

# The non-invasive measurement of faecal immunoglobulin in African equids

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## ABSTRACT

Eco-immunological research is encumbered by a lack of basic research in a wild context and by the availability of few non-invasive tools to measure the internal state of wild animals. The recent development of an enzyme-linked immunosorbent assay for measuring immunoglobulins in faecal samples from Soay sheep prompted us to optimize such an assay to measure immunoglobulin A (IgA: an antibody associated with parasitic nematode fecundity) in faecal samples from equids. We measured total IgA in domestic donkeys, wild plains zebras, and wild Grevy's zebras sharing the same landscape in central Kenya over two field seasons. Attempts to measure anti-nematode IgA more specifically, using a homogenized extract from a mixture of excreted nematodes, failed to clear background. However, we found that total IgA positively correlated with strongyle nematode faecal egg counts (FECs) in donkeys sampled during the wetter field season - a time when the donkeys were in good condition. Further, this relationship appeared among donkeys with high body condition but not among those with low body condition. Time lags of 1–4 days introduced between IgA and FEC measurements in repeatedly sampled donkeys did not yield correlations, suggesting that IgA and FEC roughly tracked one another without much delay in the wet field season. Such a direct IgA-FEC relationship did not appear for zebras in either the wet or dry field season, possibly due to higher interindividual variation in body condition among the free-roaming zebras than in the donkeys. However, Grevy's zebras had higher overall levels of IgA than either plains zebras or donkeys, potentially associated with their reportedly lower FECs at the population level. Our results suggest that equids may mount an IgA response to nematode egg production when the host is in good condition and that equid species may differ in baseline levels of mucosal IgA.

## 1. Introduction

Immune responses to parasites are typically deduced from experiments testing interactions between a single parasite species and a naïve host. Such controlled and simplified conditions have been essential for causal inference. However, it can be unclear how these experimental results can be applied to understand host-parasite interactions in the wild where coinfections by multiple parasites are the norm (Pedersen and Fenton, 2006). Wildlife immunology is hampered by a dearth of basic research as most of our understanding of the mammalian immune system derives from laboratory studies on humans and mice, and the vast majority of work on other species is restricted to a few domestic species (Jolles et al., 2015; Maizels and Nussey, 2013).

Further, immune responses are typically measured from serum, a practice with limited feasibility in the context of wildlife research, especially when threatened species are the subjects. Recent advances in the non-invasive measurement of antibodies in Soay sheep have

demonstrated potential for widespread application of faecal analysis in eco-immunological studies (Nussey et al., 2014; Watt et al., 2015). The use of faecal samples for immune measurements has the added benefit of reflecting immune molecules in the gut mucosa, which may be more representative of local immune responses to the many parasites that inhabit the host's gastrointestinal tract than immune molecules found in serum.

Equids commonly have chronic infections with a diversity of gastrointestinal nematodes (GINs), particularly strongyles, with prevalence often approaching 100% in untreated populations (Bucknell et al., 1996; Klei and Chapman, 1999; Seyoum et al., 2015; VanderWaal et al., 2014). Although some of the more pathogenic *Strongylinae* are now rare in domestic equids due to intervention programs, cyathostomins are extremely prevalent and heavy burdens can lead to damaged mucosa due to plug feeding or, on rare occasions, to larval cyathostominosis, a highly fatal condition more common in younger horses (Corning, 2009; Herd, 1990; Kolk and Velduis Kroeze, 2013).

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Immunological research on wild equids trails far behind that on domestic equids. However, some patterns have emerged in plains zebras (*Equus quagga*) in Etosha National Park, Namibia. In this population, immune responses geared towards extracellular parasites (components of the T-helper 2 immune response: specifically, circulating IgE, IgG<sub>b</sub>, and interleukin-4) rose in the wet season, coinciding with seasonal surges in nematode reproductive activity (Cizauskas et al., 2014). The Etosha plains zebra data were obtained from whole blood and seral samples drawn from captured animals, but non-invasive techniques of assessing immune responses to GINs would be valuable for advancing the understanding of equid host-parasite interactions in nature, especially for endangered equids like the Grevy's zebra (*Equus grevyi*). We set out to develop a modified protocol of the non-invasive Soay sheep faecal immunoglobulin assay (Nussey et al., 2014; Watt et al., 2015) for equids, and to evaluate its application for eco-immunological research on wild equids.

We focused on measuring IgA as it is the most active immunoglobulin in the gut mucosa (in ruminants Hendawy, 2018; in equids Klei and Chapman, 1999; Wells et al., 1981). In contrast, IgE is present only in low concentrations in the gut mucosa and was often undetectable in faecal samples from Soay sheep (Watt et al., 2015). Although IgA has a diversity of functions including tissue repair and tagging pathogenic bacteria (Bice et al., 2017; Palm et al., 2014), it is reportedly the immunoglobulin that most effectively degranulates eosinophils (Behm and Ovington, 2000), which can severely damage or kill GINs *in vivo* (in sheep Balic et al., 2006; in horses Steuer et al., 2018). Further, local IgA responses have been associated with reduced growth rates and fecundity in GINs parasitizing sheep (Halliday et al., 2007; reviewed in McRae et al., 2015). IgA is therefore a particularly relevant immune metric to relate to faecal egg counts (FECs), a commonly used non-invasive measure of the reproductive output of the nematode population infecting a host.

In this study, we optimized a faecal IgA enzyme-linked immunosorbent assay (ELISA) for equids and analysed the relationship between IgA, FECs, and demographic and environmental factors, in wild and domestic African equids. We studied two zebra species in central Kenya, the plains zebra and the Grevy's zebra. We also ran assays on samples from domestic donkeys (*Equus africanus asinus*) that shared the same landscape because individual donkeys could be reliably sampled at regular intervals to obtain longitudinal data. We employed a combination of individual- and population-level analyses to seek to understand the underlying host-parasite dynamics. Using these data, we investigated whether mucosal IgA measured non-invasively from faecal samples was indicative of an immune response to GINs in domestic and wild equid species and, if so, under what demographic or environmental circumstances.

## 2. Materials and methods

### 2.1. Field methods

We systematically drove predetermined routes along roads around Mpala Research Centre (36°53'E, 0°17'N, elevation 1,700–2,000m above sea level), Laikipia District, Kenya to find plains zebras and Grevy's zebras June 13–July 29, 2017 and June 12–July 28, 2018. The routes and direction of travel were alternated to cover the area evenly and minimize spatial and temporal sampling bias. The study site consists of a semi-arid savanna woodland landscape harbouring a diverse fauna including several large and medium-sized herbivores in addition to the zebras, such as elephant (*Loxodonta africana*), Cape buffalo (*Syncerus caffer*), impala (*Aepyceros melampus*), and Grant's gazelle (*Gazella granti*). The wildlife share their habitat with ranchers and local herdsmen that use the land to graze cattle and donkeys. Rainfall peaks roughly twice a year in this area. Our field seasons each fell during a time of year that typically spans the end of the long rains and the beginning of the more sporadic continental rains, but the 2017 field

season followed a drought and rainfall was somewhat sparser and later (94.7 mm over the two months, falling mostly in July) than the 2018 season (136.4 mm, falling almost entirely in June) (Caylor et al., 2018). FECs in equids typically peak with the onset of seasonal conditions that favour nematode egg survival, development, and larval migration onto pasture: late winter and early spring in temperate regions and the wet season in the tropics (Cizauskas et al., 2014; Getachew et al., 2008; Matthee et al., 2002; Nielsen et al., 2007).

Faeces were collected opportunistically from individuals of each species as soon as possible after defecation was observed, timing collection to minimize disturbing the animals, but collecting faeces only when it could be done within 20 min of defecation. A photo of the individual was taken and notes on the photo number, GPS location, date, time, species, sex, and age (juvenile – under two years of age - or adult) were taken for each individual. While sampling was opportunistic, sampling effort was focused on adults. Upon collecting 2–3 boluses of faeces, the sample was sealed in a Ziploc bag, labelled, and kept on ice packs in a cooler until we returned to the research station later that day. Photos of the sampled zebras were run through stripe recognition software (HotSpotter, now called WILDBOOK; Crall et al., 2013; Parham et al., 2018) to identify each individual and determine which were repeatedly sampled.

Through both field seasons, we collected faeces from donkeys from a herd kept at the same study site. The donkeys were individually recognizable on sight based on numbers spray painted on the rump on the first day of sampling in each field season as well as ear markings, scars, and other physical features, and were easily followed as the herd moved through the landscape until they defecated. We repeatedly sampled 13 donkeys in 2017 (including 1 juvenile female, 3 juvenile males, 4 adult females, and 5 adult males) and 11 in 2018 (2 juvenile males, 4 adult females, 5 adult males). Nine individuals were sampled in both field seasons; the herd lost one adult female, a juvenile female, and a juvenile male but gained an adult female between field seasons. Donkey faeces was collected at least once a week, but more frequently (on a daily basis for as many individuals as possible) in the first and last four days of each field season. Each donkey was photographed in profile as well as from behind at least once in a field season. These photographs were used to estimate body condition using a commonly used body condition scoring chart for donkeys published by The Donkey Sanctuary that runs from scores of 1 (emaciated) to 5 (obese), including half scores (Milner and Rougier, 2014; Polidori and Vincenzetti, 2017; The Donkey Sanctuary, 2018).

### 2.2. Laboratory methods – faecal egg counts

We used a modified McMaster technique, with a lower detection limit of 50 eggs/gram of faeces, to measure the density of strongyle nematode eggs in faecal samples by salt flotation the same day that they were collected (Ezenwa, 2003; Herd, 1992). A saline solution was prepared by heating water to a boil, removing it from heat and adding table salt (NaCl) while stirring until it would no longer dissolve. This saturated saline solution was left to cool before use and had a specific gravity of around 1.2. We homogenized the samples by massaging the collection bags thoroughly and a subsample of 3 g was placed in a measuring cup. Water was added to a total volume of 42 ml and the sample was mixed into solution with a tongue depressor. The slurry was then strained through a metal sieve into a plastic cup, using the tongue depressor to press down and release as much liquid as possible. The filtrate was swirled briefly by moving the cup in a circular motion and used to fill a 15 ml tube. After centrifugation at a rcf of 1,534 g for 5 min, the supernatant was discarded and saturated saline solution was added to fill the tube halfway. A wooden stick was used to break apart the pellet and mix it with the solution and the tube was then filled to the top with more saturated saline solution. The tube was capped and inverted a few times to homogenize the sample. A pipette was immediately used to draw solution from the middle of the tube to fill one

of the chambers of a McMaster slide (Chalex Corp.). After inverting the tube a few more times, a second subsample was drawn to fill the second chamber and the slide was left to sit for at least 2 min to allow eggs time to float to the top of the slide. Eggs were counted within the McMaster slide grid of each chamber under  $100\times$  total magnification with a compound microscope. FECs were calculated by multiplying the sum of the two counts from the McMaster slide chambers by 50 to obtain an estimate of nematode egg density in eggs per gram of faeces. In horses, FECs have been found not to be affected by time of day sampled and subsample effects on counts are weak compared to the effects of horse identity, but they are not negligible even after sample homogenization (Carstensen et al., 2013; Denwood et al., 2012). Although performing counts on multiple subsamples of each sample is recommended in some cases (e.g., to confirm FECs of zero; Nielsen et al., 2010), this practice is labour-intensive and would have cut our sample size drastically as we were already operating at capacity in the field. Instead, we capitalized on previous findings that drawing a sample from a larger amount of homogenized dung exponentially reduces FEC variability (e.g., increasing the homogenized sample from 3g to 20g reduces the inter-sample coefficient of variation from 0.28 to 0.1; Denwood et al., 2012), and collected at least 30g of dung, including multiple boluses, all of which was homogenized before extracting the 3g used to perform the count.

### 2.3. Laboratory methods – faecal IgA

We conducted sandwich ELISAs to measure total immunoglobulin A in equid faecal samples. A modified assay was developed from the protocol recently used for Soay sheep (Nussey et al., 2014; Watt et al., 2015). An important consideration for the measurement of immunoglobulins from faecal samples is that surface antigens on parasites in the gut may be altered by enzymatic activity and natural immune responses in the gastrointestinal tract are therefore thought to be polyclonal – consisting of a mix of antibodies with affinities for different epitopes of a given antigen (Russell and Kilian, 2015). The use of polyclonal antibodies is therefore more effective than monoclonal or monospecific antibodies at capturing mucosal IgA in ELISAs.

On the same day that samples were collected and after sample homogenization in the collection bags, a subsample of roughly 5g was extracted and frozen in a small Ziploc bag. Before running ELISAs, these subsamples were prepared by thawing them out on ice, adding  $0.5\text{ g} \pm 0.05\text{ g}$  to a 2 ml tube and adding 750  $\mu\text{l}$  of pre-chilled phosphate-buffered saline (PBS) containing protease inhibitor (Roche complete minitabs 04 693 116 001) to reach a ratio of 1.5:1 protease inhibitor solution to weight of faecal sample. We waited for 5 min while the solution softened the sample on ice, after which a clean toothpick was used to break up the faeces and mix it thoroughly with the solution. Samples were left to stand for 20 more minutes on ice, centrifuged at a rcf of 15,000 g for 5 min, and the supernatant was extracted with a pipette and used as the sample extract. Each sample was then diluted to 1:64 with PBS + protease inhibitor as this was the dilution found to most closely approach the inflection point in calibration curves during assay optimization. When optimizing the assay, sample concentrations were tested in doubling dilutions starting from a high-concentration sample of faecal extract diluted to 1:2 in PBS + protease inhibitor and ending with a low-concentration sample of 1:256 PBS + protease inhibitor (eight concentrations tested in duplicate through several assay runs). After sample dilution, samples were frozen until ready for analysis.

To measure total IgA, immunosorbent microplates (Nunc Maxisorp, Thermo Fisher Scientific) were coated with 50  $\mu\text{l}$  per well of purified horse IgA (Cedarlane CLFA10) diluted to 2  $\mu\text{g}/\text{ml}$  in carbonate buffer. The plates were covered with clingfilm and incubated overnight or for up to 3 days at 4 °C. They were then emptied and washed three times with PBS with 1% Tween (PBST) using a squeeze bottle aimed directly at the bottom of each well and moving steadily across the wells without

causing them to overflow. Each plate included a calibration series of five dilutions of purified horse IgA, each diluted to half of the concentration of the last (2, 1, 0.5, 0.25, and 0.125  $\mu\text{g}/\text{ml}$  in PBS + protease inhibitor), which served both to produce a standard curve and as positive controls, as well as two negative controls (one with PBS + protease inhibitor and one with PBS only) and samples in duplicate. Samples, standards, and controls were added at 50  $\mu\text{l}$  per well. All samples and controls were thawed on ice and kept cold while plates were loaded, and calibrators were prepared anew each day because they degrade with repeated freeze-thaw cycles. Samples and controls were distributed in rapid succession using a multichannel pipette, with calibrators positioned in the first two columns, samples duplicated in neighbouring columns, and the negative controls in the last row of the last two columns of each plate. Plates were then covered with clingfilm and incubated for 1 h at 37 °C. After this, plates were washed five times with PBST and loaded with 50  $\mu\text{l}/\text{well}$  of detection antibody diluted to 0.125  $\mu\text{l}/\text{ml}$  in PBST (HRP-conjugated goat anti-horse IgA; Bethyl Laboratories Inc. A70-103P). They were then covered and incubated again for an hour at 37 °C, after which they were washed five times with PBST. ABTS peroxide substrate was then added at 100  $\mu\text{l}/\text{well}$  (KPL Inc. 50-62-00) and plates were developed in the dark at 37 °C for 5 min and read immediately at a wavelength of 405 nm in a plate reader (Thermo Scientific Multiskan FC, model 51119000).

Any samples that did not clear background or for which the duplicates gave readings that were so different that they brought the plate's overall correlation between replicate 1 and replicate 2 below a Pearson's  $r$  of 0.80 were re-run. These repeatability procedures are similar to the methods in Nussey et al., (2014), but they performed this between replicate plates and reran the entire plate if  $r$  fell below 0.80. We further removed any samples for which the percent difference between the two replicates was more than 15% (calculated by dividing the absolute difference between the replicate IgA measures by their mean value), as this is a quality check often employed for seral immunoglobulin assays.

We additionally attempted to measure a population of IgAs more specific to GINs than total IgA. Crude somatic nematode antigen was prepared from a mixture of nematode species by harvesting worms from zebra faeces *en masse*. Antigen preparation from a mix of cyathostomin species has successfully captured IgG from seral samples of donkeys in Egypt (Abo-Aziza et al., 2017), but this approach has yet to be attempted on faecal samples. We washed the collected worms three times with saline. We then homogenized them in a tube with PBS and sterilized steel balls, embedding the tube in a clean paint can filled with pre-chilled silicon beads to secure the tube in place and keep it cool and subjecting the can to 30 min of shaking in an industrial paint shaker (a sample homogenizer was not available to us in the field). While this process produced sufficient concentrations of protein in solution for potential antibody detection (confirmed with a Qubit protein assay), none of our assays using the nematode antigen as capture antigen cleared background controls even after two more attempts at making fresh nematode antigen. We were thus restricted to measuring total IgA using commercially available purified horse IgA as the capture antigen.

### 2.4. Statistical analyses

Eco-immunological studies on seral immunoglobulins often calculate Ig concentrations using a standard curve determined from running serial dilutions of antibodies of known concentration on each plate (Budischak et al., 2017). To ensure that non-linearities in optical density (OD: the IgA concentration reading from the plate reader) values did not affect our results, we calculated IgA concentration using this technique. For each plate, an exponential model was fitted to the standards, including the mean of the negative controls and blanks as the zero mark, and we used the 'predict' command in the core R package *stats* to calculate IgA concentrations from raw ODs, which were then multiplied by the dilution factor (64) to obtain IgA concentration in ng/

ml (R Core Team, 2019). The average concentration for each sample's replicates was used as our IgA concentration metric.

To evaluate the relationship between IgA and FEC at the population level, we ran a linear mixed model for predicting IgA concentration in donkeys and another for zebras using 'lmer' in the *lme4* package (Bates et al., 2015). We square root-transformed the IgA concentrations (sqrtIgA) to obtain normality in model residuals, as verified both graphically and with a Shapiro-Wilk test. The order of sample distribution on the plate had an effect on the readings due to slight differences in reaction time, with OD declining slightly in later columns (Kruskal-Wallis chi-square = 14.76, df = 5,  $p = 0.011$ ), so column number for the first replicate of each sample (the second replicate was always in the next column) was included as a random effect in each model, as was the plate ID. For the donkey model, individual identity was included as an additional random effect, but too many individual zebras were sampled to do the same in the zebra model. Fixed effects were log-transformed FEC (logFEC), sex, and age, and for the zebra model species was also included. We also included season, defining a given month as 'dry' if rainfall in the previous month did not exceed 100 mm and 'wet' if it did, following Ezenwa (2004) where this cut-off corresponded to significant differences in vegetation greenness at Mpala. For the donkey model, year replaced season as this decreased AIC. Because equid FECs are typically highly seasonal, rising in the rainy season in tropical areas (Cizauskas et al., 2014; Getachew et al., 2008), we included a log-FEC\*Season or logFEC\*Year interaction in the models.

Model selection proceeded using the information theoretic AIC approach (Burnham and Anderson, 2002). AIC values were computed for all possible combinations of the fixed effects, including the random effects-only null model, using the 'dredge' function in the *MuMIn* package (Bartoń, 2018). The lowest-AIC model and any models within 4 AIC points of the lowest-AIC model were chosen for the candidate model set (Richards et al., 2011). Model fit was assessed with the *DHARMA* package (Hartig, 2018), and among candidate models that fit the data, model-averaged results using the zero-method were calculated (if a factor did not appear in a given candidate model, its values were counted as '0' for that model rather than discounting the model in the factor's average values).

To consider time lags in the IgA-FEC relationship, we plotted individual timelines in logFEC and plate-adjusted mean IgA ODs (so that plate effects were adjusted for in the visualization) for donkeys sampled in 2018. This was the dataset for which we had the most individual-level data over a sampling period. We did the same for seven Grevy's zebras repeatedly and intensively sampled over the same month-long period in 2018, but did not have sufficient sample sizes from our efforts to repeatedly sample individual plains zebras. The number of days between samples was irregular for both donkeys and Grevy's zebras. However, potential lags in timing between the fluctuations of IgA and FEC were analysed by determining whether Pearson's correlations were improved when IgA measurements from 1, 2, 3, and 4 days before or after FEC measurements from the same donkey were correlated (using all data available that fit each time interval), compared to the correlation without a time lag. For Grevy's zebras, we were able to test for correlations with a time lag in IgA of only 1 day before and 1 day after FEC measurements as sample sizes fell too much to allow us to test for greater time lags. As in the models, sqrtIgA and logFEC values were used for these correlations.

Finally, although an insufficient number of body condition scores were obtained on sampled donkeys to include this factor in the model predicting IgA concentration, we ran simple linear regressions to determine whether logFEC and sqrtIgA were correlated for donkeys in low body condition (with a body condition score of 2.5 or lower) and for donkeys in high body condition (with a score of > 2.5) using the *lm* function in base R. All statistical analyses were conducted using R version 3.5.3 (R Core Team, 2019).

**Table 1**

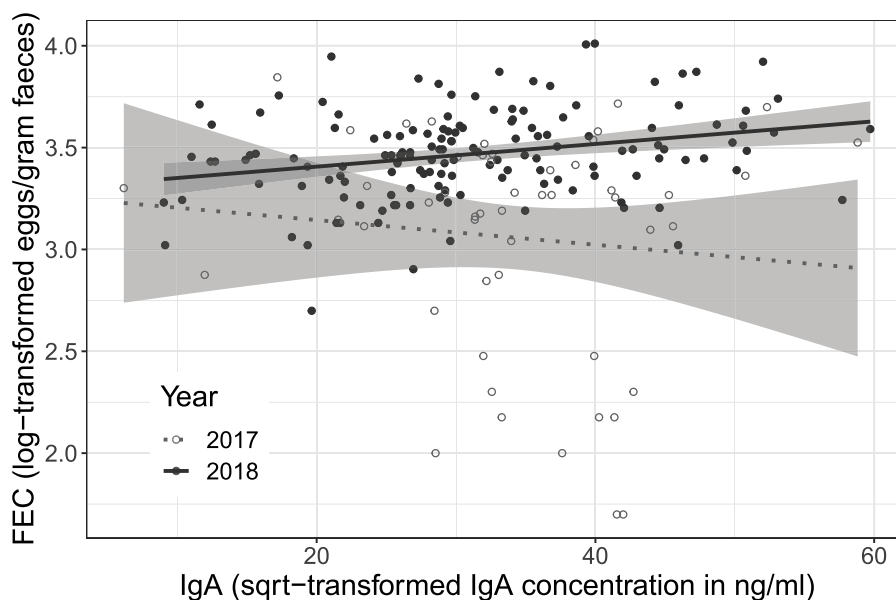
Donkey model-averaged results for predicting square-root transformed IgA concentration ( $n = 199$ ). Details of random effects were derived from the global models.

Variable	Estimate	SE	z value	p value	Random Effects
<b>Intercept</b>	43.78	7.05	6.17	< 2 x 10 <sup>-16***</sup>	<b>ID (n = 19 IDs)</b> Intercept var = 5.34
<b>Age_juvenile</b>	0.65	1.92	0.34	0.74	Intercept SD = 2.31
<b>Sex_male</b>	0.34	1.49	0.23	0.82	<b>Plate (n = 15 plates)</b> Intercept var = 61.62
<b>Year_2018</b>	-39.92	12.30	3.22	0.0013**	Intercept SD = 7.85
<b>logFEC</b>	-3.13	2.13	1.46	0.14	<b>Column (n = 6 columns)</b> Intercept var = 0.74
<b>Year_2018 x logFEC</b>	11.16	3.57	3.10	0.0019**	Intercept SD = 0.86 Residual var = 46.47 Residual SD = 6.82

### 3. Results

For donkeys, IgA was significantly higher in 2017 than 2018 and a positive correlation between IgA and logFEC was present in 2018, the year with higher rainfall (Table 1, Fig. 1). This was confirmed with a Spearman rank correlation between logFEC and sqrtIgA concentration in the 2018 donkey data subset ( $\rho = 0.30$ ,  $p = 2.06 \times 10^{-4}$ ). In 2017, there was markedly higher variance in logFEC and no correlation with sqrtIgA levels (Fig. 1). For zebras, the model-averaged results yielded a species difference: Grevy's zebras had higher IgA concentrations than plains zebras (Table 2). Interspecific differences in IgA, including donkey data, were verified through a simpler ANOVA and a subsequent Tukey's *post hoc* test but these revealed differences only between donkeys and zebras, suggesting that the interspecific difference in IgA between Grevy's and plains zebras emerged only after other factors were accounted for in a model ( $F = 9.02$ ,  $p < 0.001$ ; mean sqrt-IgAs: GZ = 36.40, PZ = 34.63, DK = 31.95; GZ-PZ difference  $p = 0.33$ , GZ-DK difference  $p < 0.001$ , PZ-DK difference  $p = 0.36$ ; Fig. 2). Interspecific differences in FEC in our dataset did not emerge between the two zebra species, but donkeys had higher FECs than either zebra species (Kruskal-Wallis chi-squared = 16.46,  $p < 0.001$ ; mean FECs: GZ = 2330.7, PZ = 2063.5, DK = 2958.8; significance of differences: GZ-PZ difference  $p = 0.18$ , GZ-DK difference  $p < 0.001$ , PZ-DK difference  $p < 0.001$ ; Fig. 2). A similar proportion of samples came from juveniles in each study species (24% for DK, 23% for GZ, 17% for PZ).

At the individual level, the plotted data for both the donkey and Grevy's zebra timelines seemed to show rough tracking between IgA and FEC (S1 and S2). The lack of a time lag was reinforced by the deterioration of the correlation between sqrtIgA and logFEC in the 2018 donkey data when either lagging or leading IgA levels were correlated to FEC (Table 3). While fewer samples could be included when testing for longer time lags, sample sizes remained high for time lags of a couple of days and showed a complete lack of a correlation when any lag was introduced. The results indicate rough synchrony (lags of less than one day) and confirm that analysing the IgA-FEC relationship within the same sample is most appropriate. The Pearson's tests did not produce a significant correlation between logFEC and sqrtIgA concentrations for Grevy's zebras sampled in that same month in 2018, whether or not a time lag in IgA was implemented (Table 4). While the dataset was smaller for these wild equids that were difficult to track and sample repeatedly, we suspect that if the same correlation were present



**Fig. 1.** In donkeys, IgA increased with logFEC but only in 2018, when rainfall was higher. Shading represents standard error, significant correlations are shown in solid lines, and non-significant trends in dotted lines.

**Table 2**

Zebra model-averaged results for predicting square-root transformed IgA concentration (n = 101 Grevy's zebras and 37 plains zebras). Details of random effects were derived from the global models.

Variable	Estimate	SE	z value	p value	Random Effects
<b>Intercept</b>	33.66	9.40	3.55	0.00039***	<b>Plate (n = 15 plates)</b>
<b>Age_juvenile</b>	1.61	1.52	1.05	0.30	Intercept
<b>Sex_male</b>	1.26	1.25	1.00	0.32	var = 75.16
<b>Season_wet</b>	1.12	13.70	0.081	0.94	Intercept
<b>Species_plains</b>	-3.40	1.42	2.37	0.018*	SD = 8.67
<b>logFEC</b>	0.63	2.89	0.22	0.83	<b>Column (n = 6 columns)</b>
<b>Season_wet x logFEC</b>	-0.40	4.23	0.09	0.93	Intercept
					var = 0.76
					Intercept
					SD = 0.87
					Residual
					var = 40.05
					Residual
					SD = 6.33

in Grevy's zebras, the dataset with no time lag should have had a large enough sample size to at least approach significance. This points to either an interspecific difference in FEC-IgA dynamics or greater interindividual variability in the traits that mediate these dynamics (e.g., body condition) in the free-ranging zebras than in the domestic donkeys, all of which were sampled from the same herd.

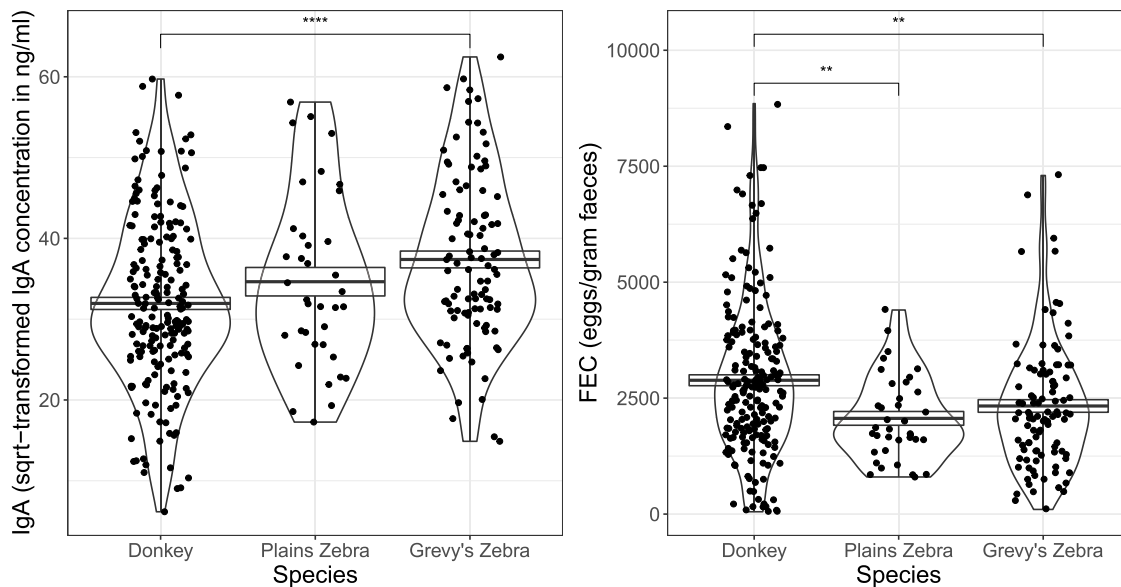
We found evidence that body condition is indeed one such mediating trait. For donkeys, body condition was significantly higher in 2018 than 2017 (whether tested with an unpaired Wilcoxon rank sum test  $W = 135, p < 0.001$ ; or a paired Wilcoxon rank sum test using the first body condition obtained for each individual in each field season  $V = 0, p < 0.01$ ). We found that donkeys in high body condition had a significant positive correlation between logFEC and sqrtIgA ( $p < 0.05$ , adjusted  $Rsq = 0.18$ ) while no relationship was apparent in donkeys in low body condition ( $p = 0.28$ , adjusted  $Rsq = 0.0051$ ; Fig. 3). This raises the possibility that differences in body condition contributed to the differences in the FEC-IgA relationship between years for the donkeys and that variation in body condition potentially affected our ability to detect a relationship in zebras.

**4. Discussion**

Our modified ELISA protocol successfully produced total IgA measures that cleared background from equid faecal sample extracts. Despite our measures being restricted to total IgA rather than GIN-specific IgA and despite the many potential functions that IgA can take on, we found associations with nematode FECs under certain circumstances, indicating that responding to GINs may be a principal role of mucosal IgA in equids in good condition and that faecal IgA may be a useful indicator of immune response to GINs.

FECs and total IgA were positively correlated in donkeys sampled in 2018, but not in 2017. The heavier and earlier rains in the 2018 field season likely led to improved vegetation quality at the time of sampling. Rainfall far exceeded long-term averages in the wet season leading up to sample collection in 2018, whereas the 2017 field season followed a severe drought and lower-than-average rainfall almost every month for a full year prior (Caylor et al., 2018). Donkeys had higher body condition in 2018, perhaps enabling them to mount IgA responses, whereas in 2017 many donkeys may have been physiologically conserving resources. IgA may therefore be a costly immune response, mounted only when energy or nutritional reserves permit. In line with this reasoning, Cizauskas et al. (2014) found serral IgE, IgGb, and eosinophils to rise with the amount of rainfall individual plains zebras experienced over the two months prior to sampling. In Soay sheep, total IgA was found to positively correlate with body weight, also in line with the notion that IgA represents a potentially costly form of immunity (Nussey et al., 2014). Once a response is mounted, the correlation between FECs and IgA in our 2018 donkey samples suggests that an IgA response is mounted only in proportion to need.

The time series data on repeatedly sampled individuals, our comparisons of correlations with time lags between IgA and FEC, and the positive nature of any IgA-FEC correlations that emerged suggest that the correlations arise from an IgA response that quickly follows an increase in nematode reproductive activity in the gut. The converse – for FEC to rise promptly in response to increases in mucosal IgA – is improbable. While IgA can take on a number of possible roles, the association with FECs in particular indicates that a high proportion of the total mucosal IgA may be dedicated to the depression of reproduction in the nematode community harboured by the host when in good body condition. The apparent break down of the IgA-FEC correlation in



**Fig. 2.** IgA was higher in Grevy's zebras than donkeys and, when other factors were taken into account in the zebra model, Grevy's zebra IgA was higher than plains zebra IgA. Conversely, FEC was higher in donkeys than the other two species (and at the population level plains zebras have higher FEC than Grevy's zebras; DIR and KJT unpublished data, Rubenstein et al., 2010). Horizontal bars indicate means, horizontal box edges standard errors, and asterisks and symbols above the brackets denote level of significance for differences that emerged in simple tests (ANOVA with Tukey *post hoc* for sqrtIgA, Kruskal-Wallis with Dunn's *post hoc* for FEC;  $p < 0.05^*$ ,  $p < 0.01^{**}$ ,  $p < 0.001^{***}$ ).

**Table 3**

Comparison of Pearson's correlations in the 2018 donkey data between logFEC and sqrt-transformed IgA with time lags in IgA. Only the correlation with no time lag was significant.

Time-lag in IgA	Pearson's <i>r</i>	<i>t</i>	df	<i>p</i>	95% CI
4 days prior	0.58	1.22	3	0.31	−0.622, 0.967
3 days prior	0.098	0.36	13	0.73	−0.436, 0.581
2 days prior	0.106	0.57	29	0.57	−0.258, 0.443
1 day prior	0.016	0.11	47	0.91	−0.266, 0.296
No time lag	<b>0.27</b>	<b>3.37</b>	<b>143</b>	<b>0.00097***</b>	<b>0.113, 0.416</b>
1 day after	0.081	0.55	47	0.58	−0.205, 0.354
2 days after	0.13	0.69	28	0.50	−0.242, 0.468
3 days after	−0.012	−0.041	12	0.97	−0.539, 0.522
4 days after	−0.58	−1.25	3	0.30	−0.968, 0.615

**Table 4**

Comparison of Pearson's correlations in the 2018 Grevy's zebra data between logFEC and sqrt-transformed IgA with time lags in IgA. Only a time lag of 1 day could be tested and no correlations were significant.

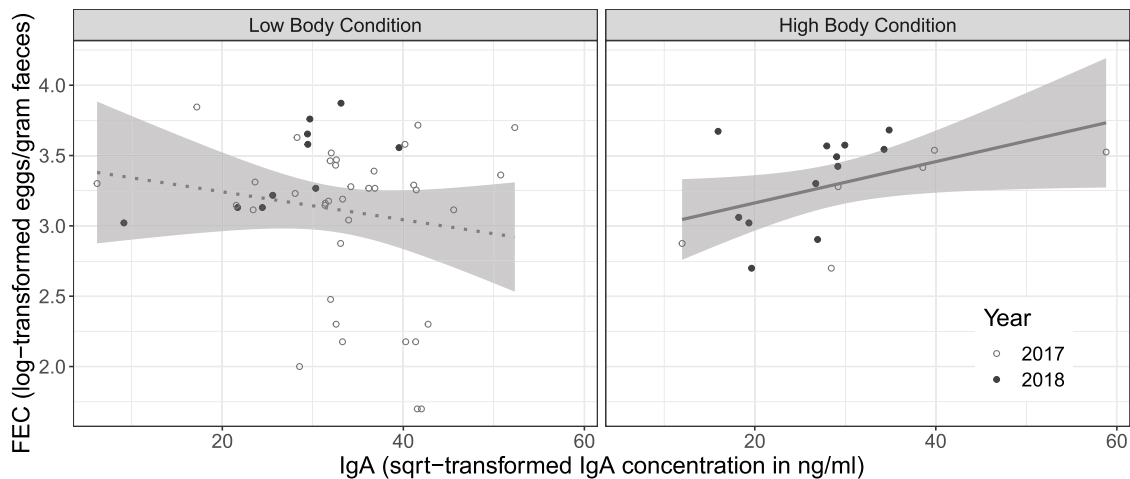
Time-lag in IgA	Pearson's <i>r</i>	<i>t</i>	df	<i>p</i>	95% CI
1 day prior	0.37	1.38	12	0.19	−0.200, 0.753
No time lag	−0.028	−0.23	67	0.82	−0.263, 0.210
1 day after	−0.21	−0.76	12	0.46	−0.669, 0.357

donkeys with low body condition suggests that this immunoglobulin type is not a principal response to nematodes when nutritional resources are restricted.

Whereas a direct relationship between FEC and IgA did not appear in our zebra models, the species-level difference in IgA that arose in the zebra model may still suggest a possible link to FEC. Grevy's zebras had higher IgA than plains zebras, and although FECs did not differ between the zebras in our dataset, Grevy's at a population level typically have lower FECs than plains (Rubenstein, 2010; DIR and KJT unpublished data). Grevy's zebras may have higher baseline investment in IgA, or an IgA response that is more easily triggered than in plains zebras, and their tendency for lower FECs could result from this immune strategy. The donkeys we sampled supported the idea of opposing interspecific

differences in IgA and FEC: they had significantly lower IgA than Grevy's zebras and higher FECs than both zebra species. But what might explain the lack of a correlation between IgA and FEC for the zebras? The free-roaming zebras in our study may have been less uniformly affected by seasonal variation in local food resources than the donkeys we sampled, all of which were from the same herd and constrained in their movements and group spread by the herders. If the FEC-IgA relationship is dependent on body condition, and if interindividual variation in body condition within a given season was higher among zebras than donkeys, correlations between FEC and IgA may have been masked for the zebra datasets. The proportion of samples originating from juveniles in each species was similar and age bias was unlikely to have contributed to the interspecific differences we found; age was also not a significant factor in either the donkey or zebra model. It is also possible that zebras have more interindividual variability in immune strategies than donkeys, and that the FEC-IgA relationship is only present at the population level in the latter species. Still, the seasonal nature of the FEC-IgA relationship, its apparent dependence on body condition in donkeys, and the positive correlation between IgA and body weight in Soay sheep (Nussey et al., 2014) indicate that factoring body condition into further eco-immunological studies of IgA may be important in revealing host-parasite dynamics.

In Soay sheep faecal samples, total IgA was not related to FECs; nematode-specific Ig measures were the only immune metrics found to relate to FECs. Total IgA correlated with anti-*Teladorsagia circumcincta* (anti-Tc) IgA in the sheep (Sparks et al., 2018; Watt et al., 2015), raising the possibility that the relationship we observed between total IgA and FECs in donkeys emerged indirectly from total IgA relating to nematode-specific IgAs, particularly if the proportion of total IgA that is anti-nematode in the gut mucosa is higher in equids than in ovids. Our attempts at measuring anti-nematode IgA using antigen derived from a mixture of zebra nematodes may have failed for a number of reasons. Different helminth species can interact with different aspects of the immune system – for example, tissue eosinophilia suppresses *Haemonchus contortus* but not *T. circumcincta* larvae in sheep (Henderson and Stear, 2006). Our genetic and morphological analyses of a subset of nematodes we extracted from zebras revealed a species from the *Strongylinea* family and a wide diversity of cyathostomin species, some



**Fig. 3.** In donkeys, FEC and IgA are correlated among individuals in high body condition (with a body condition score > 2.5), in data pooled across years. Shading represents standard error, significant correlations are shown in solid lines, and non-significant trends in dotted lines.

of which may not interact with IgA (DIR and KJT, in preparation). The nematode antigen mixture we derived from the species expelled in dung at the time of this study may therefore have been too low in antigens that interact with IgA to clear background. The successful use of nematode antigen mixtures in measuring IgG in donkey seral samples suggests that circulating Ig's, or perhaps IgG isotypes, may be more amenable for mixed antigen capture (Abo-Aziza et al., 2017). Further work using monospecific nematode antigens will help to determine the specificity of IgA responses to mixed helminth infections in equids.

FECs are thought to be a crude indicator of parasite burden at best (Nielsen et al., 2010). This measure more accurately represents the total nematode reproductive output at a given time, but it is a measure of parasite activity that is particularly likely to relate to IgA due to the documented effects of this immunoglobulin on worm fecundity (Hendawy, 2018; Stear et al., 1995). Because the energy and materials used to produce eggs originates from host blood and mucosa in the case of equid strongyles, which are blood-suckers or plug-feeders (Jacobs, 1986), and because worm length and therefore metabolism correlates with the number of eggs it produces (Kuzmina et al., 2012), FECs are also a reasonable measure of the costs, in terms of resource extraction, that the adult strongyle nematode burden imposes on its host. Moreover, while measures of additional immune molecules and fitness-related indicators would help to ascertain the full costs of nematodes to equid hosts, measures that focus on nematode reproduction have the benefit of more directly addressing questions of evolution and epidemiology.

Both FECs and faecal Ig measures are typically plagued by high variability, making it difficult to see patterns in the data (Denwood et al., 2012; Nussey et al., 2014). For FECs in horses, the main source of variation appears to be individual host identity, but the subsample and, to a lesser extent, the faecal pile and faecal bolus from which the counts are drawn also contribute to FEC variability (Carstensen et al., 2013; Denwood et al., 2012). Drawing samples from more than 20g of homogenized dung as we did should greatly reduce FEC variability, and analysing multiple subsamples per sample in future studies can further reduce noise in FEC data. The inherent variability in both FECs and Ig measures make finding patterns more difficult; the correlation we found between FECs and Igs in donkeys in 2018 and in donkeys in high body condition across years is therefore likely to be even stronger than our results indicate.

Non-invasive methods for taking immune measurements are just beginning to be used for wildlife research (Hayward et al., 2014; Nussey et al., 2014; Watt et al., 2015). These techniques hold great promise for expanding available data on the internal state of animals for which

obtaining blood samples is logistically or ethically difficult, such as endangered species or species vulnerable to capture-related injury and death. The immune system is exceedingly complex and we measured only a single immunoglobulin type, yet relationships between IgA and nematode egg density emerged in donkeys, broad differences in IgA levels across equid species corresponded with broad differences in FECs, and the fluctuations in IgA and FEC roughly tracked each other without a considerable time lag in repeatedly-sampled donkey individuals. Many more eco-immunological studies will be required to unveil the intricacies of the immune strategies of wild equids. We hope that a variety of non-invasive techniques will emerge to complement FECs and ELISAs on faecal samples to strengthen our toolkit to study host-parasite relationships in the wild.

#### Declaration of competing interest

The authors of have no conflicts of interest associated with this manuscript to report.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://>

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