coefficient of variation (CV). Testing for parallelism is important when commercial kits are used with fecal samples as these kits are designed for use with serum, and endogenous compounds in the fecal matrix may interfere with the binding of the IgA to the antibody (Tu & Bennett, 2017). Second, in a small set of samples, we assessed experimentally whether sample collection and storage conditions in the field affect IgA concentrations. Third, we tested whether long-term storage in a freezer (up to 22 months) altered the observed IgA concentrations. Fourth, because of the prohibitive costs of the commercial IgA, we also developed an in-house ELISA assay. We validated the custom ELISA by comparing the IgA concentrations obtained with that assay to the IgA concentrations obtained with the commercial ELISA. We then used the fecal IgA concentrations determined by the in-house ELISA for our last two goals. Fifth, we assessed whether fecal IgA concentrations reflected defense against gut parasites by evaluating the relationship between parasite burden and fecal IgA in wild baboons. Relationships between parasites and the immunoglobulins that resist them are complex. On the one hand, parasitism may stimulate IgA production, leading to a positive relationship between fecal IgA and parasite burden. On the other hand, if IgA is effective in reducing parasite burdens, we may expect a negative relationship between fecal IgA and parasites. While both relationships are possible, there seems to be more support for a negative relationship between IgA and parasite burden, especially in studies conducted in wild populations (Albery et al., 2019; Clerc, Devevey, Fenton, & Pedersen, 2018; Watt et al., 2016). Therefore, we predicted that baboons with high fecal IgA will have lower *Trichuris trichiura* (whipworm) egg count and lower parasite richness. We chose these measures of parasitism because in our population they are linked to reproductive costs in female baboons (Akinyi et al., 2019). Sixth and finally, we tested how IgA concentrations varied in wild baboons as a function of sex, age, and reproductive effort. While it is commonly hypothesized that males invest less in immune

TABLE 1 Research goals and samples used to attain each goal

	Research goal	Sample sizes
1	Validate the commercial IgA kit by assessing parallelism, accuracy, and intra- and inter-CV	Parallelism, accuracy, and intra-assay CV were assessed using a fecal pool from 20 samples from wild, Amboseli baboons. Inter-assay CV was assessed using three samples from captive baboons.
2	Test the effects of field storage conditions on IgA concentrations	Four fecal samples from four captive baboons, partitioned into six aliquots (Figure 1a)
3	Test the effects of long-term storage on IgA concentrations	19 of the 310 fecal samples collected from Amboseli baboons
4	Develop and validate an in-house IgA assay	206 of the 310 fecal samples from Amboseli baboons (i.e., those collected in 2013 and 2014 and assayed with both the commercial and in-house assay)
5	Test the relationship between IgA concentrations and *Trichuris trichiura* egg count in wild baboons.	31 samples of the 310 fecal samples from Amboseli baboons (i.e., those collected in 2013, 2014, and 2015 and assayed with the in-house assay) also had parasite data
6	Test how IgA concentrations vary in wild baboons as a function of sex, age, and reproductive effort	310 samples collected from 84 males and 84 females in Amboseli (i.e., those collected in 2013, 2014, and 2015 and assayed with the in-house assay)

Abbreviations: CV, coefficient of variation; IgA, immunoglobulin A.

function than females (Folstad & Karter, 1992; Nunn, Lindenfors, Pursall, & Rolff, 2009; Roberts et al., 2004; Rolff, 2002), this prediction has received mixed empirical support, with contradictory results depending on the measure of immune function (Prall & Muehlenbein, 2014). Most research on IgA finds no sex differences (Dion et al., 2004; Huang et al., 2014; Lantz et al., 2018; Watt et al., 2016; but see Pihl & Hau, 2003; Yin et al., 2015); therefore, we predicted that male and female baboons would have similar fecal IgA concentrations. We also predicted effects of age, as follows: the immune system, undeveloped at birth, progressively matures during development and into adulthood, before declining in old age (reviewed in Martin et al., 2006; Simon, Hollander, & McMichael, 2015; Weiskopf, Weinberger, & Grubeck-Loebenstein, 2009). This pattern has been shown for cell-mediated as well as humoral immunity and in particular for IgA (Evans, Goldsmith, & Norris, 2000; Lantz et al., 2018; Terao, 2009; Weber-Mzell et al., 2004; Yin et al., 2015), leading us to predict that younger and older baboons will have lower fecal IgA concentrations than adults. Finally, according to the hypothesis that in food-limited environments, reproductive effort leads to tradeoffs with immunity in both males and females (Lochmiller & Deerenberg, 2000; McDade et al., 2016), we expected to observe: (a) lower IgA concentrations in pregnant and lactating females compared to cycling females, with the lowest IgA concentrations in lactating females, and (b) lower IgA concentrations in males that spent more time in reproductive effort via mate-guarding.

2 | METHODS

2.1 | Subjects

The fecal samples collected for this study came from wild and captive baboons (Table 1). The wild baboons were individually identified members of the Amboseli baboon population in Kenya. Specifically, we collected 310 fecal samples from 84 male and 84 female baboons (1–6 samples per individual; mean ± standard deviation [*SD*]: 1.84 ± 1.12) that were between 7 months and 24.5 years of age (mean ± *SD*: 8.57 ± 4.95 years of age). Subjects and samples were chosen and collected opportunistically (e.g., samples were collected whenever an individual defecated while under close observation). For a subset of these samples (31 samples), we also collected a fecal sample for parasite analyses. This population consists of yellow baboons, *Papio cynocephalus*, that experience some admixture with neighboring populations of olive baboons, *Papio anubis* (Alberts & Altmann, 2001; Charpentier et al., 2012; Tung, Charpentier, Garfield, Altmann, & Alberts, 2008). Demographic, behavioral, and life-history data (births, emigration, immigration, and maturation events) on this population have been collected on a near-daily basis for over four decades (e.g., Alberts & Altmann, 2012; Gesquiere et al., 2007; see www.amboselibaboons.nd.edu for a complete bibliography and the Baboon Project Monitoring Guide, which outlines the data collection protocols).

In addition, to explore the effects of field storage conditions on IgA concentrations (e.g., lack of refrigeration), we performed experimental storage treatments (Goal 2) on four fecal samples collected from four captive olive baboons (*Papio anubis)* that were present at the Division of Laboratory Animal Resources, Duke University Medical Center (Durham, NC) at the time of the experiments. All four animals were sexually mature adult males.

All data collection procedures were noninvasive, adhered to the laws and guidelines of Kenya and were approved by the Animal Care and Use Committee at Princeton University (IACUC 1821), Duke University (IACUC A018-13-01, A028-12-02), and the University of Notre Dame (IACUC 16-10-3415). This study complied with the American Society of Primatologists Principles for the Ethical Treatment of Non-human Primates.

2.2 | Fecal collection and extraction

2.2.1 | Fecal collection and storage

To validate the commercial kit, develop and validate the in-house assay, and to measure natural variation in IgA (Goals 1, 3, and 4; Table 1), we collected fecal samples from wild baboons opportunistically, immediately after defecation, from known individuals in Amboseli. Samples were homogenized, and an aliquot of each sample was placed in an empty tube, stored in a cooler with ice packs, brought to our camp site (within 8 hr), and stored in camp at −20°C. Samples were then sent to University of Nairobi where they were freeze dried and stored at −20°C until shipment to the United States. In this study, we analyzed fecal IgA concentrations of 310 fecal samples from 168 baboons collected in May–June 2013, May–Jul 2014, and May–June 2015. The samples collected in years 2013 and 2014 (206 samples) were analyzed using the commercial kit, and all samples from year 2013 to 2015 (310 samples) were analyzed using the in-house ELISA. For a small subset of these samples (31 samples), we also collected an aliquot in a tube containing 10% of buffered formalin for parasite determination.

To explore whether field storage conditions affected measured IgA concentrations and to test inter-assay variation of the commercial kit (Goal 2 and part of Goal 1; Table 1), we collected one fecal sample from each of four captive baboons held at the Division of Laboratory Animal Resources, Duke University Medical Center. Samples were collected immediately following defecation, after which each sample was homogenized and divided into six aliquots: one was collected in 95% ethanol and five were collected in empty tubes; all six aliquots were frozen at −20°C. The aliquots were then shipped on dry ice to Princeton University and stored at −20°C until processed as indicated in Goal 2 below to simulate processing of field samples in Kenya (See Figure 1a). Processed samples were freeze dried and sifted to a fecal powder (Gesquiere et al., 2008; Khan, Altmann, Isani, & Yu, 2002), before being extracted and assayed.

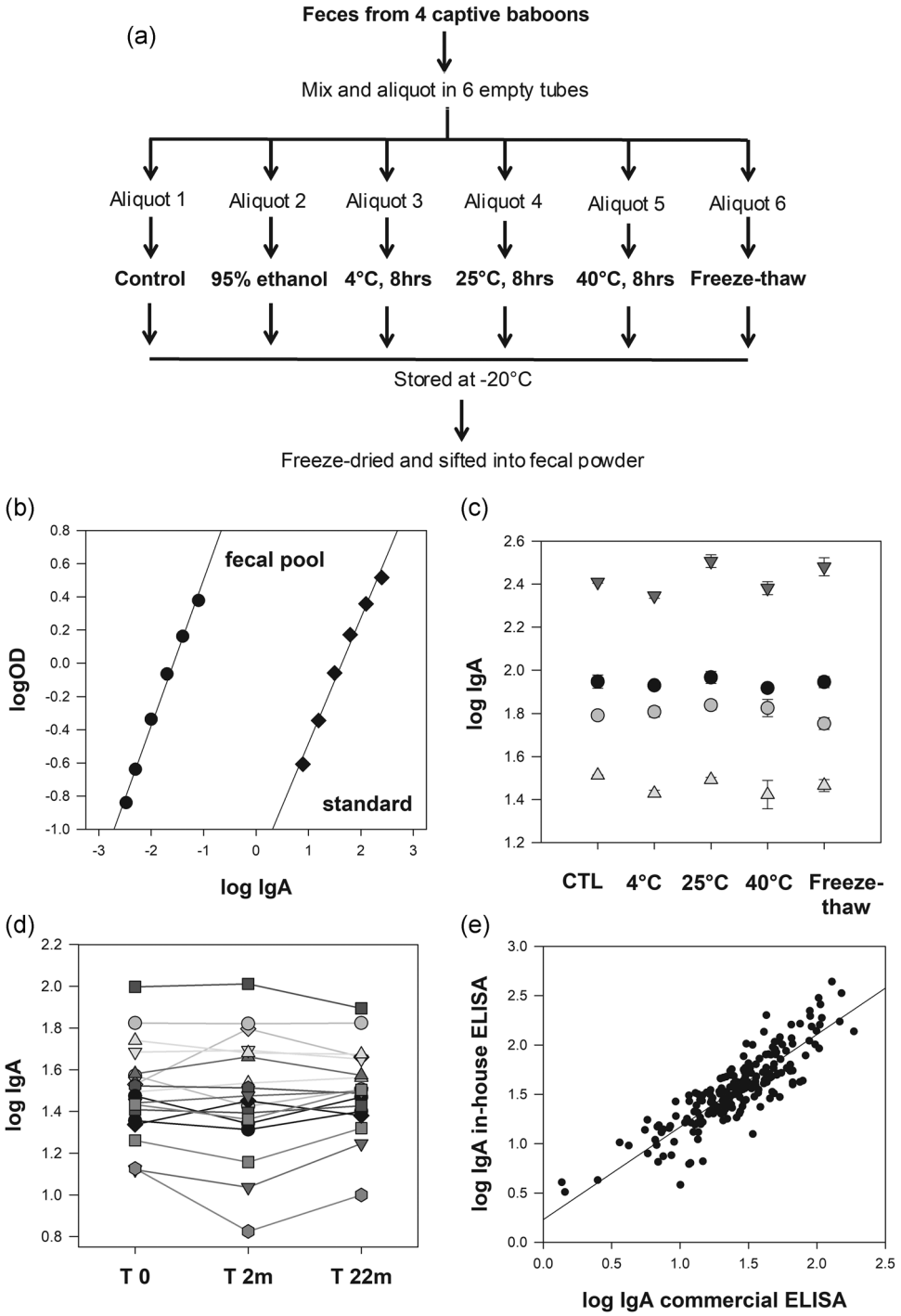

FIGURE 1 Results of the validation of the commercial and in-house ELISA. (a) Experimental design using feces collected from four captive baboons to test the effect on IgA concentrations of fecal collection and storage in the wild (described in the methods for Goal 2). (b) Comparison of a standard curve using the standards from the Monkey IgA kit with a serial dilution of a baboon fecal pool plotted on logit-log scale standards: $y = 0.81 \times -1.31$, $R^2 = 0.994$, fecal pool: $y = 0.88 \times + 1.39$, $R^2 = 0.993$. (c) Comparison of the IgA concentrations (on a logarithmic scale) of four fecal samples, aliquoted and treated under different conditions (see Figure 1a). (d) Comparison of IgA concentrations (on a logarithmic scale) of 19 wild baboon fecal samples right after extraction (at time 0), and after 2 months, and 22 months of storage at −20°C in the kit buffer. (e) Correlation between of IgA concentrations (on a logarithmic scale) determined with an in-house ELISA versus the values obtained with the commercial kit. IgA, immunoglobulin A

2.2.2 | IgA extraction

Freeze dried samples were first sifted through fine mesh (~40 mesh) into a fecal powder. 0.1 g of fecal powder (from captive or wild baboons) was then extracted into 2 ml of the IgA diluent from the Monkey IgA kit (Life Diagnostics, Inc., Cat# 5016-3) by mixing the samples on a multipulse vortexer for 30 min, and then centrifuging the samples for 20 min at 3,200 rpm. Supernatant from each sample

was collected in Eppendorf tubes and then further centrifuged for 30 min at 10,000 g. After centrifugation, supernatant was collected from each sample and stored at −20°C until it was assayed.

2.3 | Validation of a commercially available ELISA kit for fecal IgA determination and testing the effects of sampling conditions and storage

2.3.1 | Validation of the commercial assay: Parallelism, accuracy, precision (Goal 1)

To validate IgA concentrations using the Monkey IgA ELISA kit (Life Diagnostics, Inc., Cat# 5016-3), we made a fecal pool by mixing IgA extracts from 20 of the 310 wild baboon samples. The fecal pool had an IgA concentration of 31 μg/g feces. We then used this pool to determine parallelism, accuracy, and precision of the assay. Specifically, to test whether the fecal matrix interferes with IgA measurement, we assessed parallelism by running serial dilutions (12.5, 25, 50, 100, 200, and 300) of the fecal pool and testing whether its slope is parallel to that of the standard curve. We tested for assay accuracy (concentration observed/concentration expected × 100) by spiking the IgA standards with the fecal pool and running the spiked standards as samples. The intra-assay CV was evaluated by running the fecal pool 10 times through the assay. The inter-assay CV was assessed by running three of the fecal IgA extracts from captive baboons on seven of the assay plates (extracts from captive baboons had IgA concentrations of 71, 85, and 307 μg/g feces).

Samples were run at a 1:50 dilution. The ideal dilution was determined by running wild fecal samples at different dilutions, and by using the dilution that gave us an optical density in the middle of the range of the optical density obtained with the standards.

2.3.2 | Effect of fecal collection and storage on IgA concentration (Goal 2)

Fecal samples collected for IgA measurement are typically frozen at − 20°C immediately following collection, to avoid degradation by bacteria (Hau et al., 2001; Pihl & Hau, 2003). However, in field conditions, access to a freezer is usually not an option, and samples are commonly stored in a cooler or at ambient temperature for several hours (Khan et al., 2002; Lynch, Khan, Altmann, Njahira, & Rubenstein, 2003; Whitten, Brockman, & Stavisky, 1998). Hence, we explored how common collection and storage conditions in the field affected IgA concentrations using the aliquots collected from the four captive baboons (Figure 1a). *Aliquot 1*, stored at −20°C without ethanol, represented the optimal conditions of collection and storage in a lab setting and was used as "control." *Aliquot 2*, stored in 95% ethanol at −20°C, tested the effect of storage in ethanol 95% on IgA concentrations. *Aliquots 3*, *4*, and *5* were stored without ethanol for 8 hr at 4°C (*Aliquot 3*), at 25°C (*Aliquot 4*) or at 40°C (*Aliquot 5*) before being stored at −20°C. These treatments tested field conditions in which samples were kept in a cooler containing ice packs until return to the camp site (*Aliquot 3*), or when samples were kept at ambient temperature (which can sometimes reach 40°C) until return to the camp site (*Aliquots 4 and 5*). *Aliquot 6* was stored without ethanol and subjected to two freeze-thaw cycles before being stored at −20°C. This aliquot tested for the effect of repeated freeze-thaw on IgA concentrations (Figure 1a).

2.3.3 | Effects of long-term sample storage on fecal IgA concentrations (Goal 3)

To test the stability of fecal IgA concentrations through time when extracted samples were stored at − 20°C in the kit buffer, we re-assayed 19 samples after 2 months and 22 months of storage and we compared these concentrations to the fecal IgA concentrations obtained right after extraction (Time 0).

2.4 | Development of an in-house ELISA for fecal IgA determination (Goal 4)

While commercial kits are convenient, they are also expensive, and therefore, we developed an in-house ELISA using commercially available component reagents, for more cost-effective long-term measurement of IgA in baboon feces. In brief, microtiter plates (Nunc; Fisher Scientific) were coated with 50 μl/well of 4 μg/ml polyclonal monkey IgA (LSBio Lifespan Biosciences Inc.) dissolved in carbonate buffer (0.06 M, pH 9.6), and incubated overnight at 4°C. Plates were used within 24 hr of coating. When ready for use, wells were emptied and 100 μl of blocking buffer (Tris Buffered Saline (1×) with 0.5% Tween 20 (TBST 1×) + 2% powdered milk) was added to each well, and incubated for 2 hr at 37°C. Coated plates were washed with TBST (1×), and 100 μl of standard (Monkey Immunoglobulin A range 3.91–250 ng/ml from Life Diagnostics) or diluted sample (1:60 and 1:120) was added to each well. After 2 hr of incubation at 37°C, the plates were washed (TBST 1×), and 100 μl of the peroxidase labeled goat anti-monkey IgA (Monkey IgA HRP 0.2 μg/ml from KPL Inc.) was added to each well. After 2 hr of incubation at 37°C, the plates were washed and 100 μl of ABTS peroxide substrate was added to each well and incubated for 20 min at 37°C. The optical density was measured at 450 nm. All samples were run in duplicate. Any duplicates with a CV > 15% were rerun, but overall the average intra-CV across all samples, and for all data included in the analyses below, was low (<5%). The inter-plate CV was assessed by comparing, for each assay plate, the wild fecal pool (31 μg/g) run at three different concentrations: high (1:50 dilution of the pool), medium (1:100 dilution of the pool), and low (1:200 dilution of the pool).

The fecal IgA concentrations obtained with the in-house ELISA were compared to the concentrations obtained with the commercial Monkey IgA ELISA by reassaying the 206 fecal samples from wild baboons obtained in years 2013 and 2014.

2.5 | Assessement of fecal IgA as a measure of gut mucosal immunity (Goal 5)

Using 31 fecal samples from wild baboons for which we had both parasite data and IgA concentrations (obtained with the in-house ELISA), we tested the relationship IgA concentrations and two measures of parasitism linked to costs in female baboons: *Trichuris trichiura* egg counts and parasite richness (i.e., the count of distinct parasite species in the sample). *T. trichiura* is the most common parasite in our population, occurring in 92.5% of the female fecal samples, and in 96% of the male samples (Akinyi et al., 2019; Habig et al., 2019). Furthermore, previous research in our population has found that both *T. trichiura* egg counts and parasite richness are associated with reduced fertility (i.e., long interbirth intervals) in female baboons (Akinyi et al., 2019). *T. trichiura* burdens and parasite richness were estimated by counting parasite eggs in fecal samples via fecal flotation and sedimentation (Bowman, 2014; Gillespie, 2006). For each sample, two flotation slides were analyzed from a 4 g sample of fecal matter, and five sedimentation slides were analyzed from 2 g of fecal matter. For detailed protocols see Akinyi et al. (2019).

2.6 | Assessment of IgA variation in wild baboons (Goal 6)

We also assessed whether IgA concentrations varied by sex, age, and reproductive effort in wild baboons, using the IgA concentrations obtained with the in-house ELISA for the 310 fecal samples collected from Amboseli baboons between 2013 and 2015. Below we describe how each of these predictor variables is collected.

2.6.1 | Sex

In baboons, sex is obvious from external genital morphology.

2.6.2 | Age

For all females ($N = 84$), and for males born in study groups ($N = 64$ of 84 male subjects), age was based on known birth dates. For immigrant males ($N = 20$ male subjects), age was estimated based on coat condition, degree of scarring, body carriage and canine tooth condition when they first joined the study population (see Alberts & Altmann, 1995a, for details).

2.6.3 | Female life history and reproduction

The onset of puberty in females occurs at menarche, marked by the female's first sex-skin swelling in her first menstrual cycle. Females experience menarche at a median age of 4.5 years; at that time, they are still investing in their own growth (Altmann & Alberts, 2005; Onyango, Gesquiere, Altmann, & Alberts, 2013). We consider females to be adolescent from the onset of menarche until first conception. At this point, typically 1–1.5 years following menarche, we consider females to be adult (Charpentier, Tung, Altmann, & Alberts, 2008; Onyango et al., 2013). Female reproductive state (cycling, pregnant, or lactating) is assigned based on records of female sexual swelling state (turgescent or deturgescent) and size, the presence of menstrual blood, and the color of the paracallosal skin (Altmann, 1973; Gesquiere et al., 2007). Menstrual cycles in female baboons are easy to identify by the successive turgescence (follicular phase) and deturgescence (luteal phase) of the sexual skin. Failure to cycle after 40 days and the absence of menstrual blood usually indicates that the female is pregnant (Beehner, Nguyen, Wango, Alberts, & Altmann, 2006; Beehner, Onderdonk et al., 2006). Pregnancy is then confirmed by the change in color of the paracollosal skin from black to pink, approximately 2 months after conception (Altmann, 1973). The average gestation length for female baboons is 177 days (Altmann, 1980). After birth, the female remains in post-partum amenorrhea for an average of 1 year unless her infant dies, in which case the female usually resumes cycling about 3 weeks after the infant's death (Altmann, Altmann, & Hausfater, 1978; Gesquiere et al., 2018).

2.6.4 | Male life history and reproduction

The onset of puberty in males is defined as the first day of the first month during which observable scrotal rounding associated with testicular enlargement was recorded (Alberts & Altmann, 1995b). Males experience testicular enlargement at a median age of 5.41 years (Onyango et al., 2013). Males with enlarged testes produce viable sperm and are sexually mature (Bercovitch & Goy, 1990; Plant, 1994). However, they are not reproductively active for another 2 years after testicular enlargement. During this period, which we define as adolescence, males experience a growth spurt, at the end of which males are twice as large as adult females and can agonistically challenge adult males (Altmann & Alberts, 2005). Adulthood is defined by attaining a dominance rank among adult males, which occurs at a median age of 7.45 years in Amboseli (Alberts & Altmann, 1995b; Altmann & Alberts, 2005; see also Akinyi et al., 2017). After attaining an adult dominance rank, males begin to consort (i.e., mate guard) and copulate with females (Alberts & Altmann, 1995b). Male baboons also disperse from the natal group; in Amboseli, this may occur before or after they attain adult rank and begin their reproductive lives (Akinyi et al., 2017; Alberts & Altmann, 1995a, 1995b).

2.6.5 | Male consortships

We used sexual consortships to measure male reproductive effort. Sexual consortships are an easily observable form of mate guarding, during which an adult male maintains close spatial proximity to, follows, grooms, and mates with an estrous female while also keeping

other males away from the female (Alberts, Watts, & Altmann, 2003; Bercovitch, 1987b; Hausfater, 1975). Consortships are energetically costly for males because they reduce foraging and resting time (Alberts, Altmann, & Wilson, 1996; Rasmussen, 1985). As part of our regular monitoring of each study group, all occurrences of sexual consortships were recorded (Alberts & Altmann, 2012; Gesquiere et al., 2007; see Baboon Project Monitoring Guide at www.amboselibaboons.nd.edu). Each record includes the onset and termination of the consortship (or, for censored bouts, the start time and/or end time of the day's observations). For each adult male, we calculated the total time spent in consortship in the 1 month-period preceding sample collection, by adding the duration of all consortships for that male. We excluded samples for males that were present less than 28 days of the 31-day period.

2.7 | Statistical analysis

All our analyses were conducted using IBM SPSS version 25. Fecal IgA concentrations were logarithm-transformed in order for the model residuals to attain a normal distribution. Significance levels for all the tests were set at $p < .05$.

Parallelism between standard curves and serial dilutions of fecal pools was determined by a test of the equality of two slopes (Neter, Wasserman, & Kutner, 1990). To assess the effect of collection and storage in the field, as well as the impact of long-term storage of extracted samples on fecal IgA concentrations, we used General Linear Models (GLMs) with repeated measures (we note that our sample size for field storage conditions is very small, with just five treatments applied to four samples, so these results should be interpreted with caution). The equality in variance across the different repeated measures (i.e., sphericity) was verified by conducting a Mauchly's test of sphericity. In the case of short-term storage, sphericity was violated; therefore, we used the Greenhouse-Geisser correction. Fecal IgA concentrations obtained by the in-house versus commercial ELISA were compared using a paired *t* test as well as a Pearson correlation.

We tested whether *T. trichiura* egg counts and parasite richness in both sexes were associated with fecal IgA concentrations by running generalized linear mixed models with Poisson error distribution and log link function including fecal IgA concentration as a fixed effect and baboon identity as a random effect. Because previous work in our group has shown that *T. trichiura* egg count is strongly predicted by age, both in males and females (Akinyi et al., 2019; Habig et al., 2019), we also added age as a fixed effect in the *T. trichiura* egg count model.

Finally, we tested whether variation in fecal IgA concentrations was consistent with biologically expected values based on sex, age, and reproductive effort. Specifically, we ran three linear mixed models one that included both sexes and one each to examine the relationship between IgA and reproductive effort in males and females, respectively. Subject identity was included as a random effect in all models. To test the effects of sex and age on fecal IgA, we ran a model including the following fixed effects: subject sex and age, entered as a quadratic term ($n = 310$ samples from 168 individual baboons). We also added an interaction between sex and age as well as sex and the quadratic effect of age but neither interactions were significant and therefore, were removed. To test the relationship between female reproductive effort and fecal IgA concentrations, we restricted the data to sexually mature female subjects and included whether the female had reached adulthood, and her reproductive state (cycling, pregnant, or lactating) as fixed effects ($n = 92$ samples from 58 individual baboons: 39 samples for cycling females, 20 samples for pregnant females and 33 samples for lactating females). To test the relationship between male reproductive effort and fecal IgA concentrations, we restricted the data set to sexually mature males ($n = 113$ samples from 55 individual baboons). Fixed effects were whether the male had reached adulthood (ranked above an adult male) and how much time he spent in consortship. Note that a male's rank was not added to the model as all the adolescent males' ranks were necessarily lower than all adult males' ranks, and therefore, these two variables were highly correlated. To test if rank had more explanatory power than male maturation, we ran an alternative model that included male rank as a fixed effect, instead of whether or not the male had reached adulthood; this model had a higher Akaike's Information Criteria and therefore, was not retained.

3 | RESULTS

3.1 | Goal 1: IgA assay validation: Parallelism, accuracy, and precision

The slope of a serial dilution of a baboon fecal pool showed strong parallelism with the slope of the standard curve ($F = 1.632$, $p = .242$, $N = 11$; see Figure 1b), indicating that there was no matrix effect when measuring IgA from fecal extracts. The assay was highly accurate; when we spiked each standard with an aliquot of the baboon fecal pool, the accuracy was 98.0% ± 5.1 (mean ± SE; $N = 6$). The intra-assay CV was 6.25% ($N = 10$) for the fecal pool (IgA concentration = 31 μg/g feces). The inter-assay CVs for seven plates were 15.3%, 8.16%, and 12.60%, respectively, for three fecal extracts with IgA concentrations of 71, 85, and 307 μg/g feces.

3.2 | Goal 2: Field collection and storage

Ethanol is not a suitable preservative for IgA as fecal IgA concentrations were below detection levels when the samples were collected in 95% ethanol (Aliquot 2). In contrast, fecal samples stored without ethanol and frozen immediately after collection (Aliquot 1) had a mean IgA concentration of 110 μg/g feces (range 33–257 μg/g feces). When fecal samples are stored for 8 hr at 4°C (Aliquot 3), 25°C (Aliquot 4), or 40°C (Aliquot 5) or freeze-thaw repeatedly (Aliquot 6), it seems to have little effect on fecal IgA concentrations when compared to samples that were immediately frozen ($F_{1.66, 4.99} = 2.292$, $p = .196$; see Figure 1c). However, with a small sample size of $n = 4$, statistical analyses should be treated

with caution. Note that prior studies have also found that fecal IgA is quite stable to freeze-thaw cycles and storage at ambient temperature (Lantz et al., 2018; Peters et al., 2004).

3.3 | Goal 3: Long-term storage

Storage of the extracted samples at −20°C for 2 months and 22 months did not significantly affect fecal IgA concentrations ($F_{2, 34} = 0.976$, $p = .387$; Figure 1d).

3.4 | Goal 4: Comparison of IgA concentrations obtained with in-house or commercial ELISA

We found that fecal IgA concentrations determined with the in-house IgA ELISA were highly correlated to the IgA concentrations determined with the commercial kit (Pearson's correlation coefficient = 0.860, $p < .001$, $N = 207$; Figure 1e). However, not surprisingly, because the antibodies used were of different sources, the absolute fecal IgA concentrations given by the two methods varied (Paired *t* test: $T = -10.721$, $p < .001$, $N = 207$). The IgA concentrations given by the in-house ELISA were lower to those determined with the commercial kit. Note that all subsequent analyses (Goals 5 and 6) relied on the in-house ELISA.

3.5 | Goal 5: Assessment of fecal IgA as a measure of gut mucosal immunity

After correcting for baboon's age, we found a statistically significant association between *T. trichiura* burden and fecal IgA concentrations but no significant relationship between IgA and parasite richness. Specifically, baboons exhibited higher *T. trichiura* egg counts when they had lower fecal IgA concentration ($F_{1, 28} = 95.921$, $p < .001$; Table 2 and Figure 2b); for each 10 µg/g decrease in fecal IgA a corresponding 16.5% increase of the mean of *T. trichiura* egg counts was observed. As has been reported previously (Akinyi et al., 2019), *T. trichiura* egg counts were also higher in older baboons ($F_{1, 28} = 6.668$, $p = .015$; Table 2 and Figure 2a); for each 1 year increase in baboon's age, there was a corresponding 14.2% increase of the mean of *T. trichiura* egg counts. For parasite richness, there was no significant relationship with IgA ($F_{1, 29} = 1.599$, $p = .216$; Table 3). The relationship between age and parasite richness was not significant in this analysis, nor in two larger studies (Akinyi et al., 2019; Habig et al., 2019) and is not included the model of parasite richness.

3.6 | Goal 6: Variation in fecal IgA concentrations in wild baboons as a function of age, sex, and reproductive effort

In our population, the average fecal IgA concentrations was 60.5 ± 53 µg/g feces (mean ± *SD*) with a range between 1.4 and 438.5 µg/g feces and a CV of 87.6%. As predicted, fecal IgA concentrations did not vary across sexes ($F_{1, 148} = 0.035$, $p = .852$; Figure 3a, see also Table S1). Age entered as a squared term did not significantly predict fecal IgA (age^2: $F_{1, 172} = 1.955$, $p = .164$), but there was a trend for the linear term of age ($F_{1, 179} = 3.304$, $p = .071$; Figure 3b). We also added an interaction between sex and

TABLE 2 Model results for the association between *Trichuris trichiura* egg counts and IgA concentrations, correcting for age, in wild baboons

Variables	Estimate	SE	*t* value	*p*	Lower CI	Upper CI
Intercept	2.559	0.695	3.683	.001	1.136	3.982
Age	0.189	0.073	2.582	.015	0.039	0.338
IgA	−0.022	0.002	−9.794	<.001	−0.026	−0.017

Abbreviations: IgA, immunoglobulin A; SE, standard error.

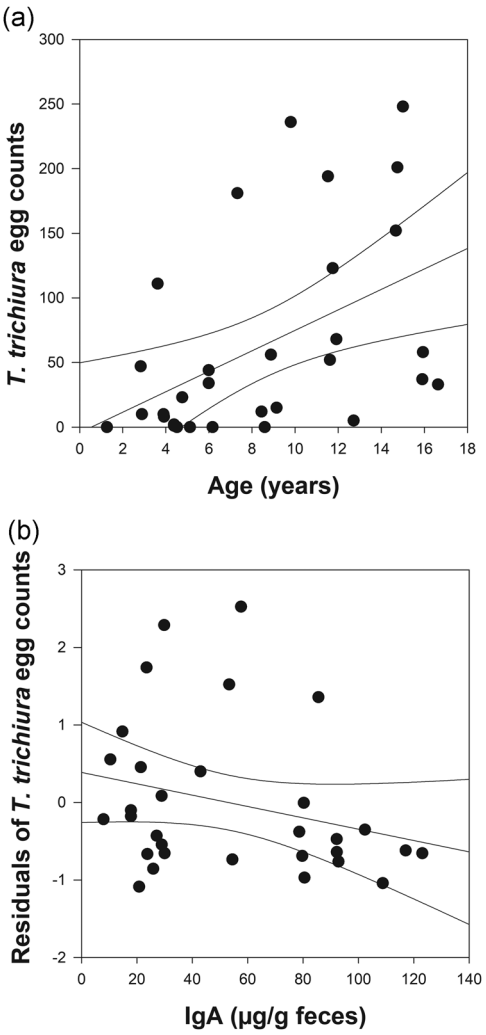

FIGURE 2 Predictors of *Trichuris trichiura* egg counts in wild baboons. (a) Plot showing variation in *T. trichiura* egg counts as a function of host age. (b) Plot showing variation of the residual values of *T. trichiura* egg counts (from GLMM correcting for age) in function of fecal IgA concentration. 95% CI are also included in the graphs. IgA, immunoglobulin A

TABLE 3 Model results for the association between parasite richness and IgA concentrations, in wild baboons

Variables	Estimate	SE	*t* value	*p*	Lower CI	Upper CI
Intercept	0.579	0.230	2.521	.017	0.109	1.049
IgA	0.004	0.003	1.265	.216	−0.003	0.011

Abbreviations: IgA, immunoglobulin A; SE, standard error.

age but the interaction was not significant, and therefore, was removed.

When considering only females that had reached menarche (i.e., adolescent and adult females), fecal IgA concentrations were not different between the two age categories ($F_{1, 48} = 0.158$, $p = .692$; Figure 3d), but female reproductive state did predict fecal IgA ($F_{2, 70} = 3.769$, $p = .028$; Figure 3c, see also Table S1). Specifically, fecal IgA concentrations were significantly lower in lactating females than in pregnant females ($p = .008$), while cycling females did not significantly differ from lactating or pregnant females (cycling vs. lactating: $p = .455$; cycling vs. pregnant: $p = .095$). The difference between lactating and pregnant females represented 16% of the mean fecal IgA.

Among sexually mature males, adults had higher fecal IgA concentrations than adolescents ($F_{1, 69} = 8.271$, $p = .005$; Figure 3d, see also Table S1), this difference represented 15% of the mean fecal IgA. However, we found no evidence that male reproductive effort, measured by time spent in consortships, predicted IgA concentrations ($F_{1, 104} = 0.768$, $p = .383$; see also Table S1).

4 | DISCUSSION

Methods to measure mucosal immunity noninvasively are valuable for understanding variation in animal immune responses and trade-offs between immunity and other life-history processes in wild populations. In this study, we validated the measurement of fecal IgA in baboons using a commercial kit and developed a custom IgA ELISA that provides data highly correlated to the commercial kit, but at a much lower cost. These lower costs make in-house assays better suited to field studies, which often analyze large numbers of samples.

Storage in 95% ethanol (a method commonly used to preserve steroid hormones) resulted in denatured IgA (as reported for other proteins, see Herskovits, Gadegbeku, & Jaillet, 1970; Schubert & Finn, 1981), but storage of the extract at −20°C for nearly 2 years did not significantly alter IgA concentrations. Other studies have similarly reported the stability of antibodies to very long-term storage at −20°C (Henderson, Ownby, Klebanoff, & Levine, 1998) as well as stability of fecal IgA to freeze-thaw cycles and storage at ambient temperature (Lantz et al., 2018; Peters et al., 2004). The heat-tolerance of fecal IgA makes it a great tool for field studies, because in remote field conditions, samples are often kept at ambient temperature for several hours, and sometimes for several days, before being frozen.

When we simultaneously measured IgA concentration and *T. trichiura* egg counts in wild baboon feces, we found that samples with high egg counts also had low fecal IgA concentrations. This pattern suggests that reduced IgA levels are linked to reduced mucosal immunity, and therefore, higher egg counts. Several other studies conducted in wild populations have similarly found a negative

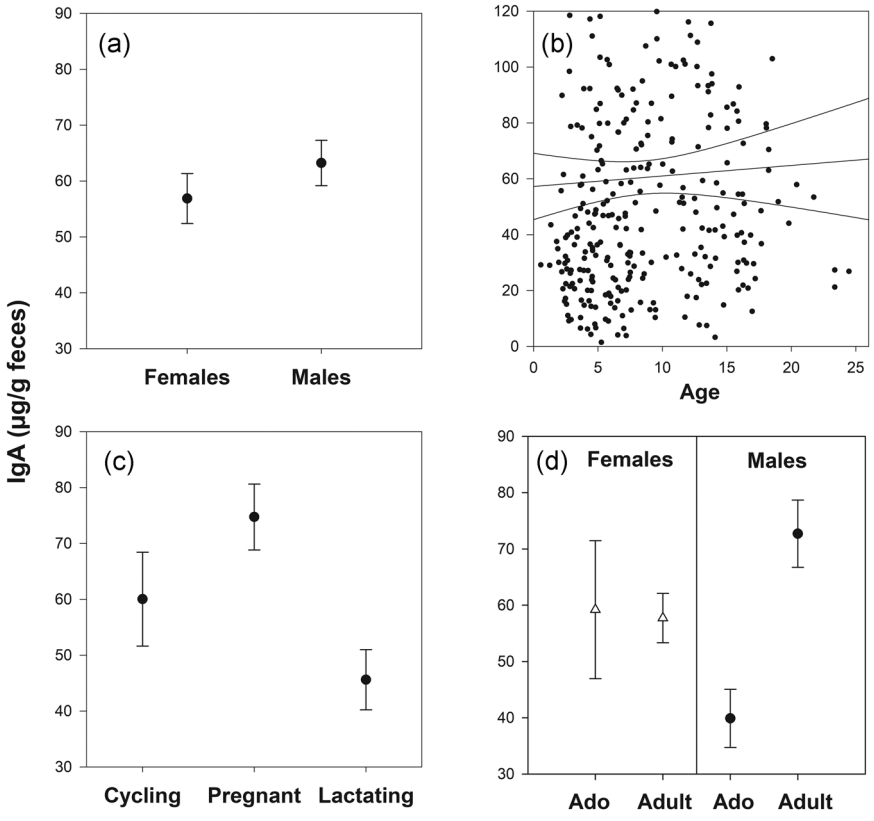

FIGURE 3 Predictors of IgA concentrations in wild baboons. (a) Mean (±SE) fecal IgA concentrations for males and females of all ages; (b) variation in fecal IgA concentrations with age categories, including both sexes; (c) mean (±SE) fecal IgA concentrations for sexually mature females in different reproductive states; and (d) mean (±SE) fecal IgA concentrations for adolescent females vs. adult females and for adolescent males vs. adult males. ado, adolescent; IgA, immunoglobulin A; SE, standard error

relationship between fecal IgA and parasite burden (Albery et al., 2019; Clerc et al., 2018; Watt et al., 2016). Our result suggests that fecal IgA can be used to estimate gut mucosal immunity or resistance against gut parasites in the wild.

Male and female IgA concentrations did not differ significantly from each other, indicating that our data, consistent with data from other studies of fecal IgA, failed to support the hypothesis that males invest less in immune function than females (Huang et al., 2014; Lantz et al., 2018; Pihl & Hau, 2003; Watt et al., 2016). Baboons in Amboseli are exposed to numerous gastrointestinal parasites (Akinyi et al., 2019; Habig et al., 2019), and it may be too costly for males to reduce their sIgA production because of its important role in defense. While male baboons seem to have a comparable gut mucosal immunity to females, their immune response in other mucosal tissues (i.e.,eye, nose, respiratory) or their systemic immune response may be different from females' response (Folstad & Karter, 1992; Grossman, 1985; Nunn et al., 2009; Roberts et al., 2004; Rolff, 2002). Specifically, the immune system is comprised of multiple arms that vary in their costs and benefits (Lee, 2006; Martin et al., 2006), and the immunosuppressive effect of testosterone may occur in some tissues or in some immune components, but not others (Braude, Tang-Martinez, & Taylor, 1999; Soma, 2006).

The effect of age on fecal IgA concentrations is more complex; while we found no evidence of a decrease in fecal IgA concentration in the oldest individuals, we found a nonsignificant trend for an increase in fecal IgA with age. When examining mature males and mature females separately, the effect of age became significant for males but not females. Specifically, adult males had higher fecal IgA concentrations than adolescent males. This sex difference may be linked to differences in the growth and development of male and female baboons (Altmann & Alberts, 2005). Upon reaching sexual maturity, adolescent male baboons produce viable sperm but remain reproductively inactive for another 2 years after testicular enlargement (Bercovitch & Goy, 1990; Plant, 1994). During that time, they go through a growth spurt, at the end of which they are twice as large as adult females (Altmann & Alberts, 2005). Adolescent female baboons do not experience a growth spurt and hence are investing in growth to a lesser extent than adolescent males (Altmann & Alberts, 2005). Therefore, the lower IgA concentration in adolescent males compared to adult males may be a consequence of their heavily investment in growth and development, to the detriment of their immune system (reviewed in Martin et al., 2006; Rauw, 2012). In contrast to other studies (reviewed in Martin et al., 2006; Simon et al., 2015; Weiskopf et al., 2009; see also Evans et al., 2000; Yin et al., 2015), we did not find evidence for immunosenescence in our population, but this may be due to our very small sample size ($n = 13$) for old individuals.

The lower fecal IgA concentrations in lactating females compared to pregnant females suggest a stage-dependent tradeoff between reproductive effort and gut mucosal immune function. During lactation, female baboons in Amboseli are more energetically constrained than they are during pregnancy; in addition to milk production, lactating females must carry their infants almost constantly during the first 2 months of life, and then intermittently until they are 8 months or older, by which time infants may weigh 15% of their mother's body mass (Altmann & Samuels, 1992). Evidence that lactation is highly costly in our population has been demonstrated by the significant loss of body fat in lactating females, and the delay in cycle resumption until females regained a positive energy balance (Bercovitch, 1987a; Gesquiere et al., 2018). Lactating female baboons also take longer to heal than pregnant or cycling females (Archie et al., 2012). This intriguing pattern of lower IgA concentrations in lactating females compared to pregnant females parallels experimental evidence that lactation has stronger negative impacts upon immune function than gestation in other mammalian species, including rats (Jones, Sakkas, Houdijk, Knox, & Kyriazakis, 2012) and sheep (Gonzalez-Garduno, Torres-Acosta, & Chay-Canul, 2014).

However, the possibility of a general tradeoff between reproduction and immunity in female baboons is obscured by the fact that cycling females were intermediate in fecal IgA concentrations between lactating and pregnant females, and were statistically indistinguishable from both. The lack of a significant difference could result from the relatively low power of this small data set. Indeed, we were unable to control for important factors, such as the phase of the cycle or the time into pregnancy or lactation, which could affect IgA concentrations. Another possibility is that if mucosal immunity has relatively low energetic costs, tradeoffs might only occur with the most energetically costly aspects of reproductive effort (during lactation but not pregnancy). Furthermore, pregnant females are known to alter their immunity from cell-mediated (Th1) to humoral (Th2) to reduce energetic costs and reduce inflammatory risks to the fetus (Baker, Schountz, & Wang, 2013; Downs, Boan, Lohuis, & Stewart, 2018; Shurin, Lu, Kalinski, Stewart-Akers, & Lotze, 1999; Wegmann, Lin, Guilbert, & Mosmann, 1993; see also review by Lee, 2006). The shift from Th1 to Th2 immunity may alleviate the inflammatory costs of Th1 on fertility, and therefore, promote fecundity (e.g., as suggested by Blackwell et al., 2015). Pregnancy may thus boost antibody titer, such that maternal immunosuppression only becomes apparent after delivery and the onset of lactation.

In conclusion, we have established that IgA can be measured in baboon feces, providing a valuable noninvasive measure of gut mucosal immunity. While fecal IgA is unlikely to reflect systemic immune responses or the mucosal immune response of another tissue (i.e., upper-respiratory tract, genital tract; Haneberg et al., 1994; Watt et al., 2016), it is an especially relevant measure of immunity for wild populations that are constantly exposed to gastrointestinal pathogens. Indeed, measures of antibodies are sometimes more predictive of individual health and fitness than measures of parasite burden. For example, in wild Soay sheep of St. Kilda, Scotland, the high repeatability of antibody titers (at the individual scale) and the normal distribution of antibody data (at the population scale) have combined to lend power to analyses of life history variables; indeed, nematode-specific antibodies have proven to be more sensitively associated with survival of the sheep than have nematode fecal egg counts (which have low individual-scale repeatability and negative binomial distributions at the population scale; Hayward et al., 2019). Further research is needed to determine whether fecal IgA is likewise a predictor of baboon fitness. Finally, in

the Amboseli baboon system, fecal IgA provides a nice complement to measures of wound healing that largely reflect the innate and cell-mediated arms of the immune system (see Archie et al., 2012, 2014). In contrast, IgA reflects the humoral component of the immune system. Together these two measures will lend insight into how ecological factors modulates tradeoffs between different arms of the immune system and other energetically costly processes in the wild.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

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