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Noninvasive Assessment of Stress and Reproduction in Captive Lions (*Panthera leo*) Using Fecal Hormone Analysis

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ABSTRACT

Assessing steroid hormones through feces provides invaluable insight on the stress and reproductive physiology of wildlife, and has been broadly applied to monitor the health and welfare of wild animals managed under human care. This study utilized fecal hormone monitoring to evaluate adrenal and gonadal activity in 18 captive lions (*Panthera leo*) across five Spanish zoological institutions, focusing on how biological and management factors affect these metrics. We analyzed fecal glucocorticoid metabolites (FGM) concentrations and, additionally in females, fecal progesterone (FPM), estradiol (FEM), and androgen (FAM) metabolites in relation to their reproductive status. Results indicated significant variability in FGM levels among individuals, with no consistent trends influenced by sex or zoo environment, including changes in the frequency of providing environmental enrichment at two zoos. Importantly, reproductive status significantly impacted adrenal and gonadal hormone levels; ovariectomized lionesses showed lower concentrations of FGM, FPM, and FEM compared to intact and deslorelin-implanted females, the latter of which exhibited higher and more variable FAM levels. These findings advance our understanding of hormone patterns in lions and suggest implications for their management in captivity.

1 | Introduction

Noninvasive hormone monitoring is extensively utilized to assess endocrine function in wildlife, providing insights into stress, welfare, and reproductive statuses. This approach significantly enhances management practices and conservation efforts for endangered species (Schwarzenberger 2007; Schwarzenberger and Brown 2013; Kersey and Dehnhard 2014). Hormone measurements in feces are particularly advantageous as they allow for long-term monitoring without disturbing the animals, reflecting hormonal activity over a species-specific intestinal transit time (Möstl and Palme 2002; Millspaugh and Washburn 2004; Palme 2019). Consequently, fecal analysis of steroid hormone metabolites serves as a practical method to evaluate the activity of the hypothalamic-pituitary-gonadal (HPG) and adrenal (HPA) axes in wildlife.

In captive settings, numerous management and environmental conditions have been documented to impact adrenal and reproductive functioning, as demonstrated for several felid

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species (Wielebnowski et al. 2002; Wielebnowski et al. 2002; Dembiec et al. 2004; Wells et al. 2004; Fanson and Wielebnowski 2013; Vaz et al. 2017; Fazio et al. 2020; Panchal et al. 2022). Yet, the effect of environmental and husbandry factors on the physiology of lions (*Panthera leo*) remains poorly explored. Research on the species' management conditions has primarily relied on behavioral data, with a main focus on enrichment (Bashaw et al. 2007; Ncube and Ndagurwa 2010; Martínez-Macipe et al. 2015; Quintavalle Pastorino et al. 2017; Soriano et al. 2018; Regaiolli et al. 2019; Finch et al. 2020) and, to a lesser extent, on housing conditions and environment changes (Kohari et al. 2017; Galardi et al. 2021; Williams et al. 2021; Seyrling et al. 2022). In 2007, a multi-institutional survey was conducted on the social, management, and environmental conditions of African lions in North American zoos, revealing several husbandry practices associated with reproductive success for the subspecies (Daigle et al. 2015). More recently, researchers explored the link between personality and stress as a tool to improve individual animal welfare during challenging husbandry practices. Results revealed a negative relationship between agreeableness and fecal glucocorticoid metabolites (FGM) levels, suggesting that lions with carefree traits may better overcome stressful situations (Vaz et al. 2022). To our knowledge, only two other studies on lions have investigated adrenocortical activity in relation to zoo husbandry. Goswami et al. (2021) evaluated the behavioral and physiological responses of Asiatic lions to a series of enrichment interventions and described improved behavioral parameters and lower glucocorticoid concentrations post-enrichment. In addition, previous work by our research group showed how a decrease in daily handling coupled with a more stable social environment resulted in a decrease in adrenocortical activity in African lions (Serres-Corral et al. 2021).

Lions breed reasonably well in captivity compared to other felids (Bertschinger et al. 2008; Putman et al. 2015), to the extent that contraception has been widely applied as a management tool to limit reproduction in zoo collections (Seal et al. 1976; Asa et al. 2012). A thorough understanding of the reproductive biology of the species is essential for *ex situ* conservation efforts. However, until the last decade, only a limited number of endocrinological studies on lions had investigated various aspects of their reproduction (Schmidt et al. 1979; Brown et al. 1993; Schramm et al. 1994; Graham et al. 1995). With the development of noninvasive fecal steroid hormone assays, longitudinal monitoring of sex hormones made it possible to characterize ovarian steroid profiles in females (Umapathy et al. 2007; Putman et al. 2015; Callealta et al. 2024) and to investigate changes in steroid excretion patterns with age in males (Putman et al. 2019). Additionally, several studies have investigated the impact of hormonal contraception on the HPA and HPG axes in females, focusing mainly on the efficacy and reversibility of the different contraceptive drugs (Bertschinger et al. 2008; Moresco et al. 2014; Putman et al. 2015; Braga et al. 2020; Kawase et al. 2021).

This multi-institutional study explores adrenocortical activity in lions in relation to several biological factors and management practices to expand knowledge on the stress physiology of lions in captivity. Besides, we add to the understanding of reproductive physiology in lions by comparing sex hormone concentrations between intact, hormonally contracepted, and ovariectomized females. In addition to fecal progesterone metabolites (FPM) and fecal estradiol metabolites (FEM), this study is the first to examine fecal androgen metabolites (FAM) in lionesses and investigate how concentrations differ with reproductive status. Androgens have been traditionally understood solely as male hormones (Gesquiere et al. 2014; Pavitt et al. 2016), being reduced to simple precursors of estrogens in females (Gleicher et al. 2011; Fürtbauer et al. 2013). In recent years, however, more attention has been drawn to the biological significance of these hormones in the opposite sex, including their potential roles in female sexual behavior and aggression (Gesquiere et al. 2014; Setchell et al. 2015).

Taking into account the above, the specific objectives of the present study were to: (1) characterize individual FGM profiles in lions and explore the physiological response to a change in the environmental enrichment program at two zoological institutions; (2) evaluate the impact of biological factors (i.e., sex, age, and reproductive status) and zoo on baseline FGM concentrations; and (3) examine FPM, FEM and FAM concentrations in females in relation to reproductive status.

2 | Materials and Methods

2.1 | Study Animals and Sample Collection

This study included 18 lions of the African (*Panthera leo*, $n = 14$) and Asiatic subspecies (*Panthera leo persica*, $n = 4$) housed at five Spanish zoological institutions: Barcelona Zoo (BCN), Bioparc Valencia (BPV), Parque de la Naturaleza de Cabárceno (CA), Zoobotánico Jerez (JZ) and Zoo Aquarium de Madrid (MAD) (Table 1). All lions were in good health and under stable social and environmental conditions, except for two females (see Section 2.5.2).

Hormonal contraception in females consisted of deslorelin acetate implants (Suprelorin, Virbac, France). In all cases, reproduction was downregulated for more than 3 years at the time of sampling. Two implants were given to each female every 12 to 24 months in accordance with the species-specific recommendations for 4.7 and 9.4 mg Suprelorin. In addition, a female at BPV (BPV4) was diagnosed with a bilateral ovarian leiomyoma, thus the reproductive status for this lioness was defined as a reproductive disease.

Fecal samples were collected for 1 to 2 months between March and April 2019 (BCN) and between April and June 2021 (BPV, CA, JZ, and MAD). Feces were collected daily during the morning cleaning routines and immediately stored at −20°C until steroid hormone extraction. In those zoos where individual identification of feces was not possible with usual management routines (i.e., individual housing), nontoxic shredded colored waxes (Giotto-bebe, Fila Iberia S.L., Spain) were used as fecal markers as previously described for the species (Serres-Corral et al. 2021). The fecal markers were evident in feces within 24-48 h after ingestion, indicating the gut transit time for the species.

TABLE 1 | Zoological institution, biological information (subspecies, sex, age, and reproductive status), and number of fecal samples collected (n) from the 18 lions studied.

Zoo	Subspecies	Lion	Sex	Age (years)	Reproductive status	*n*
BCN	African	BCN1	Male	2.9	Intact	14
		BCN2	Male	14.6	Intact	6
		BCN3	Female	14.5	Contracepted	12
		BCN4	Female	14.6	Contracepted	11
		BCN5	Female	14.5	Contracepted	7
BPV	African	BPV1	Female	15.6	Ovariectomized	13
		BPV2	Male	5.0	Intact	15
		BPV3	Female	6.0	Ovariectomized	8
		BPV4	Female	12.8	Reproductive disease	11
		BPV5	Female	10.0	Ovariectomized	13
CA	African	CA1	Female	20.9	Intact	6
		CA2	Male	5.7	Intact	14
		CA3	Female	13.0	Intact	12
		CA4	Female	13.0	Intact	6
JZ	Asiatic	JZ1	Male	8.1	Intact	15
MAD	Asiatic	MAD1	Female	7.3	Contracepted	15
		MAD2	Male	10.8	Intact	23
		MAD3	Female	6.7	Contracepted	16

Abbreviations: BCN, Barcelona Zoo; BPV, Bioparc Valencia; CA, Parque de la Naturaleza de Cabárceno; JZ, Zoobotánico Jerez; MAD, Zoo Aquarium de Madrid.

2.2 | Housing and Husbandry Conditions

A survey was completed by each zoological institution to obtain accurate information on their housing and husbandry conditions, including details of the outdoor and indoor enclosures, management routines, social structure, nutrition, and environmental enrichment program (Table 2).

2.2.1 | Change in the Environmental Enrichment Programs in MAD and JZ

In addition to the regular behavioral enrichment program described in Table 2, MAD and JZ implemented changes in their respective programs during the study. For JZ, the change consisted of increasing the frequency to a daily placement of novel stimuli/items (i.e., scents, hidden food, and objects) on the third week of the study. In the case of MAD, the frequency was also increased to three-to-four times a week for two consecutive weeks in the middle of the study. Therefore, to evaluate the effect of these changes on FGM concentrations, three study phases were established: (1) Pre-environmental enrichment phase (Pre-EE): the lions were in basal conditions and were provided with the regular enrichment program of the zoo; (2) Environmental enrichment phase (EE): the specific changes in the regular enrichment program are implemented as described above for each zoo; and (3) Post-environmental enrichment phase (Post-EE): return to the basal enrichment conditions established in their programs.

2.3 | Fecal Steroid Hormone Extraction

Steroid hormone extraction was conducted following a methanol-based protocol previously described by our research group for the species (Serres-Corral et al. 2021). Briefly, all fecal samples were dried in an oven (Memmert Universal oven UF55, Memmert, Germany) at 60°C until completely dry (four to six days). Then, samples were pulverized using a Mixer Mill (MM2 type; Retsch, Germany) and sieved with a mesh strainer to remove undigested and/or fibrous materials (Jepsen et al. 2021). Subsequently, 5.5 mL of 55% methanol was added to 0.3 g of powdered sample, and the mixture was vortexed (Vortex Mixer S0200-230 V-EU; Labnet International Inc., USA) for 30 min. Samples were then centrifuged (Hermle Z300K; Hermle Labortechnik, Germany) at 7750× g for 15 min and the supernatant was transferred into 1.5 mL microcentrifuge tubes. The resulting fecal extracts were frozen at −20°C until steroid hormone determination.

Feces from BCN were collected as part of a previous study on lions (Serres-Corral et al. 2021). Therefore, steroid hormone extraction for those samples was conducted in 2019 and fecal extracts have been stored frozen (− 20°C) since then. For the present study, BCN samples were analyzed again together with all the samples collected in 2021 (BPV, CA, JZ, and MAD). Before data analysis, the impact of storing fecal extracts for 3.5 years on measurable FGM concentrations was evaluated. The initial concentrations obtained (2019) and the concentrations obtained in the present study did not differ statistically (Two-tailed pairwise t test: $t = 1.22$, $df = 49$, $p > 0.05$) and correlated strongly (Pearson correlation: $r = 0.70$, $p < 0.001$).

TABLE 2 | Details on the housing and management conditions of the lions at each zoo.

Zoo	Outdoor enclosure size	Housing conditions at night	Feeding regime	Environmental enrichment program
BCN	1090 m^2	Split group[†]	3–6 kg horse/beef bone-in meat (evening); fasting once a week	Sporadically; scents, noises, food, and objects
BPV	360 m^2	Group	2.5–3 kg chicken/beef bone-in meat (evening); no fasting	Daily; food
CA	16000 m^2	Individual	3–5 kg horse/beef bone-in meat (evening); half diet once a week	No
JZ	150 m^2	Alone[‡]	4–5 kg beef boneless meat (evening); fasting once a week	Sporadically; scents, food, and objects
MAD	1600 m^2	Split group (Male; females)	7–8 kg chicken/beef bone-in meat (evening); fasting once a week	Sporadically; scents, noises, food, and objects

Abbreviations: BCN, Barcelona Zoo; BPV, Bioparc Valencia; CA, Parque de la Naturaleza de Cabárceno; JZ, Zoobotánico Jerez; MAD, Zoo Aquarium de Madrid.
[†]Both males (BCN1 and BCN2) were permanently separated to avoid cohabitation conflicts. At night the group was divided into two: BCN1, BCN4, BCN5 and BCN2, BCN3.
[‡]There was only a single lion in the zoo.

2.3.1 | Sample Selection for Sex Steroid Hormone Quantification in Females

For each female, a small number of fecal extracts (n = 5 per lioness) were selected from the complete set of samples for the quantification of FPM, FEM, and FAM concentrations. Generally, a weekly sample was selected, thus representing the entire month of sampling.

2.4 | Steroid Hormone Analysis

Steroid hormone concentrations were measured with commercial enzyme immunoassays (EIA) specific for each hormone. Before analysis, fecal extracts were diluted with the buffer solution provided by the EIA kit as necessary for each hormone: 1:5 for FGM and 1:100 for FPM, FEM, and FAM (sample volume:total volume).

Concentrations of FGM were measured with a cortisol EIA kit (Neogen Corporation, Ayr, UK) previously validated for the species and matrix (Serres-Corral et al. 2021). Intra and inter-assay coefficient of variation (CV) were reevaluated for this study, and were 9.25 ± 1.42% and 11.76%, respectively.

Concentrations of FPM, FEM, and FAM were determined with progesterone, estradiol, and testosterone commercial EIA kits (Neogen Corporation, Ayr, UK), respectively. All assays were analytically validated by verifying: 1) precision within the assay by calculating the intra-assay CV of pooled samples read in duplicate or triplicate; 2) specificity (linearity of dilution test) by diluting pooled samples at multiple ratios between 1:2 and 1:10 with the buffer solution provided by the EIA kit; and 3) accuracy (spike-and-recovery test) by adding known amounts of pooled samples to known amounts of different pure standard hormone solutions provided by their respective EIA kits, and calculating the difference between the expected and observed concentrations (Table 3).

2.5 | Statistical Analysis

R software (R-project, Version 4.3.0, R Development Core Team, University of Auckland, New Zealand) and Graph Pad Software Inc. (GraphPad Prism, version 8.0.2; Graph Pad Software Inc., San Diego, CA, USA) were used for statistical analysis and graphical representation of the data. The statistically significant level was settled at a p value < 0.05 in all tests performed. In addition, given the small sample size, post-hoc pairwise comparisons showing a tendency ($p < 0.1$) were also described.

2.5.1 | Individual FGM Profiles

Levels of FGM were first evaluated longitudinally for each lion by identifying baseline and peak concentrations. An iterative process was conducted in which concentrations greater than the mean + 1.5 standard deviations (SD) were systematically eliminated until no values exceeded that cut-off (mean + 1.5 SD). All values above

TABLE 3 | Analytical validation of the progesterone, estradiol, and testosterone Neogen EIA kits. Cross-reactivity of the hormone antibodies as reported by the manufacturer for the respective EIA kits is also shown.

EIA kit	Precision (%CV)	Specificity (R^2; mean error)	Accuracy (mean ± SD recovery)	Cross-reactivity[†]
Progesterone	11.27%	0.99; 8.89%	120.48 ± 16.19%	Progesterone 100.0%, deoxycorticosterone 2.5%, corticosterone 2.0%, pregnenolone 2.0%, androstenedione 1.0%, 17-hydroxyprogesterone 0.40%, testosterone 0.29%, cortisol 0.20%, cortisone 0.20%, dehydroepiandrosterone 0.20%, estradiol 0.20%, estriol 0.20% and estrone 0.20%.
Estradiol	4.01%	0.99; 10.22%	105.91 ± 7.08%	17ß-estradiol 100.0%, testosterone 1.0%, estriol 0.41% and estrone 0.10%.
Testosterone	7.07%	0.98; 4.07%	96.97 ± 7.86%	Testosterone 100.0%, dihydrotestosterone 100.0%, androstenedione 0.86%, bolandiol 0.86, testosterone enanthate 0.13%, estriol 0.10% and testosterone benzoate 0.10%.

Abbreviations: EIA, enzyme immunoassay; CV, coefficient of variation.
[†]Steroids with a cross-reactivity < 0.06% are not shown.

the cut-off were then classified as peaks. Individual FGM profiles were summarized using descriptive statistics. Kruskal–Wallis tests were conducted to compare overall and baseline FGM levels among lions in each zoo, except for JZ where only a single male was present. When statistically significant differences were detected, post hoc pairwise comparisons using the Wilcoxon rank-sum test with Holm correction were used.

The effect on FGM levels resulting from changes implemented in the environmental enrichment programs in MAD and JZ was examined for each lion (MAD1, MAD2, MAD3, and JZ1). Individual Kruskal–Wallis tests were conducted to evaluate overall and baseline FGM levels between the studied phases (Pre-EE, EE, and Post-EE). Wilcoxon rank-sum tests with Holm correction were utilized for post hoc pairwise comparisons.

2.5.2 | Influence of Biological Factors and Location on Baseline FGM Concentrations

Data was not normally distributed (Shapiro–Wilk test: $p < 0.05$) even after log transformation, thus non-transformed baseline FGM concentrations were analyzed using generalized linear mixed models (GLMM) with gamma distributions and log link functions. First, candidate models were built to assess the influence of age, sex, zoo, and their interactions on baseline FGM concentrations, including individual as random factor in all models. Akaike's information criterion corrected for small samples (AICc) was used to select the best-fit model. A second GLMM was then built to evaluate the influence of reproductive status on baseline FGM concentrations in females, with individual as random factor. The Walds $\chi 2$ test was utilized to assess the marginal significance of the fixed variables in both final GLMMs and, when statistically significant, Tukey-adjusted post hoc pairwise comparisons were conducted. Two females were excluded from statistical analysis as they were not considered to be in basal conditions. Female BPV4 was diagnosed with a reproductive disease, and CA1 was segregated from the group.

2.5.3 | Effect of Reproductive Status on Sex Steroids in Females

Female BPV4 had a reproductive disease (bilateral ovarian leiomyoma), and thus was excluded from the statistical analysis of sex steroid hormones in females. Hormone data was log-transformed to achieve a normal distribution (Shapiro-Wilk test: $p > 0.05$). Three LMM were fitted to examine the relationship between FPM, FEM, or FAM and reproductive status (intact, contracepted, and ovariectomized), considering individual as random factor. In the case of FAM analysis, two values from contracepted females were considered outliers and were removed from the data set before analysis. These two values were more than 2 SD above the mean of their group and had a strong influence on the data set and residual distributions. The significance of reproductive status was evaluated using Wald's $\chi 2$ test, and post hoc pairwise comparisons were conducted using the Tukey adjustment method.

3 | Results

3.1 | Individual FGM Profiles

Concentrations of FGM ranged from 4.23 ng/g to 79.06 ng/g, with individual mean (± SD) overall and baseline FGM levels ranging from 9.05 ± 2.94 ng/g to 35.48 ± 14.43 ng/g and 7.87 ± 1.95 ng/g to 35.48 ± 14.43 ng/g, respectively. Kruskal–Wallis tests revealed significant differences in overall FGM concentrations between individuals in BCN and CA ($p < 0.05$) and baseline FGM levels between lions in CA and MAD ($p < 0.05$) (Table 4).

Longitudinal evaluation of individual FGM profiles showed a proportion of peaks ranging from 0% to 53%, with great variability between individuals within each zoo (Figure 1).

3.1.1 | Change in the Environmental Enrichment Programs in MAD and JZ

Among all peaks detected in the individual FGM profiles, a 3.5 and twofold increase from the baseline mean in MAD1 and MAD2, respectively, coincided with the first days of implementing a change in the environmental enrichment program (EE phase) (Figure 1e).

In MAD, overall and baseline FGM levels did not differ significantly between phases in two lions (MAD2: $\chi^2 = 1.13$ and 2.50, df = 2, $p > 0.05$; MAD3: $\chi^2 = 0.95$ and 4.97, df = 2, $p > 0.05$) (Figure 2). In the other female (MAD1), significant differences were found in overall FGM concentrations between enrichment phases ($\chi^2 = 8.01$, df = 2, $p < 0.05$). Specifically, overall FGM concentrations significantly increased in the EE phase compared to the Pre-EE phase (mean ± SD = 30.73 ± 16.60 ng/g and 14.30 ± 3.18 ng/g, respectively).

Regarding the male in JZ, overall and baseline FGM concentrations did not differ significantly ($\chi^2 = 3.57$, df = 2, $p > 0.05$ and $\chi^2 = 3.14$, df = 2, $p > 0.05$, respectively) between the environmental enrichment phases studied (Figure 2). Nevertheless, a progressive reduction in the proportion of peaks was observed between phases, decreasing from 83% in the Pre-EE phase to 40% and 25% in the EE and Post-EE phases, respectively (Figure 1d).

3.2 | Influence of Biological Factors and Location on Baseline FGM Concentrations

The final GLMM explained 63% (conditional R^2) of the variance in FGM concentrations, including sex, zoo, and the interaction of sex and zoo as fixed factors. Sex alone had no significant influence on baseline FGM concentrations ($\chi^2 = 0.27$, df = 1, $p > 0.05$). A significant effect of zoo and sex:zoo interaction was detected ($\chi^2 = 149.11$, df = 4, $p < 0.001$ and $\chi^2 = 46.27$, df = 3, $p < 0.001$, respectively), suggesting that the influence of sex on baseline FGM concentrations differed between zoos. Post hoc pairwise comparisons revealed higher baseline FGM concentrations in females compared to the male in CA ($p < 0.001$), a tendency for higher baseline levels in the male compared to females in MAD ($p = 0.06$), and no significant differences between sexes in BCN and BPV ($p > 0.05$). Within the sexes, BPV females exhibited significantly lower baseline FGM levels than females from other zoos ($p < 0.001$). Likewise, males from CA and BPV had statistically lower FGM concentrations than other males ($p < 0.05$) (Figure 3a).

TABLE 4 | Kruskal–Wallis tests and post-hoc pairwise comparisons (Wilcoxon rank-sum test with Holm correction) of overall and baseline FGM concentrations among lions in each zoo. Only significant ($p < 0.05$) post-hoc pairwise comparisons and comparisons showing a tendency ($p < 0.1$) are presented. Significant differences ($p < 0.05$) are denoted in bold.

Zoo	FGM	Chi-square (df)	*p* value	Post-hoc comparisons
BCN	Overall	13.50 (4)	**0.009**	**BCN4 versus BCN1: *p* = 0.034**
				BCN4 versus BCN3: *p* = 0.051
	Baseline	8.62 (4)	0.071	
BPV	Overall	6.86 (4)	0.144	
	Baseline	5.12 (4)	0.275	
CA	Overall	17.63 (3)	**< 0.001**	**CA1 versus CA2: *p* = 0.002**
				CA2 versus CA3: *p* = 0.007
	Baseline	17.00 (3)	**< 0.001**	**CA1 versus CA2: *p* < 0.001**
				CA1 versus CA3: *p* = 0.070
				CA1 versus CA4: *p* = 0.078
				CA2 versus CA3: *p* = 0.048
				CA2 versus CA4: *p* = 0.078
MAD	Overall	5.11 (2)	0.078	
	Baseline	10.72 (2)	**0.005**	**MAD1 versus MAD2: *p* = 0.004**

Abbreviations: FGM, fecal glucocorticoid metabolites; df, degrees of freedom; BCN, Barcelona Zoo; BPV, Bioparc Valencia; CA, Parque de la Naturaleza de Cabárceno; JZ, Zoobotánico Jerez; MAD, Zoo Aquarium de Madrid.

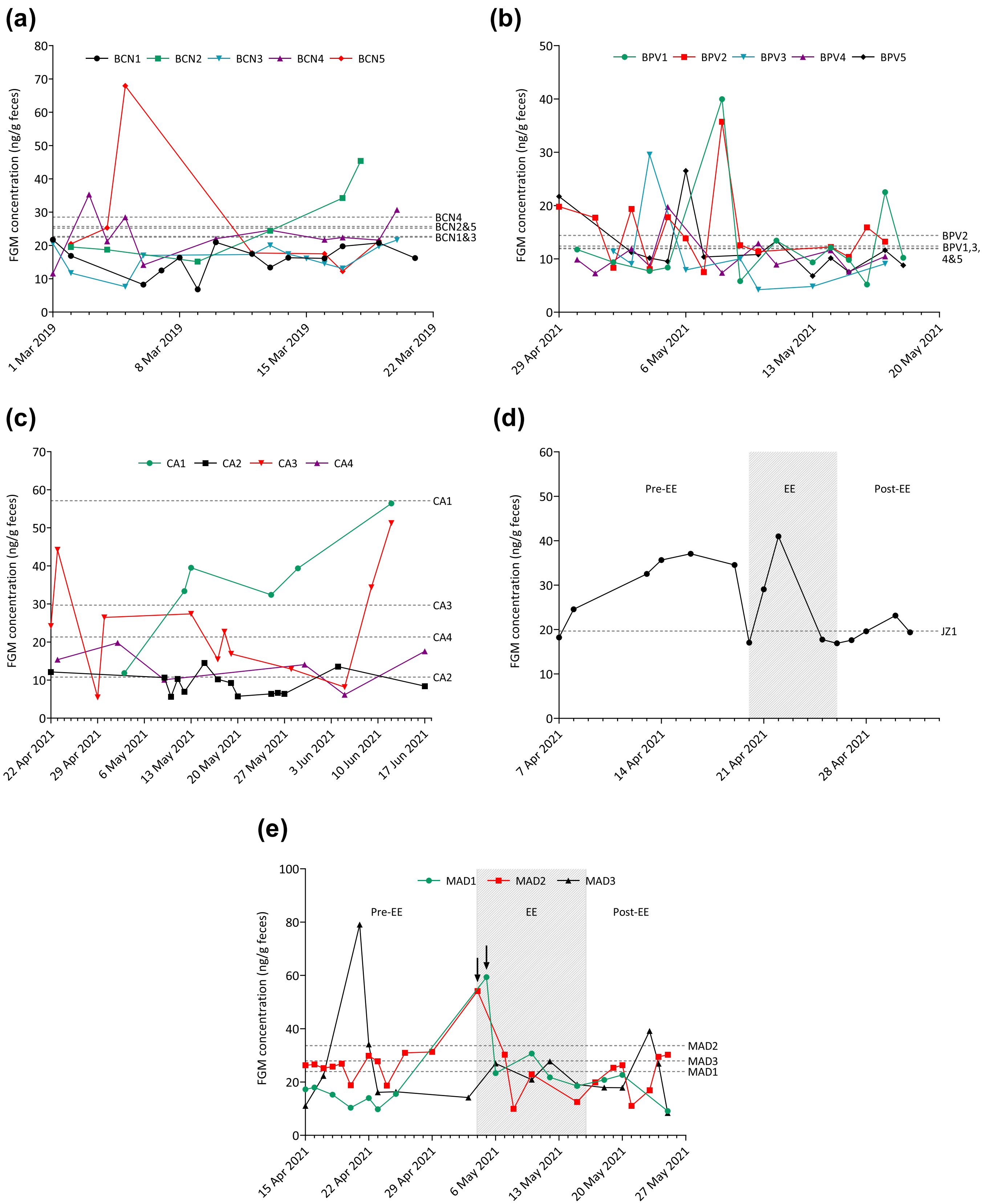

FIGURE 1 | Individual fecal glucocorticoid metabolites (FGM) profiles of lions at (**a**) Barcelona Zoo (BCN), (**b**) Bioparc Valencia (BPV), (**c**) Parque de la Naturaleza de Cabárceno (CA), (**d**) Zoobotánico Jerez (JZ), and (**e**) Zoo Aquarium de Madrid (MAD). Horizontal discontinuous lines symbolize individual cut-offs (mean + 1.5 SD), with all values above the line classified as peaks. The gray areas in MAD and JZ differentiate the environmental enrichment phase (EE) from the basal enrichment conditions before (Pre-EE) and after (Post-EE). Black arrows in MAD denote two marked peaks on the first days of the EE phase. [Color figure can be viewed at wileyonlinelibrary.com]

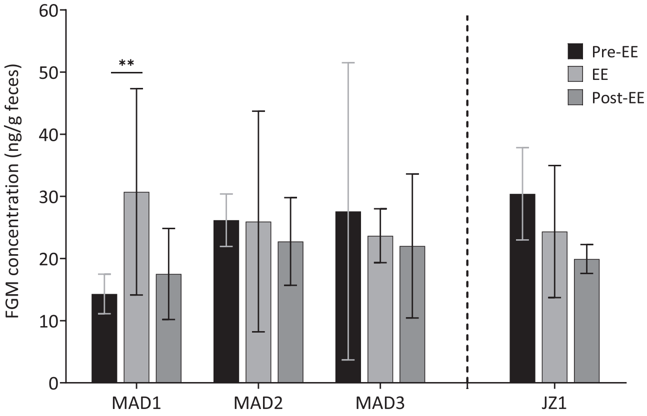

FIGURE 2 | Mean (±SD) of overall fecal glucocorticoid metabolites (FGM) concentrations for the lions at Zoo Aquarium Madrid (MAD1, MAD2, and MAD3) and for the male at Zoobotanico de Jerez (JZ1) for the three phases studied: Initial enrichment conditions before implementing changes in the program (Pre-EE), increase in the weekly frequency of placement of novel stimuli (EE), and return to the initial enrichment conditions (Post-EE). Asterisks (**) denote significant differences ($p < 0.05$) between phases.

Reproductive status in females significantly influenced baseline FGM concentrations ($\chi^2 = 60.58$, df = 2, $p < 0.001$), with ovariectomized females having lower FGM levels than intact and contracepted females (Figure 3b).

3.3 | Effect of Reproductive Status on Sex Steroid Hormones in Females

A significant influence of reproductive status was detected in all sex hormones (FPM: $\chi^2 = 9.37$, df = 2, $p < 0.01$; FEM: $\chi^2 = 42.82$, df = 2, $p < 0.001$; FAM: $\chi^2 = 7.29$, df = 2, $p < 0.05$). Post-hoc tests revealed ovariectomized females having significantly lower FPM and FEM concentrations than intact and contracepted females, and significantly lower FAM levels than contracepted females ($p < 0.05$) (Table 5).

4 | Discussion

The present study contributes to broadening knowledge of the stress and reproductive physiology of lions in captivity by characterizing fecal steroid hormone patterns in relation to various individual factors and husbandry practices. Biologically relevant variations in adrenocortical and gonadal activity were observed, demonstrating the validity of the chosen assays to reliably monitor all main steroid hormones in feces for the species. In brief, reproductive status in females significantly influenced the activity of the HPG and HPA axes. Sex had a variable effect on FGM concentrations based on the zoo, with no clear trend. Furthermore, individual FGM profiles were investigated more in depth, and variable stress responses to a number of specific conditions were detected.

4.1 | Individual Hormonal Profiles

A great variability in FGM concentrations and proportion of peaks was detected among the hormonal profiles of lions.

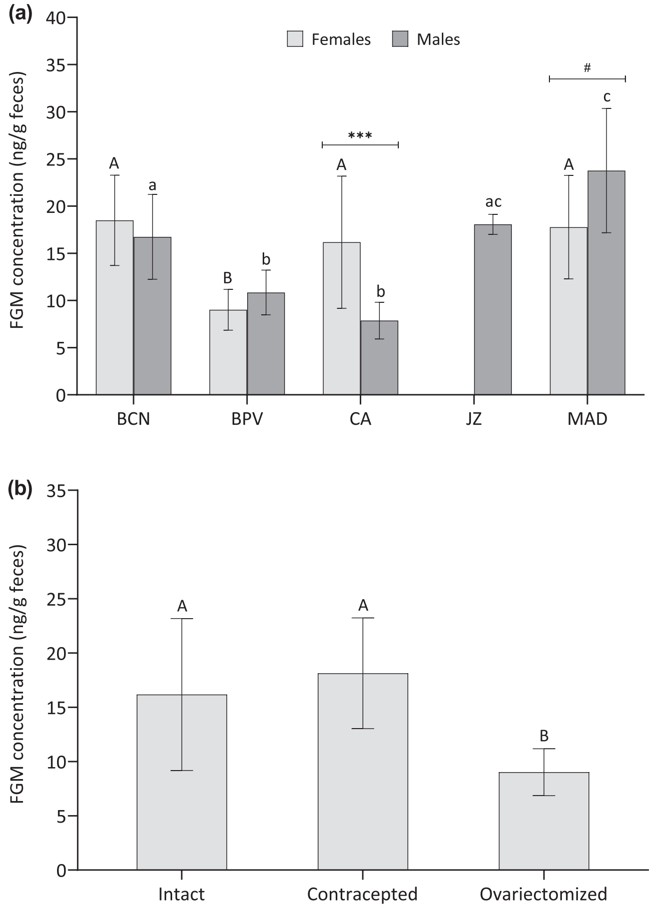

FIGURE 3 | Mean (±SD) of baseline fecal glucocorticoid metabolites (FGM) concentrations clustered by sex and zoo (**a**), and by reproductive status in females (**b**). Asterisks (***) denote significant differences ($p < 0.001$) and hash (#) a tendency ($p = 0.06$) between sexes within a zoo (**a**). Different upper-case letters indicate significant differences ($p < 0.001$) in females between zoos (**a**) and between reproductive status (**b**). Different lower-case letters indicate significant differences ($p < 0.05$) in males between zoos (**a**). *Abbreviations*: BCN, Barcelona Zoo; BPV, Bioparc Valencia; CA, Parque de la Naturaleza de Cabárceno; JZ, Zoobotánico Jerez; MAD, Zoo Aquarium de Madrid.

This was clearly exemplified in CA, where the oldest female (CA1) had relatively higher overall and baseline FGM concentrations than her conspecifics. These differences in GC excretion are consistent with the information provided by the zoo personnel indicating that this female had been repeatedly attacked by the male (CA2) in an attempt to evict her from the group. It is well known that aggressive interactions can elicit marked and persistent stress responses and that, eventually, defeated members tend to exhibit higher levels of GCs than dominant members (Zayan 1991; Creel 2001; Sapolsky 2005). Furthermore, agonistic encounters occur more frequently in captivity, where conflicts cannot be avoided as effectively as in the wild (Creel 2001). Accordingly, although there is no consensus on the hormonal profile of chronically stressed wild animals (Dickens and Romero 2013), the elevated baseline levels of FGM along with the context provided by zoo staff appear to be indicative of exposure to chronic social stress for this lioness.

TABLE 5 | Mean (±SD) concentrations and coefficient of variation (CV) of fecal progesterone (FPM), estrogen (FEM), and androgen (FAM) metabolites (ng/g feces) in females by reproductive status. Within each hormone, different upper-case letters indicate significant differences ($p < 0.05$) in females between reproductive status.

Reproductive status	FPM Mean ± SD	FPM CV	FEM Mean ± SD	FEM CV	FAM Mean ± SD	FAM CV
Intact	8228.63 ± 4983.65^{A}	61%	588.23 ± 313.15^{A}	53%	32.59 ± 13.78^{AB}	42%
Contracepted	8391.97 ± 5785.27^{A}	68%	435.62 ± 125.13^{A}	29%	38.71 ± 26.98^{A}	70%
Ovariectomized	4146.42 ± 916.05^{B}	22%	237.95 ± 71.04^{B}	30%	19.20 ± 4.82^{B}	25%

4.1.1 | Change in the Environmental Enrichment Programs in MAD and JZ

For the most part, the detected peaks of FGM could not be attributed to any recorded event. Yet, two of the three lions in MAD (MAD1 and MAD2) experienced a relevant peak on the first sample collected after increasing the frequency of days in which environmental enrichment was given. This is in agreement with previous studies in which exposure to novel enrichment items has been associated with increased GC concentrations (Baugh et al. 2017; Hambrecht et al. 2021). Still, the changes implemented in MAD had no evident impact on FGM concentrations. Only one female (MAD1) showed significantly higher overall FGM concentrations in the EE phase compared to baseline conditions (pre and post-EE). As for the changes applied in JZ, although nonsignificant, a moderate decrease in FGM levels coupled with a progressive reduction of peaks between phases was observed. Many factors can influence how an animal copes with changes or novel enrichment items, including age and sex differences in the stress response (Poessel et al. 2011) and distinct personality traits (Goswami et al. 2020; Vaz et al. 2022). Accordingly, a wide range of studies have described a certain degree of individual variability in the stress response to the same environmental enrichment routines (Cummings et al. 2007; Vasconcellos et al. 2009; Poessel et al. 2011; Rafacz and Santymire 2014; Coelho et al. 2016; Hambrecht et al. 2021). In contrast to our results, a previous study in Asiatic lions demonstrated a positive effect on behavior and stress physiology of food, sensory, and manipulable enrichment interventions (Goswami et al. 2021). In our study, the changes applied were potentially insufficient to elicit a measurable effect on adrenocortical activity. Nevertheless, results should be interpreted cautiously, as there are some intrinsic limitations of the study, including the small sample size and/or the short duration of the different phases studied. Furthermore, incorporating behavioral data into the study would have allowed for a more robust determination of the effects of a change in the environmental enrichment program.

4.2 | Influence of Biological Factors on Adrenal and Gonadal Steroids

4.2.1 | Baseline FGM Levels in Relation to Sex and Zoo

It is well known that factors intrinsic and extrinsic to the individual can influence baseline FGM concentrations, hampering the interpretation of findings in stress-related studies (Millspaugh and Washburn 2004; Palme 2005). Among others, the effects of age, sex, and reproductive status on GC production have been widely described in the literature with no consistent pattern among species (Dantzer et al. 2014; Palme 2019). Therefore, these confounding factors should be investigated for each species beforehand to accurately interpret subsequent results. In our study, age was excluded from the final statistical model, and thus could not be investigated. However, all previous studies on lions concur that age does not have an impact on FGM concentrations for the species (Putman et al. 2015; Putman et al. 2019; Vaz et al. 2022).

Sex had a variable influence on FGM levels depending on the zoo. In CA, the male presented significantly lower baseline FGM levels than females, while in MAD there was a tendency toward the opposite. In the remaining zoos, sex had no influence on GC secretion. In agreement with the latter, a recent study in lions found no sex-related differences in FGM levels, although authors suggested that it may be due to the reproductive status of the females studied (i.e., hormonally contracepted) (Vaz et al. 2022). When it comes to other Felidae, variable results have been documented between species and even within species. Namely, while a number of studies have not detected any effect of sex on FGM concentrations [tiger (Vaz et al. 2017; Jepsen et al. 2021); leopard (Panchal et al. 2022); jaguar (Mesa-Cruz et al. 2014); fishing cat (Fazio et al. 2020); cheetah (Terio et al. 2004)], others have reported that females exhibit higher FGM levels than males [tiger (Narayan et al. 2013; Parnell et al. 2014); Canada lynx (Fanson et al. 2012); clouded leopard (Wielebnowski et al. 2002)] and, a single study, described males having higher FGM concentrations than females [jaguar (Conforti et al. 2012)]. The mechanisms behind sex differences in GC concentrations may be explained by differences in the metabolism and excretion of GCs (Goymann 2012; Palme 2019), but also by differences in the reactivity of the HPA axis to stressors or variable stages of the reproductive cycle in females (Touma and Palme 2005; Parnell et al. 2014).

In our study, the non-consistent effect of sex on adrenocortical activity between zoos suggests that other confounding factors not accounted for may, in part, contribute to the variability found. For instance, social structure and rank have been described to affect baseline GC secretion (Zayan 1991; Creel 2001), although there is a lack of consensus among species on whether dominant or subordinate individuals experience a greater amount of the so-called social stress (Sands and Creel 2004; Behringer and Deschner 2017). Besides, the social structure of lions itself posed a limitation to this study. In the wild, lions live in fission-fusion social units with typically less than ten related females, their offspring, and one or few unrelated males (Matoba et al. 2013; Sogbohossou et al. 2014). In zoo settings, this structure is generally maintained, housing multiple females with one or two males (AZA Lion Species Survival Plan 2012). Accordingly, in the present study, such an unbalanced sex ratio implied having a very limited number of males, thus results should be interpreted with caution, as individual variability in males may have greatly contributed to those findings.

Likewise, when stratified by sex, non-consistent differences in adrenocortical activity were observed among zoos. In females, only BPV lionesses presented lower FGM concentrations than females in other zoos, which potentially reflects their reproductive status (i.e., ovariectomized) rather than differences between zoos. Regarding males, more variability in FGM levels was found among zoos (Figure 3a), but in line with our previous explanation, the inherent variability associated with a small sample size in males precludes drawing conclusions on whether the zoo had a real impact on FGM concentrations.

4.2.2 | FGM, FPM, and FEM Levels and Reproductive Status in Females

Among females, reproductive status had a significant effect on adrenocortical function, with ovariectomized females having lower baseline FGM concentrations than intact and hormonally contracepted females. Furthermore, as expected, ovariectomized lionesses also presented significantly lower FPM and FEM concentrations than the other groups. However, the observed differences in reproductive status must be interpreted with caution, as the zoo could be a potential confounding factor. Notably, with the exception of contracepted females, each reproductive status group was represented by lionesses from a single zoo.

It is well known that the HPA and HPG axes are tightly related, with one modulating the activity of the other and vice versa (Toufexis et al. 2014; Ghosal et al. 2023). In basal conditions, GCs have a metabolic role in energy regulation (Busch and Hayward 2009) and, for this reason, it is argued that the varying energetic demands of the different reproductive phases in females may partly explain the reported variability in FGM concentrations (Fanson et al. 2012; Glaeser et al. 2020). As such, reproductive phases of high metabolic demand like gestation and lactation have been associated with increased GC production in other mammals [e.g., tiger (Jepsen et al. 2021); pied tamarin (Price et al. 2019); roan antelope (Kamgang et al. 2022); Asian elephant (Glaeser et al. 2020)]. Furthermore, changes in GC concentrations have been described during the estrus cycle in association with the typical fluctuations in sex steroid hormones, particularly in estrogen concentrations (Knott et al. 2013; Koester et al. 2017). In line with that, a number of studies have described lower GC concentrations in gonadectomized females (Saltzman et al. 1998; Seale et al. 2004; Hydbring-Sandberg et al. 2021), potentially mediated by the loss of circulating estrogen (Da Silva 1999). A study in Canada lynx also found that spayed females had lower FGM concentrations than intact females, but similar to those of males, thus further highlighting the potential correlation of ovarian activity with GC production (Fanson et al. 2012).

Surprisingly, no significant differences were found in FGM, FPM, and FEM concentrations between hormonally contracepted and intact females. In all cases, contraception consisted of deslorelin acetate implants, a gonadotropin-releasing hormone (GnRH) agonist that reduces the secretion of pituitary gonadotropins, thus suppressing ovarian function (Moresco et al. 2014). Given that deslorelin downregulates reproduction, we expected concentrations of adrenal and gonadal steroid hormones to be more similar to those of ovariectomized females. Previous studies on the efficacy of deslorelin implants in lions concurred that ovarian steroid hormone concentrations were generally decreased for long periods of time (28 to 48 months) (Bertschinger et al. 2008; Putman et al. 2015; Braga et al. 2020). Additionally, Putman et al. (2015) also found a significant decrease in FGM concentrations in lionesses after initiating treatment with deslorelin. It is unclear why our studied females had fecal steroid hormone concentrations similar to those of intact females. The small sample size coupled with a certain degree of individual variability in the response to treatment may have contributed to our findings. For instance, lack of complete suppression of ovarian activity (Putman et al. 2015; Guthrie et al. 2021) and early resumption of FEM peaks and estrous behavior (Bertschinger et al. 2008) have been previously reported in felids on several occasions. Yet, these signs of ovarian activity do not necessarily reflect implant failure or reversal, as deslorelin has been previously associated with infertile follicular development (Cecchetto et al. 2017; Guthrie et al. 2021).

Alternatively, the time frame and number of samples analyzed in our study may have been insufficient to detect differences between intact and contracepted females. Our sampling approach did not confirm whether the intact females were cycling and/or ovulating. Given the short duration of the estrus, typically 2 to 9 days in lionesses (Umapathy et al. 2007; Putman et al. 2015), and the length of the interestrus period, FEM peaks could easily have been missed with our sporadic sampling approach. Additionally, elevated FPM concentrations are expected only in females that have recently ovulated (either induced or spontaneously) or pregnant females (Andrews et al. 2020). Moreover, while it remains unclear whether lions undergo an age-associated decline in reproductive performance (Putman et al. 2015), the three intact females in our study were older (13 to 20 years old), which may have affected their reproductive cyclicity. Overall, longer and more frequent sampling would have been necessary to assess and corroborate reproductive cycling in these females. However, regardless of the hormone levels, the contracepted lionesses had been treated with deslorelin for more than 3 years at the time of sampling and were housed with intact males, but no pregnancies occurred, thus suggesting that females were properly contracepted.

4.2.3 | FAM Concentrations and Reproductive Status in Females

In the present study, we conducted a first validation step to non-invasively monitor female androgens by demonstrating that the assay kit employed reliably measures FAM concentrations in lionesses. As in males, female androgens are produced in the gonads and adrenal glands and are involved in a wide range of physiological functions including reproduction (Staub and De

Beer 1997; Ghosal et al. 2023). For example, changes in androgen concentrations have been described during pregnancy (Dloniak 2004; Gesquiere et al. 2014; Setchell et al. 2015; Pavitt et al. 2016) and in relation to ovulation or breeding season (Staub and De Beer 1997; Fürtbauer et al. 2013). In the present study, and in line with our results for the other steroid hormones, we expected to find the lowest FAM concentrations in ovariectomized females. However, FAM levels did not differ between intact and ovariectomized females, and only contracepted lionesses presented significantly higher FAM levels than ovariectomized females. Remarkably, higher variability was observed in FAM concentrations in contracepted females (Table 5: CV = 70% vs. 42% and 25% for intact and ovariectomized females, respectively), even after two extreme values (> 2 SD above the mean of the group; 249.29 and 236.83 ng FAM/g feces) were removed from the data set. There is no clear explanation as to why this greater variability in hormone concentrations was observed only in contracepted females, but punctually high FAM levels were detected in three of the five contracepted females (BCN3, BCN5, and MAD1). Therefore, the observed findings did not constitute an isolated case but were rather consistent among deslorelin-implanted lionesses. Along similar lines, Mitchell et al. (2022) documented six cases of ovarian transdifferentiation in adult captive lionesses, which included mane development and clitoromegaly, presumably driven by excessive testosterone production of ovarian origin. Interestingly, five of the six females in their study had received deslorelin treatment for several years, which led to permanent suppression of the ovarian function, and subsequent masculinization four to seven years after the last contraceptive treatment. The authors suggested that deslorelin-induced sterility may play a role in ovarian transdifferentiation and masculinization in lionesses, presumably characterized by increased testosterone levels. These findings could help explain the higher variability in FAM levels observed only in the deslorelin-treated lionesses in our study. Together, these studies highlight the need for further investigations into the potential impact of GnRH agonist implants on female androgen levels and the long-term effects of this contraceptive drug.

5 | Conclusions

This study significantly advances our understanding of steroid hormone profiles in captive lions and elucidates how these profiles are influenced by everyday husbandry practices and housing conditions. We observed substantial variability in FGM concentrations among lions, pinpointing specific conditions that trigger heightened stress responses. These observations underscore the necessity for tailored monitoring approaches, reinforcing the value of fecal hormone analysis in ongoing welfare assessments.

Interestingly, neither sex nor zoo consistently affected baseline adrenocortical activity, whereas reproductive status markedly influenced both glucocorticoid and sex hormone levels, highlighting the complex relationship between stress and reproductive physiology. Ovariectomized females showed lower steroid hormone concentrations, whereas females treated with deslorelin—a contraceptive—exhibited hormone levels comparable to those of intact females.

Moreover, the successful validation of an EIA for detecting female androgens opens new research avenues into their roles in behavior, extending beyond lions to other social species. Moving forward, it is imperative that future research explores specific husbandry practices influencing lion stress and reproduction, aiming to enhance ex situ management strategies and conservation efforts for the species.

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Ethics Statement

Ethical approval was not required for this study as it involved the opportunistic and noninvasive collection of feces from lions during routine management practices, in accordance with Spanish national legislation (Real Decreto 53/2013).

Data Availability Statement

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

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