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Short communication

Exploring immunoglobulin A as a stress biomarker in lions (*Panthera leo*): Validation of an immunoassay for its measurement in feces

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ABSTRACT

Immunoglobulin A (IgA) has been investigated as a stress biomarker with the potential to complement glucocorticoid measurements in welfare assessments. This study aimed to develop the methodology and validate an enzyme immunoassay (EIA) for quantifying IgA in feces (FIgA) of lions (*Panthera leo*), investigate excretion patterns of FIgA under baseline conditions in captive lions, and explore its relationship with fecal glucocorticoid metabolites (FGM). Feces were collected from 11 lions housed in stable social groups at four Spanish zoos over a period of two to six weeks. FIgA was reliably quantified using a commercial EIA, with concentrations ranging from 0.28 to 794.17 μg IgA/g feces, showing substantial intra- and inter-individual variability. Females had significantly higher FIgA concentrations than males (113.10 vs 54.96 μg IgA/g feces; $p < 0.01$). Additionally, FIgA concentrations varied across zoos ($p < 0.001$). Positive correlations were found between FIgA and FGM for all samples combined (rho = 0.43, $p < 0.001$) and across individual means (rho = 0.70, $p < 0.05$), but not consistently when examining each lion separately. This study demonstrates for the first time that IgA can be reliably quantified in lion feces, paving the way for its application in welfare studies.

Traditionally, glucocorticoids (GCs) measurement has been the gold standard method for assessing stress responses in animal welfare studies. However, GCs solely reflect arousal (i.e., low to high activation) without effectively reflecting valence (i.e., whether a given stimulus is associated with a positive or negative impact on the animal's affective state) (Beaulieu, 2023; Edwards et al., 2019b). Therefore, new trends in animal welfare research seek to incorporate additional biomarkers alongside GCs to provide a more comprehensive measure of the stress response, leading to more robust assessments of animal welfare (Whitham and Wielebnowski, 2013).

In recent decades, the secretory form of immunoglobulin A (IgA) has been investigated as a welfare indicator in humans, laboratory and domestic animals and, more recently, in a limited number of wildlife species (Edwards et al., 2019a; Staley et al., 2018; Whitham et al., 2023). In addition to its important role in mucosal immunity, acting as a first barrier against pathogens at mucosal surfaces, changes in IgA levels have been linked with the stress system (Behringer et al., 2021; Tsujita and Morimoto, 1999). However, understanding how IgA relates to stress is not so straightforward, as up- or down-regulation may depend on several factors including the type of stressor (i.e., acute vs chronic), the animal's prior exposure to stress and valence of the stimulus (Staley et al., 2018). While short-term fluctuations in IgA concentrations can vary between studies, long-term changes in IgA levels due to chronic stress appear to be more consistent, resulting in decreased IgA levels (Lantz et al., 2018; Staley et al., 2018).

Before incorporating IgA into animal welfare assessments, thorough

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validation of the methodology and assays for the species under study is essential. Additionally, understanding baseline excretion patterns and concentrations is necessary. Therefore, the present study aimed to: 1) Develop a reliable method for extracting IgA from feces and biochemically validate a commercial enzyme immunoassay (EIA) for the quantification of fecal IgA (FIgA) in lions (*Panthera leo*); 2) Examine patterns of excretion of FIgA in captive lions considered to be in stable social and environmental conditions; and 3) Explore the relationship between FIgA and fecal glucocorticoid metabolites (FGM) concentrations.

The study included 11 adult lions (7 females, 4 males; mean ± SD age: 9.2 ± 3.5 years old, range 5–15) from four Spanish zoos that were considered in good health and in stable social and environmental conditions (Table 1). Fecal samples ($n = 110$; range 5–15 samples per lion) were collected for two to six weeks between April and June of 2021 and stored at −20 °C. Nontoxic shredded colored waxes (Giotto-bebe®, Fila Iberia S.L., Spain) were used as indigestible markers when necessary for individual identification of feces (Serres-Corral et al., 2021).

Fecal samples were first dried at 60 °C for four to six days, grinded into fecal powder and sieved with a mesh strainer to remove undigested materials. For FIgA extraction, a saline-based protocol was adapted from Edwards et al. (2019). In brief, 3 mL of phosphate-buffered saline (PBS) with Tween (PBS-T: 0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl, 0.1 % Tween® 20, pH 7.4 ± 0.2) were added to 0.1 g of fecal powder and vortexed for 10 min. Samples were then centrifuged at 7.750 ×*g* for 10 min at 4 °C, the supernatant transferred to a new microcentrifuge tube and centrifuged again at 13000 ×*g* for 5 min at 4 °C. Fecal IgA concentrations were diluted as necessary (neat to 1:100) and measured with an IgA Cat EIA kit (Product #ab190547, Abcam®, Cambridge, UK) following the manufacturer's instructions. The assay was validated for lion feces following the criteria for an immunological validation by verifying precision, specificity and accuracy. Steroid extraction and quantification of FGM concentrations was conducted as part of a previous study aiming to evaluate adrenocortical function of lions in captivity (Serres-Corral et al., n.d., *manuscript under review*). For the present study, the original FGM concentrations were directly incorporated without additional processing to assess their relationship with FIgA concentrations.

Table 1
Information relative to sex and reproductive status of the 11 lions studied and descriptive statistics for FIgA levels (μg/g feces) including number of samples (n), mean ± standard deviation (SD), range and coefficient of variation (CV).

Lion	Sex	Reproductive status	n	Mean ± SD	Range	CV%
BPV1	F	Ovariectomized	13	55.41 ± 50.26	0.56–176.87	90.71
BPV2	M	Intact	15	24.87 ± 21.89	0.92–70.39	88.00
BPV3	F	Ovariectomized	8	33.70 ± 41.67	2.47–107.28	123.67
BPV5	F	Ovariectomized	13	56.25 ± 70.16	0.72–201.23	124.73
CA2	M	Intact	14	38.01 ± 48.87	0.28–150.82	128.55
CA3	F	Intact	12	152.84 ± 142.83	0.71–383.85	93.45
CA4	F	Intact	6	91.68 ± 132.76	5.49–356.24	144.81
JZ1	M	Intact	5	150.67 ± 143.20	47.92–396.88	95.04
MAD1	F	Contracepted	7	179.72 ± 274.88	20.35–794.17	152.95
MAD2	M	Intact	11	117.55 ± 172.57	1.80–515.06	146.80
MAD3	F	Contracepted	6	331.35 ± 147.98	67.50–525.48	44.66

Abbreviations: BPV, Bioparc Valencia; CA, Parque de la Naturaleza de Cabárceno; JZ, Zoobotánico Jerez; MAD, Zoo Aquarium de Madrid; F, female; M, male.
Two females (BPV4 and CA1) were excluded from the study for not meeting the established criteria. Female BPV4 was diagnosed with a reproductive disease and CA1 was being segregated from the group.

Data was analyzed with R software (Version 4.3.0, R Development Core Team) and graphically represented with GraphPad Prism (Version 8.0.2; Graph Pad Software Inc.). The level of significance was set at p value <0.05. A generalized linear mixed model (GLMM) with gamma distribution and log link function was used to evaluate the effects of sex and zoo on FIgA levels, including individual as random factor. Only zoos that had individuals of both sexes were included in the model, thus excluding the sole male from JZ. Square root transformation of FIgA concentrations was employed to improve the GLMM performance on the analysis of residuals. The Walds Chi-Square (χ^2) test was used to evaluate the marginal significance of the fixed factors in the model, and Tukey-adjusted post-hoc comparisons were conducted to investigate significant differences between zoos. Spearman's rank correlations of untransformed data were used to assess the relationship between FIgA and FGM concentrations.

The assay was successfully validated for measuring FIgA in lions. Precision was demonstrated by calculating the coefficient of variation (CV) within and between plates. The intra-assay CV was 4.56 % for samples determined per duplicate and triplicate on the same plate ($n = 14$). For the inter-assay CV, three pools of FIgA extracts at low and high concentrations were determined per duplicate in four plates. The calculated inter-assay CVs were 4.47 %, 8.98 % and 6.75 % (pool mean concentrations: 38.09, 98.51 and 127.93 ng FIgA/mL, respectively). Specificity was assessed through a linearity of dilution test by diluting a pool of samples at multiple ratios (1:2, 1:3, 1:4, 1:6) with the buffer solution provided by the assay. Observed IgA concentrations were compared with the expected concentrations and results demonstrated a strong correlation between obtained and expected values (Pearson $r > 0.99$, $p < 0.001$). Accuracy was measured through the spike-and-recovery test by spiking different volumes of the sample pool with different volumes of known concentrations of the Cat IgA standard provided by the assay kit (12.99, 25.73 and 99.28 ng IgA/mL). For each combination, the recovery percentage was calculated from the observed and expected IgA concentrations. The average recovery percentage obtained was 90.15 ± 6.65 % (mean ± SD), indicating that the assay was highly accurate measuring IgA in feces for the species.

Fecal IgA concentrations ranged from 0.28 to 794.17 μg/g feces. Consistent with previous studies (Behringer et al., 2021; Edwards et al., 2019a; Tress et al., 2006), marked intra- and inter-individual variability was observed (Table 1).

In other species, a number of biological factors have been identified as contributors to the high variability observed between animals in FIgA levels (e.g., age group and sex in reindeer (Yin et al., 2015), sex in Barbary macaques (Behringer et al., 2021) or reproductive status in female baboons (Gesquiere et al., 2020)). In our study, sex was evaluated and significantly influenced FIgA concentrations ($\chi^2 = 9.78$, df = 1, $p < 0.01$), with mean concentrations in females being twice as high as those in males (Fig. 1a). In most of the wild species studied, mainly non-human primates, sex seemingly has no influence on FIgA concentrations (Ferreira et al., 2021; Gesquiere et al., 2020; Huang et al., 2014; Lantz et al., 2018; Whitham et al., 2023). However, in reindeer, young females had higher FIgA levels than males of the same age (Yin et al., 2015), whereas the opposite was described in Barbary macaques, with males presenting higher FIgA concentrations than females (Behringer et al., 2021). It is argued that these differences might be attributed to trade-offs between reproduction and immunity at times of high energetic demand for both females (e.g., birth) and males (e.g., mating competition) (Albery et al., 2020; Ferreira et al., 2021; Metcalf and Graham, 2018). However, whether the sex differences found in FIgA concentrations in lions are due to a greater reproductive effort in males, leading to lower FIgA levels, is unknown.

Levels of FIgA also varied across zoos ($\chi^2 = 18.51$, df = 2, $p < 0.001$) (Fig. 1b). Post-hoc tests revealed significantly higher FIgA levels in MAD than in BPV ($p < 0.001$). Additionally, there was a statistical trend

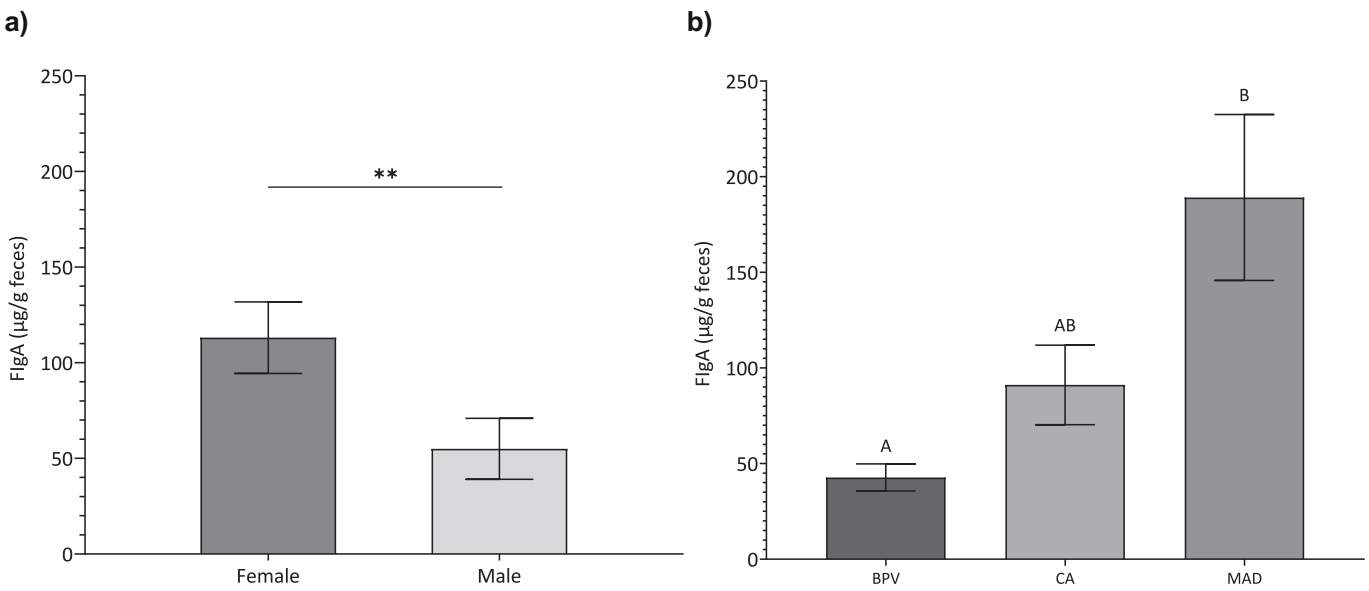

Fig. 1. Mean (±SEM) fecal Immunoglobulin A (FIgA) concentrations for (**a**) females and males and (**b**) across zoos (BPV, CA, MAD). Asterisks (**) denote significant diferences between sexes ($p < 0.01$). Different upper-case letters indicate significant differences ($p < 0.001$) between zoos.

towards higher FIgA concentrations in MAD compared to CA ($p = 0.06$), but no significant differences were observed between BPV and CA ($p > 0.05$). Although all males studied were intact, the reproductive status of females differed among zoos (Table 1). Therefore, we cannot discount the reproductive status of females as a potential confounding factor in the observed differences between zoos.

When assessing the relationship between the immune and stress systems, significant positive correlations between FIgA and FGM concentrations were found for all samples combined (rho = 0.43, $p < 0.001$, $n = 127$) and across individual means (rho = 0.70, $p < 0.05$, $N = 11$). However, applying a longitudinal approach to examine the relationship between the two biomarkers for each lion individually yielded highly variable results, with no clear trends observed among them. While in most lions ($N = 9$) no significant relationship was found between FIgA and FGM levels (rho range = −0.30 to 0.71, $p > 0.05$, n range = 6 to 14), two lions had a significant positive correlation (BPV1: rho = 0.58, $p < 0.05$, $n = 13$; BPV2: rho = 0.62, $p < 0.05$, $n = 15$). Changes in secretory IgA concentrations are in part mediated by GCs (Staley et al., 2018), thus a correlation between both biomarkers would be expected. In fact, positive correlations between FIgA and FGM concentrations have been previously described in animals in good health and standard conditions exposed only to moderate daily stressors (Huang et al., 2014; Pihl and Hau, 2003). However, as Edwards et al. (2019a) pointed out, these studies rely on single-point sampling or averaged values prior to analysis, but do not evaluate the relationship between the two biomarkers over time. In the few studies that have explored the temporal dynamics between FIgA and FGM, no apparent correlation has been found (Bundgaard et al., 2012; Edwards et al., 2019a; Kosaruk et al., 2020). Therefore, further studies monitoring FIgA and FGM concentrations in lions over short- or long-term stressful conditions would render greater value in assessing the potential role of FIgA as a stress biomarker for animal welfare assessments.

The present study validated a methodology for the extraction of FIgA and proved that the EIA used was suitable for its measurement in lions. We explored FIgA concentrations under baseline conditions and found that FIgA levels were influenced by sex regardless of zoo location. Furthermore, the relationship between FIgA levels and the stress system under baseline conditions was assessed, laying the groundwork for future research to explore how this biomarker relates to stress in biologically relevant contexts.

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CRediT authorship contribution statement

Paula Serres-Corral: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Sergi Olvera-Maneu:** Writing – review & editing, Investigation. **Vanessa Almagro:** Writing – review & editing, Resources. **Loles Carbonell:** Writing – review & editing, Resources. **Santiago Borragán:** Writing – review & editing, Resources. **Eva Martínez-Nevado:** Writing – review & editing, Resources. **Miguel Angel Quevedo:** Writing – review & editing, Resources. **Hugo Fernández-Bellon:** Writing – review & editing, Supervision, Resources, Conceptualization. **Annaïs Carbajal:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Manel López-Béjar:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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