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Establishment and Validation of Fecal Secretory Immunoglobulin A Measurement for Intestinal Mucosal Health Assessment in Wild Lemurs

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ABSTRACT

The measurement of biomarkers in blood and excreta can enable immune status assessment and provide prognostic information on individual health outcomes. In this respect, the fecal measurement of secretory immunoglobulin A (sIgA), the primary mammalian antibody for mucosal defense, has recently received increased interest in a few anthropoid primates, but a fecal sIgA assay for use in strepsirrhine primates has not yet been reported. Here, we develop and analytically validate a cost-effective in-house sandwich enzyme immunoassay for the extraction and measurement of sIgA in feces of redfronted lemurs (*Eulemur rufifrons*). We also tested a simple method for sIgA extraction that can be used under remote field conditions and undertook experiments to assess the robustness of sIgA concentrations to variation in processing and storage conditions of fecal extracts. Our analytical validation revealed that the assay recognizes immunoreactive sIgA in redfronted lemur feces, that sIgA can be measured accurately with no potential interference from the fecal matrix, and that assay reagents and performance are highly stable over time. The field-friendly extraction procedure produced sIgA results strongly correlated with those generated by a standard laboratory extraction method. Short-term storage at room temperature resulted in a slight decline in sIgA concentrations, whereas freezing extracts at −20°C kept sIgA levels stable for at least 3 months. Longer-term storage of >5 months, however, led to a significant decline of sIgA concentrations. Multiple freeze-thaw cycles did not affect sIgA levels. This study, therefore, provides the basis for measuring fecal sIgA in lemurs and possibly other strepsirrhines. When samples are processed properly and stored frozen, and when sIgA analysis can be performed within 3 months upon sample collection, fecal sIgA measurements can become a valuable tool for monitoring aspects of immunity and health in both zoo-housed and wild-living lemurs.

1 | Introduction

Recently, there has been increasing interest in the field of ecoimmunology, aiming to explore the interactions between an organism's immunity and its environment (Demas and Nelson 2012). The value of ecoimmunology, in contrast to experimental immunological studies under controlled laboratory conditions, is to identify ecological conditions and environmental stressors that affect immune responses. This approach can also reveal adaptations and trade-offs exhibited by organisms in response to

Research Highlights

- We established and analytically validated a cost-effective, in-house ELISA for measuring fecal secretory immunoglobulin A (sIgA) in redfronted lemurs.
- This method ensures accurate results even under challenging field conditions, with stable sIgA concentrations up to 3 months under frozen conditions.
- SIgA as a non-invasive biomarker provides a valuable tool for monitoring aspects of immunity and health in zoo-housed and wild lemurs.

immunological challenges, and therefore, offers a comprehensive means towards assessing an animal's overall health and fitness (French, Demas, and Lopes 2023).

Animal populations face ongoing challenges from pathogens (i.e., viruses, bacteria, parasites), with mucosal tissues being the primary entering point (Brandtzaeg 2009). Therefore, monitoring mucosal immunity is crucial for assessing wildlife pathogen exposure, susceptibility to diseases and the energetic demands of the immune system. Evaluating immune status is best accomplished by the measurement of immune markers in blood (e.g., antibodies, inflammatory markers, cytokines, major histocompatibility complex genes; Blackwell and Garcia 2022). However, blood collection is usually not an option for many wildlife studies. In those cases, using non-invasive methods based on the analysis of immune markers from urine or fecal samples, is the more suitable option to assess immune activity in natural settings. However, compared to health markers in blood, only a few health indicators for excreta exist (Behringer and Deschner 2017; Higham et al. 2015). Nevertheless, various health markers, such as neopterin (e.g., Müller et al. 2017), haptoglobin (e.g., Higham et al. 2015), C-peptide (e.g., Emery Thompson and Knott 2008), thyroid hormones (Beeby, Baden, and Higham 2023), soluble urokinase plasminogen activator receptor (suPAR: e.g., Higham, Stahl-Hennig, and Heistermann 2020), C-reactive protein (CRP: e.g., Chuang et al. 2010), and immunoglobulins (immunoglobulin M [IgM], immunoglobulin G [IgG], immunoglobulin A [IgA]; Mohandas, Balan, and Mourya 2022) have been measured in urine and/or feces to monitor immune status. These markers provide insights into various health aspects, from metabolic rate to gastrointestinal function and infection status (see Behringer and Deschner 2017 for a review). While the assessment of those physiological markers can provide longitudinal data on immune status and response, they have been mainly used in pets, livestock and a few anthropoid primates (e.g., Shaw et al. 2012; Smets et al. 2010; Higham, Stahl-Hennig, and Heistermann 2015, 2020).

The assessment of intestinal IgA is one way to assess the immune status of an animal. IgA is the most abundant mammalian antibody—responsible for first line mucosal defense against the entry of enteric toxins—and constitutes a fundamental barrier against pathogenic organisms (De Sousa-Pereira and Woof 2019). While IgA facilitates mucus surface colonization by commensal microbiota (Johansson and Hansson 2016) and regulates immune homeostasis (Pietrzak et al. 2020), it also functions to prevent infections and modulates the gut microbiome (e.g., De La Chevrotière et al. 2012; Langford et al. 2002; León and Francino 2022), making it an essential, independent component of the immune system (Woof and Kerr 2006). Given that the majority of infections originates from mucosal surfaces, particularly those of the gastrointestinal tract (Bosch et al. 2002; Brandtzaeg 2009), assessing gut secretory antibody concentrations, such as those of secretory immunoglobulin A (sIgA), provides information about intestinal mucosal health (Pietrzak et al. 2020). In fact, sIgA is considered to signal intestinal function (Corthésy 2013), with low sIgA concentrations generally indicating impaired mucosal immunity (Campos-Rodríguez et al. 2013). The concentration of sIgA is also associated with stress responsiveness. In response to acute stressors, sIgA levels typically increase (Bosch et al. 2002) but decline during chronic stress (Gleeson and Cripps 2015; Viena et al. 2012), suggesting a link between sIgA production and the intensity and duration of stressors.

Intestinal sIgA can be measured in fecal samples and was successfully validated in sheep (Shaw et al. 2012), dogs (German, Hall, and Day 1998), Asian elephants (Edwards et al. 2019), lions (Serres-Corral et al. 2024), as well as in various non-human primates (chimpanzees: Lantz et al. 2016; baboons: Gesquiere et al. 2020; macaques: Behringer et al. 2021; Paramastri et al. 2007; snub-nosed monkeys: Huang et al. 2014), and humans (Kang et al. 2020; Scholtens et al. 2008). These studies reported co-variation of sIgA with hormonal or seasonal factors as well as with an animal's parasitic status. Specifically, IgA and fecal glucocorticoid metabolites were positively correlated in feces of Barbary macaques (Behringer et al. 2021), but negatively correlated in cynomolgus monkeys (Paramastri et al. 2007). In Sichuan golden monkeys, IgA levels were higher during summer (Huang et al. 2014), while in African equids IgA was positively correlated with nematode fecal egg count during the wet season (Tombak et al. 2020). Conversely, IgA and parasite egg count, but not parasite richness, was negatively correlated in wild baboons (Gesquiere et al. 2020). This variation indicates that fecal sIgA is overall a biologically meaningful non-invasive marker of intestinal mucosal immunity.

Lemuriformes, the endemic primates of Madagascar, inhabit diverse ecosystems across the island that are all subject to pronounced seasonality (Dewar and Richard 2007), indicating varying pathogen exposure levels (Benavides et al. 2012; Bethge et al. 2022; Clough, Heistermann, and Kappeler 2010; Gillespie et al. 2010; Springer and Kappeler 2016). Physiological health was evaluated in several lemur species via analysis of blood samples (Junge et al. 2017; Junge and Louis 2005; Singleton et al. 2018), and various non-invasive methods, including the evaluation of microbiome composition (e.g., Bornbusch et al. 2022; McManus et al. 2021; Murillo et al. 2022), stress response (e.g., Balestri et al. 2014; Malalaharivony et al. 2021; Murillo et al. 2022), and energetic condition (Beeby, Baden, and Higham 2023). Moreover, attempts were made to establish markers reflecting immune status, e.g., to study CRP in feces (Defolie 2022) and to quantify IgA in blood (Damian and Greene 1972), but both studies were of limited success with low or no detection rates, resulting in fewer methods established to non-invasively assess lemur immune system activation. Given the presence of

IgA across all mammalian subgroups (De Sousa-Pereira and Woof 2019) and the homology of immunoglobulin sequences among lemurs, chimpanzees, and humans (Larsen, Campbell, and Yoder 2014), one can expect that sIgA is also part of the mucosal immunity of lemurs. However, to the best of our knowledge, there is no study that has successfully measured sIgA in any species of lemurs, and to date, no validated assay for determining sIgA concentrations in any biological samples of lemurs has been described.

Most commercially available enzyme-linked immunosorbent assay (ELISA) kits for sIgA analyses are designed to measure sIgA in humans, laboratory rodents, domestic animals, or monkeys. These mostly species-specific assays may be unlikely to effectively cross-react with lemur sIgA due to potential structural variation in the sIgA molecule across species (Larsen, Campbell, and Yoder 2014; see also Haley 2003). In an initial pilot experiment, we confirmed that a monkey IgA ELISA (Life Diagnostics, Inc., West Chester, USA; Cat. No. IgA-3) which was successfully used to measure fecal sIgA in macaques (Behringer et al. 2021) and baboons (Gesquiere et al. 2020) does not detect immunoreactive sIgA in lemur feces. Therefore, we hypothesized that for the non-invasive assessment of sIgA in lemurs, the use of a non-specific sIgA assay would be more promising. In this respect, the literature reports a self-made IgA assay based on two commercial rabbit-polyclonal anti-human IgA antibodies with low specificity in binding human IgA analogues from different mammalian species, such as cow, swine, mink, mice, and elephants (Edwards et al. 2019; Hau et al. 1990). Building on the reagents and methodology reported, in this study we established and analytically validated an enzyme immunoassay (EIA) for the measurement of immunoreactive sIgA in the feces of redfronted lemurs (*Eulemur rufifrons*).

With the ultimate goal of utilizing fecal sIgA measurements in studies of intestinal mucosal immunity in wild lemurs, we additionally investigated the potential utility of fecal sIgA for field studies. Our focus included testing the validity of a field-friendly extraction method that does not require sophisticated laboratory equipment or electricity, making it applicable under remote field conditions (Nugraha et al. 2017; Rimbach et al. 2013). Additionally, we evaluated factors that may affect sIgA concentrations in field- and transport situations, given that several studies have demonstrated impaired stability of sIgA in biological samples related to storage condition and storage duration (Ng et al. 2003; Presser, Simuyandi, and Brown 2014; Ramírez-Santana et al. 2012; but see Gesquiere et al. 2020). Specifically, we tested the impact of short-term storage of fecal extracts at ambient temperature for several days on the integrity of sIgA, to simulate situations where samples cannot be frozen immediately after extraction or where maintaining frozen conditions during transport is challenging. We further explored the effects of long-term storage, i.e., long-term frozen storage before analysis (common with field-collected samples), and the effect of multiple thawing and refreezing cycles on sIgA stability. The latter scenario simulates potential occurrences during laboratory analyses, power outage in the field, or delays in transport when frozen samples cannot be promptly shipped with sufficient cooling. We further examined whether storage time and the state of the sample before extraction (fresh or frozen) affects fecal sIgA concentrations in fecal samples of wild redfronted lemurs.

2 | Materials and Methods

2.1 | Animals and Sample Collection

To establish the assay and test whether it would detect sIgA in lemur feces, and to evaluate variation of sIgA levels under simulated field and various storage conditions (see below), we used fecal samples collected from two female redfronted lemurs housed at Magdeburg Zoo, Germany. Daily samples ($N = 8$ per animal) were collected immediately after the animals were observed defecating. Samples were placed in a polypropylene tube and stored frozen at −20°C immediately following collection. Samples were transported to the German Primate Center (DPZ) Endocrinology Laboratory, where they were extracted and processed (see Sections 2.2 and 2.3) within 3 days upon arrival to keep the time between collection and processing to a minimum.

To further assess whether and how fecal sIgA concentrations in wild redfronted lemurs vary as a function of storage time and the state of the sample before extraction (fresh or frozen), fecal samples were collected from a population of redfronted lemurs in Kirindy Forest, western Madagascar. Samples were collected between April 2022 and November 2022 from 36 individuals (22 males; 14 females) from the forest floor immediately after defecation. All samples were collected in the morning between 7 a.m. and 11 a.m. Samples were homogenized, undigested material was removed, and each sample was either placed in a polypropylene tube and directly stored frozen at the campsite, or a fresh aliquot was extracted shortly after collection. In total, we collected 422 samples (1–18 samples per individual).

2.2 | Study 1: Validation of sIgA Measurements in Fecal Extracts of Redfronted Lemurs

2.2.1 | IgA Immunoassay

For the quantification of immunoreactive sIgA in redfronted lemur fecal samples, we established an in-house EIA based on commercially available components following Edwards et al. (2019). In brief, the sandwich EIA used two rabbit polyclonal anti-human IgA antibodies, (i) a coating antibody (code #A0262, Agilent Dako, Santa Clara, USA) and (ii) a detection antibody conjugated with horseradish peroxidase (HRP; code #P0216, Agilent Dako, Santa Clara, USA). The antibodies have been reported to show both cross-reactivity and low specificity in binding human IgA analogues from different mammalian species (Edwards et al. 2019; Hau et al. 1990), and therefore, were considered promising for the detection of fecal sIgA in our study species.

For coating the EIA plate, the coating antibody was diluted to a working concentration of 0.5 mg/L in phosphate-buffered saline (PBS) (0.01 M phosphate buffer, 0.15 M NaCl, pH 7.2) and 100 µL was added to each well of the microtiter plate, which

was then incubated overnight (18–24 h) at 4°C. After incubation, the plate was washed three times with PBS containing Tween 20 (0.1%; PBS-T), before IgA standards (0.156–100 ng/mL) and high- and low-value quality controls, all prepared from human colostrum (Cat. No. I2636, Sigma Aldrich, St. Louis, USA), as well as diluted fecal extracts were added as 50 µL duplicates to the plate. The plate was subsequently incubated on a plate shaker at room temperature (RT) for 2 h in the dark. After incubation, the plate was washed three times with PBS-T, 100 µL of HRP-conjugated IgA detection antibody (diluted to a concentration of 0.325 mg/L in PBS-T) was added to each well, and the plate was incubated for 1 h under the same conditions as described above. After a final three times wash, 100 µL of TMB substrate solution was added to the wells and the plate was incubated for a further 5–7 min on a plate shaker. Finally, the color reaction was terminated by adding 50 µL of 2 M H_2SO_4 solution to each well and the absorbance was measured at 450 nm using a microtiter plate spectrophotometer.

2.2.2 | Analytical Validation of sIgA Measurements

To assess the analytical validity of our IgA assay and to test for potential matrix interference in the measurement of sIgA in redfronted lemur fecal extracts, we performed (1) a parallelism- and (2) an accuracy test (e.g., Gesquiere et al. 2020).

(1) We tested parallelism on (i) individual samples from the two zoo-housed females (one sample from each female), and (ii) separate male and female fecal extract pools, which were generated by combining sample extracts from five wild males (male pool) and five wild females (female pool). As sIgA concentrations in wild animal samples turned out to be generally low (see Section 3), we spiked the wild fecal pool samples with IgA standard (100 ng/mL) to increase the initial concentrations, and thus, ensure a sufficient number of dilutions falling within the range of the standard curve (cf. Gesquiere et al. 2018; Schaebs et al. 2019). To test for parallelism, samples were serially diluted twofold in PBS-T and sample dilution curves and the IgA standard curve were tested for differences between slopes (Cohen et al. 2002).

(2) Assay accuracy was assessed by spiking IgA standard curve preparations (3.125–100 ng/mL) with a mixed-sex fecal extract pool prepared from wild redfronted lemur samples, running the spiked standards as samples and determining the recovery of the added amounts.

2.2.3 | Comparing a Lab and a Field Friendly Extraction Method

For extraction, samples were thawed, homogenized and 0.30–0.35 g were weighed into a polypropylene tube. We compared two extraction methods with samples ($N = 14$) from the two zoo-housed females. The samples used here have been stored frozen (−20°C) for about 4 weeks before conducting the experiment. Samples were extracted using (1) a standard laboratory procedure ("laboratory extraction") and (2) a field-friendly extraction method that we tested for its suitability to extract samples under field conditions ("field extraction"; see also Rimbach et al. 2013).

For the (1) laboratory extraction, samples were combined with 2 mL PBS-T (0.01 M phosphate buffer, 0.5 M NaCl, 0.1% Tween 20, pH 7.2) and thoroughly vortexed on a multi-tube vortexer for 15 min before centrifugation at 3000 rpm for 10 min. The supernatants were decanted into 2 mL polypropylene tubes.

For the (2) field extraction method, also 2 mL PBS were added and the fecal PBS-T suspension was shaken horizontally by hand for 2 min, followed by centrifugation for 2 min at a lower speed (500 rpm) to simulate field centrifugation using a manually operated centrifuge (cf. Nugraha et al. 2017; Rimbach et al. 2013). The supernatants were then decanted into polypropylene tubes for storage until sIgA analyses. Before analyses, all fecal extracts used for the field extraction method were centrifuged at 6000 rpm to pellet any remaining particles.

2.2.4 | Data Analyses

We used a Wilcoxon signed-rank test to compare sIgA concentrations of matched samples extracted by the laboratory and field extraction methods and calculated the Spearman rank correlation coefficient to further assess the agreement between the two extraction methods. Differences between the slopes of the sample dilution curves and the IgA standard curve were tested using the significance of the difference between two slopes calculator software (Soper 2024). Assay accuracy was determined by calculating the recovery of added IgA in spiked samples as "observed concentration/expected concentration × 100" and comparing the agreement between added and recovered amounts using the Spearman rank correlation test. All statistics were performed using SigmaStat (version 12.3; Systat Software, Inc., 2013). Two-tailed exact probability tests were performed at a significance level of $p = 0.05$.

2.3 | Study 2: Testing the Stability of sIgA in Fecal Extracts

2.3.1 | Short-Term Storage of Extracted Samples at Ambient Temperature

We tested the susceptibility of fecal sIgA in extracts to degradation by storing fecal extracts at RT for up to 10 days before freezing (to simulate a situation where samples cannot be frozen within a short time window after extraction, or where it may be difficult to keep samples frozen during transport to the laboratory). Specifically, 10 fecal extracts (from five samples each from the two zoo-housed females) were divided into four aliquots of 150 µL each. While one set of aliquots was immediately frozen at −20°C (control samples), all other aliquots were stored in the dark at ambient temperature (21°C–23°C) for periods of 3, 6 and 10 days

before being stored frozen until analysis together with the matched controls.

2.3.2 | Long-Term Freezing of Extracted Samples

To investigate the stability of sIgA levels after long-term storage of fecal extracts at sub-zero temperatures (a situation commonly encountered with field samples before they can be transported to the laboratory for analysis), we prepared seven aliquots of 150 μL each from 10 fecal extracts and kept them frozen for periods of 2 days (control samples) and 1, 2, 3, 6, 9, and 12 months (test samples) before analyzing their sIgA concentrations. Since some previous studies have extracted IgA from feces and other biological samples using a buffer containing a protease inhibitor to minimize possible proteolytic degradation of sIgA (Peters et al. 2004; Tress et al. 2006), we performed the same experiment in parallel using the same fecal samples but extracted with PBS-T to which a protease inhibitor cocktail (cOmplete Mini, Roche Diagnostics, Germany; 1 tablet/10 mL PBS-T) was added. The frozen extracts were analyzed for sIgA concentrations after their respective storage durations, and the values at the different storage times were compared with the matched control values.

2.3.3 | Repeated Freeze-Thaw Cycles

To assess the effect of repeated freeze-thaw cycles (to simulate power outages or unstable power supply to a freezer, or thawing during transport for analysis) on fecal sIgA concentrations, we aliquoted 10 fecal extracts into 250 μL each. SIgA concentrations were measured directly from these samples to serve as controls. The samples were then frozen at −20°C. After 2 days of frozen storage, samples were thawed, sIgA concentrations were measured again (1st freeze-thaw cycle), and samples were again stored frozen for 2 days, after which they were thawed again for sIgA analysis (2nd freeze-thaw cycle). The procedure was repeated three more times to provide samples that had been thawed and refrozen three, four and five times, respectively.

2.3.4 | Data Analyses

Non-parametric statistics were used throughout, due to deviations from normality. The effects of the various experiments that produced repeated measures of sIgA levels over time (i.e., short-term storage at RT; long-term storage at −20°C; multiple freeze-thaw cycles) were tested using a Friedman test. We performed post-hoc paired exact Wilcoxon signed-rank tests to determine the period after which effects on values over time first became significant (cf. Higham, Stahl-Hennig, and Heistermann 2020). For all treatments, we performed a Spearman rank correlation test to determine whether treated and control values were correlated regardless of whether or not the treatment resulted in a significant change in fecal sIgA concentrations. All statistics were performed using SigmaStat (version 12.3; Systat Software, Inc., 2013) with two-tailed exact probability tests at a significance level of $p = 0.05$.

2.4 | Study 3: SIgA Measurements in Wild Redfronted Lemurs

2.4.1 | Extraction From Fresh and Frozen Samples

Fecal samples collected from known individuals of wild animals were used for analytical validation in the field. Specifically, we tested two aspects of sIgA stability under field conditions. First, we tested for an effect of long-term freezing of sample extracts at the campsite on sIgA levels. Specifically, we examined the overall probability to detect sIgA in long-term stored samples and whether the time between sample collection and sample analysis affected sIgA concentrations. Second, we assessed whether sIgA concentrations differ between samples extracted fresh on the day of their collection compared to those stored frozen at −20°C for 2 days before extraction. For this, aliquots of 19 homogenized cross-sectionally collected samples (11 males; 5 females) were used. All samples were extracted on site at the field laboratory using the field-friendly extraction method described under Section 2.2 above with the following modifications: instead of manual shaking, samples were placed on a vortexer for 2 min and then centrifuged for another 2 min using a manually operated centrifuge. Approximately 1.5 mL of the resulting supernatants were decanted into labeled polypropylene tubes, which were then stored frozen at −20°C until transport to the DPZ endocrinology laboratory for sIgA analyses. Samples had been stored frozen before analysis for 131–358 days.

2.4.2 | Data Analyses

To examine whether the probability of measurable sIgA concentrations is associated with storage duration, i.e., sIgA being "NA" (not available), we fitted a logistic regression model (glm; Baayen 2008). In this model, the dependent, binary variable indicates whether sIgA concentration (in ng/g feces) from freshly extracted samples is NA (1 if NA, 0 otherwise), and the independent variable being the storage duration, calculated from the months between sample collection and analysis. To further test if sIgA concentrations vary over storage time, we excluded all rows with missing sIgA concentrations and fitted a linear regression model (lm; Bates et al. 2015) where the dependent variable is the (log-transformed) sIgA fresh weight (ng/g) concentration and the independent variable is the storage duration. We used a Wilcoxon signed-rank test to compare sIgA concentrations measured in extracts generated from fresh and frozen samples. All analyses were performed in R (version 4.3.0; R Core Team 2020), using the package "dplyr" (version 1.1.2; Wickham et al. 2019).

3 | Results

3.1 | Study 1: Analytical Validation of sIgA Measurements

Displacement curves of the serially diluted fecal extracts of the samples from the two zoo-housed females ran parallel to the IgA standard curve, with no differences in slopes between

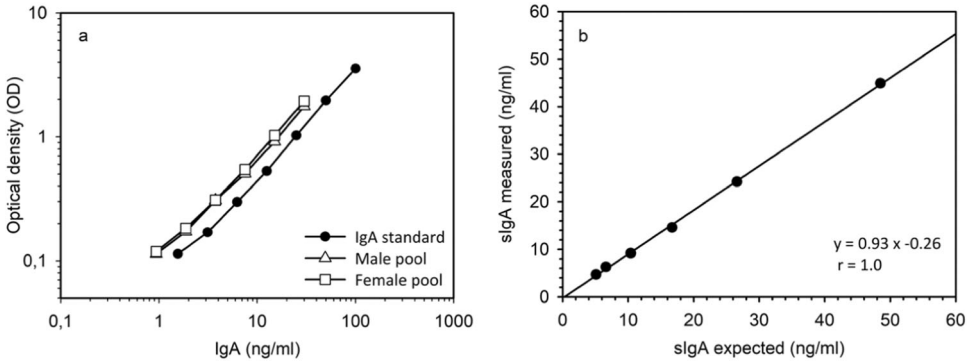

FIGURE 1 | Parallelism and accuracy tests. (a) Parallel displacement curves of serial dilutions of spiked fecal extract pools generated from samples of wild redfronted lemurs. (b) Accuracy of sIgA measurements. The figure shows the regression between expected and measured sIgA concentrations (see text for details).

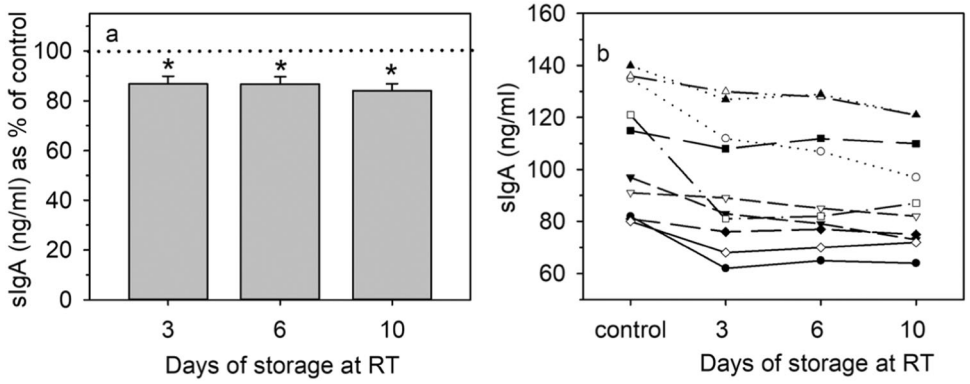

FIGURE 2 | Effect of room temperature storage for up to 10 days (d) on fecal sIgA concentrations. Data are shown as (a) percentage (mean ± SEM) of control values (= 100%; dotted line) and (b) absolute concentrations. The asterisks indicate concentrations that differ significantly from those of controls (see Section 3).

dilution curves and standard curve (sample 1: $t = 1.564$, $p = 0.193$; sample 2: $t = 1.675$, $p = 0.169$). Similarly, dilution curves of spiked pooled fecal extracts from both wild males and wild females ran parallel to the sIgA standard curve (male pool: $t = 1.545$, $p = 0.157$; female pool: $t = 0.94$, $p = 0.372$; Figure 1a).

SIgA measurements were also highly accurate, with a mean (±SD) recovery of IgA standards added to a mixed-sex fecal pool of 91.2% ± 2.9% and a correlation coefficient between added and recovered amounts of $r_s = 1.0$ ($p = 0.003$, $N = 6$; Figure 1b). The lower limit of quantification for this assay was 1.56 ng/mL. Assay variability was generally low, with intra-assay coefficient of variation (CV), calculated as the average value from the individual CVs for all of the sample duplicates, being <5% and inter-assay CVs, determined from replicate measurements of both high- and low-value quality controls, being <10% for all separate experiments evaluating sIgA stability as well as for the measurements of samples from the wild animals.

3.1.1 | Comparing a Lab and a Field-Friendly Extraction Method

SIgA concentrations measured from fecal samples processed applying the field-friendly extraction method correlated strongly with concentrations measured from samples that were extracted using an established laboratory procedure ($r_s = 0.91$, $p < 0.0001$, $N = 14$; Figure S1a). Moreover, absolute sIgA levels did not differ between the two extraction methods (laboratory extraction: 847.5 ± 249.9 [mean ± SD]; field extraction: 793.4 ± 334.4 [mean ± SD]; $W = -45$, $p = 0.1726$; Figure S1b).

3.2 | Study 2: Testing the Stability of sIgA in Fecal Extracts

3.2.1 | Short-Term Storage of Extracted Samples at Ambient Temperature

Storing fecal extracts at RT for 3–10 days resulted in a significant decrease in sIgA concentrations compared to control samples stored frozen immediately after extraction (χ^2: 19.92, $df = 3$, $p = 0.0002$). However, the decline was relatively small, with values ranging between 84.1 ± 2.8 (mean ± SEM; day 10) and 86.8% ± 3.1% (day 3) of controls (Figure 2a). Post hoc analyses showed that the decline was already significant on day 3 of storage ($W = -55$, $p = 0.002$), but also revealed that levels remained constant thereafter, i.e., sIgA levels on days 6 and 10 of storage did not differ significantly from those at day 3 (both $p > 0.3$). Despite the overall decrease in sIgA concentrations due to storage at RT, sIgA levels on each day of storage correlated strongly to those of control values (day 3: $r_s = 0.87$; day 6:

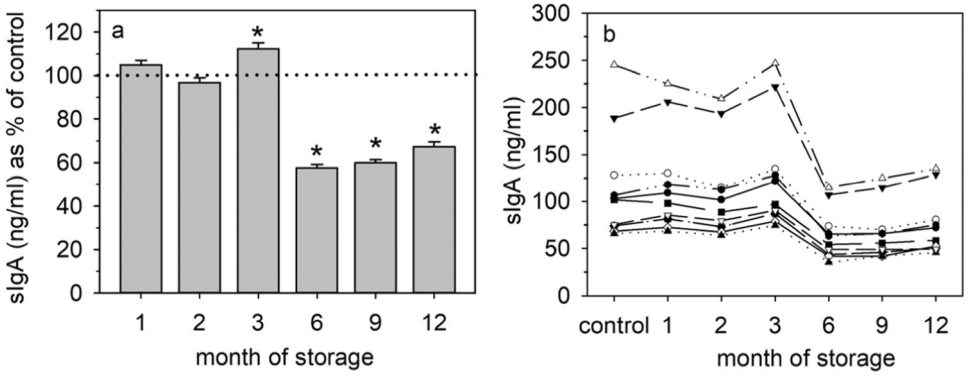

FIGURE 3 | Effect of long-term freezing on fecal sIgA concentrations. Data are shown as (a) percentage (mean ± SEM) of control values (= 100%; dotted line) and (b) absolute concentrations. The asterisks indicate concentrations that differ significantly from those of controls (see Section 3).

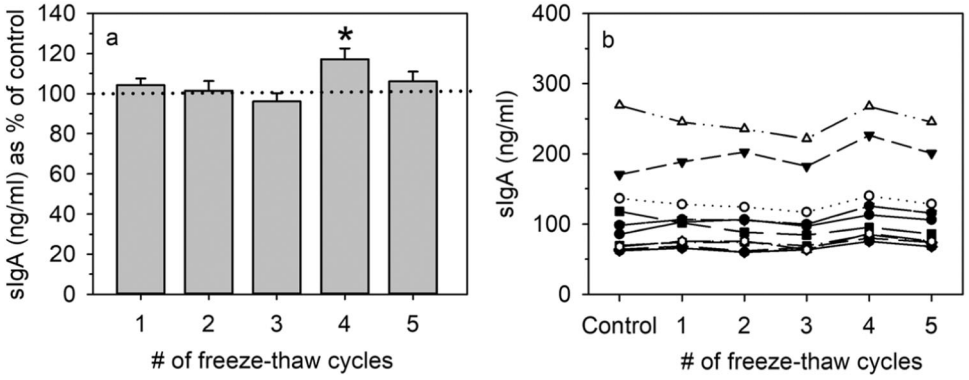

FIGURE 4 | Effect of multiple freeze-thaw cycles on fecal sIgA concentrations. Data are shown as (a) percentage (mean ± SEM) of control values (= 100%; dotted line) and (b) absolute concentrations. The asterisk indicates concentrations that differ significantly from those of controls (see Section 3).

$r_s = 0.88$; day 10: $r_s = 0.88$, all $p < 0.0001$, $N = 10$). However, the rank order of the different individual samples partly changed (Figure 2b).

3.2.2 | Long-Term Freezing of Extracted Samples

Storing fecal extracts for periods up to 12 months significantly altered sIgA concentrations over time (χ^2: 56.3871, $p = 0.0001$; Figure 3a). While sIgA levels remained consistent in the first 3 months of storage, with recorded changes in mean values across months being small (i.e., <13%; mean CV: 7.4%) and within the range of inter-assay variation, a marked and significant decrease ($W = -55$, $p = 0.002$, $N = 10$) in sIgA levels of on average > 40% was recorded between storage month 3 and 6. Given that inter-assay CV-values across the experiment were low (6.4% for QC high and 5.9% for QC low), the changes in fecal IgA concentrations over time were unrelated to variability in assay performance. SIgA levels remained at these lower values at storage months 9 and 12 ($W = -55$, $p = 0.002$, $N = 10$ for both time points), with no further decrease recorded (Figure 3a).

Because the pattern and magnitude in sIgA level changes recorded as a function of storage duration was highly consistent across test samples, the rank order of samples remained unaffected at every month of storage (Figure 3b), and sIgA concentrations correlated strongly with those of controls at each storage month ($r_s = 0.98$–0.99, all $p < 0.0001$, $N = 10$). Adding a protease inhibitor cocktail to the extraction buffer (see Section 2.3) did not prevent the decrease observed in sIgA concentrations between 3 and 6 months of storage, i.e., the pattern and magnitude of changes in sIgA levels as a function of storage duration were almost identical to those described above (χ^2: 57.4347, $p = 0.0001$; Figure S2a,b).

3.2.3 | Repeated Freeze-Thaw Cycles

There was an effect on sIgA levels when fecal extracts were subjected to multiple freeze-thaw cycles; mean sIgA concentrations ranged between 96.1% and 117.2% of those of controls across treatments (Figure 4a). These small changes were significant when all five freeze-thaw cycles were considered (χ^2: 25.6322, $p < 0.0001$). However, post hoc analyses revealed that this overall significant result was solely due to a significant increase in sIgA levels in the fourth cycle ($W = 39$ $p = 0.0488$); all other cycles (including the fifths one) showed no statistical difference in sIgA concentrations compared to those measured in the controls (all $p > 0.3$). Moreover, the rank order of samples remained unchanged after every freeze-thaw cycle (Figure 4b),

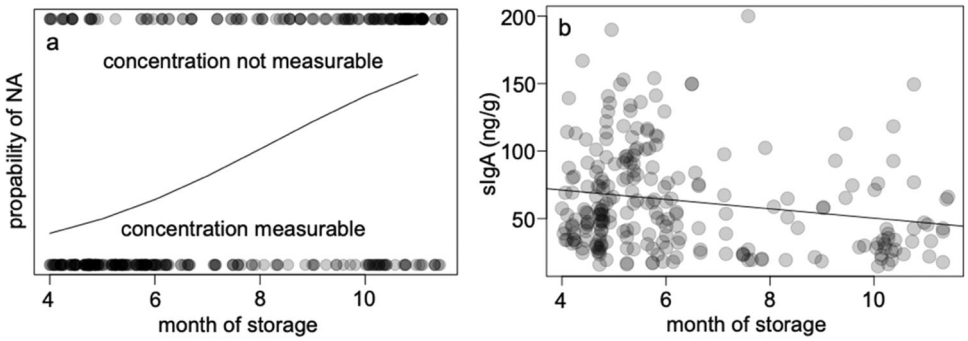

FIGURE 5 | Effect of storage time on fecal sIgA concentrations measured in wild redfronted lemurs. Data represent (a) the probability of measuring sIgA, i.e., NA, over storage time and (b) absolute concentrations over storage time. See Section 3 for statistics.

and sIgA concentrations correlated strongly with those of controls at each cycle ($r_s = 0.94–0.95$, all $p < 0.0001$, $N = 10$). Further, the mean CV of the six individual measurements per sample (controls and five freeze-thaw cycles) was < 10% across samples, indicating that all changes in sIgA concentrations recorded in response to the multiple freezing-thawing-refreezing procedures were within the range of inter-assay variability.

3.3 | Study 3: Fecal sIgA Measurements in Wild Redfronted Lemurs

The result of the logistic regression analysis is highly significant and indicates that longer storage durations are positively correlated with the probability of sIgA being not measurable ($z = 9.588$, $p < 0.001$; Figure 5a). Each additional month of storage increases this probability by approximately 57% (Estimate: 0.453, SE: 0.047). From the 422 collected samples, only 246 samples (i.e., 58,3%) had measurable sIgA concentrations. The linear model indicates a significant negative relationship between storage duration and log-transformed sIgA concentrations ($t = -4.15$, $p < 0.001$; Figure 5b), and about 6.62% of this variance is explained by storage duration (adjusted R^2: 0.062). For each additional month of storage, sIgA concentrations decreased by approximately 0.3% (Estimate: −0.079, SE: 0.019). There was a significant effect in sIgA concentrations between samples that were extracted directly on the day of sample collection and those stored frozen before extraction. Specifically, samples that were extracted on the day of collection had slightly, but significantly, lower sIgA concentrations ($W = 35$, $p = 0.014$; $N = 19$; Figure 6).

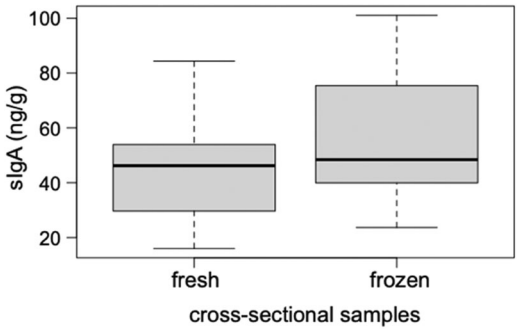

FIGURE 6 | Comparison between sIgA concentrations measured in fresh and frozen samples of wild redfronted lemurs. Data represent absolute sIgA concentrations from cross-sectional samples ($N = 19$) that were either extracted on the day of sample collection ("fresh") or after 2 days stored at −20°C ("frozen"). See Section 3 for statistics.

4 | Discussion

Non-invasive biomarkers like sIgA are crucial for assessing the health status and environmental effects on immune function. Our study validated an EIA and extraction methods for measuring immunoreactive sIgA in fecal samples of redfronted lemurs, confirming both the assay's efficacy and field-work suitability in zoo-housed and wild animals. Yet, it strongly highlights limitations of these measurements, in particular regarding sample storage conditions and storage duration.

4.1 | Validation of sIgA Measurements

While previous studies have demonstrated the feasibility of measuring sIgA in feces of several species (Edwards et al. 2019; Peters et al. 2004; Royo et al. 2004; Serres-Corral et al. 2024; Yin et al. 2015) including primates (Behringer et al. 2021; Gesquiere et al. 2020; Lantz et al. 2016), the custom monkey-specific sIgA ELISA kit failed to detect sIgA immunoreactivity in the feces of redfronted lemurs, suggesting that fecal sIgA in lemurs may differ structurally, and not only genetically (Haley 2003; Larsen, Campbell, and Yoder 2014), from that of anthropoid primates. Although key molecules involved in immune function are typically highly conserved, there is evidence for interspecific variation in IgA gene sets and the presence and/or characteristics of IgA isoforms and their subclasses (IgA1 and IgA2) as a result of different evolutionary pressures (Callard and Turner 1990; Kawamura, Saitou, and Ueda 1992). Given the evolutionary isolation and history of lemurs compared to that of anthropoid primates, it is plausible that lemur IgA differs from the forms found in higher anthropoid primates, at least at the sites where anthropoid primate specific anti-IgA detection antibodies presumably used in the commercial monkey sIgA ELISA kit are binding. However, in the absence of comparative studies on the structure of the IgA molecule within the primate taxon, this assumption remains speculative.

To overcome this potential caveat regarding sIgA analysis in our study species, we examined the validity of a literature-described

in-house made sIgA assay that used anti-human IgA antibodies with low specificity successful to bind IgA analogues from a variety of mammalian species (Edwards et al. 2019; Hau et al. 1990). Following successful establishment, our analytical validation tests of this non-specific EIA demonstrated the effectiveness of this assay in detecting sIgA in redfronted lemur feces, and that sIgA could be measured accurately and with no potential interference from the fecal matrix from both zoo-housed and wild living animals. We are confident that this assay is also likely to work for the assessment of sIgA in the feces of other lemur species and possibly of other strepsirrhine primates more generally. Because the key reagents for this assay are commercially available, as shown here, the described method can be easily established and tested for its versatility in other species.

4.2 | Testing the Stability of sIgA Concentrations Under Simulated Field Conditions

The feasibility and validity of the application of non-invasive biomarker measurements, including that of fecal sIgA, in remote and challenging field settings, depends on the stability of the compound. It has, therefore, been generally recommended to investigate the impact of sample handling, processing, and storage on biomarker concentrations, as analytes may be susceptible to degradation under varying and often less favorable conditions (Booth et al. 2009; Higham, Stahl-Hennig, and Heistermann 2020; Kalbitzer and Heistermann 2013; Kesner, Knecht, and Krieg 1995). In the case of sIgA, this is particularly important in light of several studies indicating temperature- and storage time-dependent reduced stability of this immune marker measured from different biological sources, such as colostrum (Ramírez-Santana et al. 2012), saliva (Booth et al. 2009; Ng et al. 2003; Presser, Simuyandi, and Brown 2014) and feces (Hau, Andersson, and Carlsson 2001; Krueger et al. 2019; but see Gesquiere et al. 2020). To address this issue, we initially validated a simple field extraction method without the use of electrical equipment and found that it produced comparable fecal sIgA results to those obtained using a standard laboratory extraction procedure. This confirms and extends results from studies of hormonal assessments (Nugraha et al. 2017; Shutt, Setchell, and Heistermann 2012), demonstrating that it is possible to reliably extract different types of biomarkers under challenging field conditions without the use of sophisticated equipment.

We further examined the effects of different storage conditions and durations on sIgA stability to optimize sample preservation methods. In this regard, freezing samples or sample extracts at −20°C is often considered the gold standard to minimize the risk of bacterial degradation and to stabilize the biomarker of interest (Gesquiere et al. 2020; Hau, Andersson, and Carlsson 2001; Kalbitzer and Heistermann 2013). With respect to sIgA, we were able to confirm that direct freezing of sample extracts resulted in a better stability of its concentrations compared to storage of extracts at RT for 3–10 days. The latter resulting in a consistent decrease of about 15%, which is also associated with some change in the rank order of the samples from high to low. These results confirm results from fecal IgA measurements in horses where levels decreased after 7 days of storage at RT (Krueger et al. 2019). In contrast, storage of samples for <24 h did not result in sIgA changes in baboon fecal extracts (Gesquiere et al. 2020), porcine fecal samples (Carlsson et al. 2007) and human saliva samples (Booth et al. 2009). Overall, these findings suggest that samples can be transported at ambient temperatures during the day in the field without the risk of degradation of sIgA. However, researchers should ensure that samples or sample extracts are (i) frozen within 24 h upon sample collection and (ii) are kept at sub-zero temperatures during transport to the laboratory for analysis. Thawing of samples during transport with re-freezing them within 24–48 h thereafter may be tolerated as multiple freeze-thaw cycles did not affect sIgA concentrations in our study, consistent with findings in baboons (Gesquiere et al. 2020), chimpanzees (Lantz et al. 2016), and humans (Booth et al. 2009).

Typically, samples are stored for weeks or months at field sites before being transferred to a laboratory (e.g., Rimbach et al. 2013; Shutt, Setchell, and Heistermann 2012). However, our study showed that long-term freezing of extracts for sIgA analysis from redfronted lemur feces has limitations as it is associated with a storage time dependent decline in sIgA concentrations. The decline occurred between 3 and 6 months of storage, indicating that reliable measures of absolute sIgA levels can only be obtained within a certain time window after sample collection. Nevertheless, our results show that the concentrations between 6 and 12 months of sample storage were strongly correlated with controls. Furthermore, the ranking of samples from highest to lowest remained unaffected by this decrease, suggesting that relative differences in sIgA concentrations between samples can be reliably assessed when storage periods of more than 3 months cannot be avoided. However, it is also possible that fecal sIgA in redfronted lemurs may be stable for as long as 4 or 5 months, but this remains to be evaluated. The reason for the time-dependent loss of sIgA levels is unclear, but may be related to proteolytic enzymatic processes taking place in biological samples as a result of bacterial activity (Fujiyama et al. 1985; Hau, Andersson, and Carlsson 2001), as sIgA concentrations in our frozen quality controls prepared from a purified sIgA standard preparation were extremely stable (i.e., inter-assay CV < 7%) over the 1-year measurement period. As the addition of a protease inhibitor cocktail did not prevent the observed decrease in fecal sIgA concentrations, we recommend that samples should be extracted and extracts be stored frozen on the day of collection and should be stored for no longer than 3 months before analysis.

Generally, our results align with a study on human saliva samples, where sIgA levels also decreased significantly after 3 months of storage at −30°C (Ng et al. 2003), and after 12 months in human colostrum (Ramírez-Santana et al. 2012). However, they contrast with a study on baboons (Gesquiere et al. 2020) and chimpanzees (Lantz et al. 2016), where fecal sIgA levels remained unaffected in samples stored long-term at either −20°C or ambient temperature, respectively. Together, these findings indicate potential variation among taxa in the stability of sIgA. This variation is not surprising given species differences in diet and, thus, the nature of dietary compounds and bacteria that may be involved in processes leading to sIgA degradation. Storing samples at very low temperatures, such as

−80°C (Booth et al. 2009), or freeze-drying samples with subsequent storage at −20°C (cf. Behringer et al. 2021), may be more effective in ensuring the stability of sIgA over longer durations, but this is usually not an option in the field.

4.3 | Practical Application of sIgA Measurements in Wild Lemurs

Even though we had no samples that were stored frozen for less than 4 months before analysis, the successful analytical validation of our newly established assay and successful determination of sIgA concentrations in samples from wild individuals reinforces the reliability of the assay to measure sIgA also in samples originating from wild redfronted lemurs, and generally validates its utility for studying sIgA dynamics in this species. Yet, and most importantly, sIgA assessment in samples of wild animals confirmed our experimental finding of the decrease in sIgA levels and a diminishing likelihood of measuring sIgA with prolonged storage time. We also found that wild lemur samples extracted on the same day had slightly, but significantly, lower sIgA concentrations compared to those frozen immediately and extracted after 2 days. This aligns with both a study in humans that highlighted the importance of immediate freezing saliva samples to prevent a decrease in sIgA concentration by up to 30% (Nurkka et al. 2003), as well as with our findings from zoo-housed animals, where immediate freezing maintained sIgA stability better than RT storage. Although the samples from wild animals could not be measured within the optimal time frame of 3 months to overcome potential storage effects when stored longer, the concentrations measured in the samples from the wild generally appeared to be lower than those measured in the two zoo-housed animals. Whether this is related to a higher degradation potential of sIgA present in feces from the natural setting is not known yet. SIgA concentrations in wild redfronted lemurs might also be affected by other factors such as habitat, stressors, or seasonal variation that should be considered in future studies. While our study focused on non-invasive methods, future post mortem studies in captivity may provide an opportunity to clarify whether differences in fecal composition between the zoo-housed and wild animals exist due to, for example, variation in diet and resulting microbiome composition. While investigating the consequences for the presence and/or abundance of sIgA-degrading enzymes, this approach would also aid to validate fecal measurements as an accurate indicator of mucosal immunity, by comparing fecal sIgA metrics with intestinal mucosal sIgA.

5 | Conclusions

To our knowledge, this study is the first report on the measurement of sIgA in feces of a lemur species. Given the non-specificity of the key antibodies used in our newly developed EIA, we envisage that this cost-effective assay can also be applied successfully to a broader range of lemur species, and possibly other primates. Findings on the stability of sIgA concentrations emphasized the importance of proper sample handling and storage protocols. The availability of an assay to measure fecal sIgA in both zoo-housed and wild lemurs provides a valuable tool for monitoring aspects of immunity and health. The establishment and validation of the described fecal sIgA assay represents a pivotal step in this regard and underscores the importance of non-invasive methodologies to study physiology in wildlife research.

Author Contributions

Leonie Pethig: conceptualization (equal); data curation (equal); formal analysis (equal); writing–original draft (lead); writing–review & editing (equal). **Verena Behringer:** conceptualization (equal); writing–original draft (supporting); writing–review & editing (equal). **Peter M Kappeler:** conceptualization (supporting); funding acquisition (equal); writing–review & editing (equal). **Claudia Fichtel:** conceptualization (supporting); funding acquisition (equal); writing–review & editing (equal). **Michael Heistermann:** conceptualization (equal); data curation (equal); formal analysis (equal); methodology (equal); resources (equal); writing–original draft (lead); writing–review & editing (equal).

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The datasets supporting this article are uploaded at figshare (https://figshare.com/s/51587cf8f9b74421ac34).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.