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RESEARCH PAPER

Is reproductive management for oestrus detection a stressful routine for female European mink (*Mustela lutreola*)?

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Abstract. The low reproductive rate of many mammal species is detrimental to their survival as it can lead to a decline in population size. The European mink (*Mustela lutreola*), the most endangered mammal in Europe, has difficulty reproducing in captivity due to sensitivity to maintenance and handling conditions. To improve captive breeding success, *ex situ* conservation programmes use vaginal cytology to determine the optimal time for mating. We investigated whether frequent vaginal cytology induced an increase in physiological stress response in European mink and affected the level of sex hormones metabolites. We collected faecal samples from eight females of various ages and quantified levels of faecal cortisol metabolites (FCM), faecal progesterone metabolites (FPM) and faecal oestradiol metabolites (FEM). We found that FCM, FPM and FEM levels varied during the experiment and that there was a positive correlation between the three hormones. Furthermore, FCM levels were influenced by age and individual factors, with older minks showing the highest levels. Based on our study, we conclude that frequent vaginal cytology at this conservation centre appears not to infer any added stress negatively affecting the captive breeding rate, a finding crucial for *ex situ* conservation programmes. By better understanding this species' physiology, we can help ensure its survival and contribute to the conservation of other threatened mammal species.

Key words: faecal cortisol metabolites, faecal oestradiol metabolites, faecal progesterone metabolites, steroid hormones, physiological response, vaginal cytology

Introduction

Reproduction is one of the most important biological functions for the survival and perpetuation of animal species. Understanding a species' specific reproductive and social patterns can facilitate both captive breeding and management of the species in its natural environment (Zimbler-DeLorenzo & Stone 2011), which can contribute to its propagation and conservation. Breeding species in captivity should be considered a last resort solution, used when wild populations cannot survive by other means and until more suitable conditions exist for conservation in their natural habitat (Amstislavsky et al. 2008). There are several obstacles associated with captive reproduction, however, including being able to recreate an adequate environment that meets the needs of the species (Clubb & Mason 2007), managing diseases derived from the entry of wild individuals that serve as founders or other animals housed at the

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centre (Thorne & Williams 1988) and the low genetic variability of captive populations due to inbreeding (Frankham 2003, 2005).

Homozygosity in individuals born in captivity results in the fixation of deleterious alleles, increasing susceptibility to diseases and causing reproductive problems leading to loss of fitness, high infant mortality rates and a low life expectancy (Amos & Balmford 2001, Schmalz-Peixoto & von Schmalz-Peixoto 2003). In addition, captive animals may be frequently exposed to stressful situations, such as confinement in limited spaces, restricted movement, reduced retreat space, lack of environmental stimulation, handling by caregivers and exposure to new stimuli interpreted as potentially aversive, such as loud noises (Morgan & Tromborg 2007). These factors can trigger a chronic physiological stress response in animals, negatively affecting their long-term health and well-being (Nájera 2022). The physiological response to chronic stress can affect the immune system (Webster Marketon & Glaser 2008), reproductive health (Dobson & Smith 2000), growth, development (Weary et al. 2008), and adaptive capacity of animals (Koolhaas et al. 2011).

In carnivores, the physiological stress response is regulated by a complex hormonal system responsible for the release of cortisol, which regulates reproduction through the release of sex hormones, such as testosterone, progesterone and oestrogens, in a bidirectional manner. These hormones are responsible for the maturation of the reproductive organs, the production and release of gametes, and sexual behaviour and mate selection (Morohashi et al. 2012, Jennings & Lecea 2020). Progesterone and oestrogens are female sex hormones that regulate the menstrual cycle and prepare the uterus for pregnancy (Jabbour et al. 2006, Wu et al. 2018). The hypothalamic-pituitary-adrenal (HPA) axis is a regulatory system involved in the stress response. Under stress, the hypothalamus releases corticotropin-releasing hormone (CRH), which stimulates the release of adrenocorticotrophic hormone (ACTH) from the pituitary gland. ACTH, in turn, acts on the adrenal glands to release cortisol, the primary stress hormone (Reeder & Kramer 2005, Barja 2015). The hypothalamic-pituitary-gonadal (HPG) axis is a complex feedback system that regulates the production and release of sex hormones. Here, the hypothalamus produces and releases gonadotropin-releasing hormone (GnRH), which stimulates the pituitary gland to produce and release two hormones, follicle-stimulating hormone (FSH) and luteinising hormone (LH). FSH and LH travel to the testicles in males or to the ovaries in females, stimulating the production and release of sex hormones (Dwyer & Quinton 2019). Since there is a bidirectional relationship between the HPA and HPG axis, chronic stress or distress can affect the production of sex hormones by changing their concentration. Hormone concentration measurements in animals are carried out using various techniques, using biological samples obtained either invasively (saliva, blood) or non-invasively (hair, faeces). Invasive methods require handling and immobilisation of the animal, which can alter basal glucocorticoid concentrations (Sheriff et al. 2011), while non-invasive methods avoid handling, thereby minimising measurement bias (Barja et al. 2012). A standard method used to measure glucocorticoid levels relies on quantifying metabolites in fresh faecal samples since cortisol is rapidly metabolised and eliminated from the body through faeces, with peak concentrations in mammals occurring between 12 and 48 hours after the stressor stimulus (Barja et al. 2012, Navarro-Castilla et al. 2021, Martín et al. 2023).

Ex situ conservation programmes for European mink (*Mustela lutreola*) have focused on captive breeding and reintroduction into their natural habitat. The main objective of these programmes is to maintain 85% of the original heterozygosity in the captive population for 50 years (Kneidinger et al. 2018); however, maintaining this genetic variability has its complications. Breeding pairs, specifically selected for their genetic load, have a 25% breeding success at each attempt, with breeding success depending more on males than females due to individual variation in behavioural features such as passiveness or aggressiveness (Kiik et al. 2013). The same authors verified that males born in captivity had less reproductive success than those born wild (35% and 89% success, respectively). Subsequently, Kneidinger et al. (2018) suggested that the captive environment may be having adverse effects on mink reproductive behaviour, while Blanchard et al. (2001) hypothesised that 'social stress' was a primary factor causing abnormal behaviour in captive-bred European mink. In nature, the mink is a solitary species (Youngman 1990); however, in captivity, individuals are usually placed in enclosures close to other mink, where they constantly receive signals (mainly olfactory) from neighbouring facilities that may cause chronic stress, leading to distorted reproductive behaviour (Kiik et al. 2013). In the study of Nagl et al. (2015), all captive females had normal heat cycles; thus, *a priori*, the focus is mainly on males. Nevertheless, for approximately three months before the start of the reproductive

season, captive females are usually subjected to heat detection techniques. As this entails an increase in the frequency of invasive management, these techniques may constitute a potential stress-inducing factor.

To assess whether reproductive management for oestrus detection represented a stressful routine for female European mink, we evaluated whether routine vaginal cytology in females triggers an increase in the levels of faecal cortisol metabolites (FCM) that may then be altering metabolites levels of two sex hormones (progesterone and oestradiol), thereby physiologically hindering reproduction. Our hypotheses were: 1) frequent performance of vaginal cytology increases FCM levels in female European mink, as previously confirmed in several wild and domestic felid species (Genaro et al. 2007) and other mustelid species, such as the black-footed ferret (*Mustela nigripes*), subjected to veterinary tests requiring injections and/or animal handling (Young et al. 2001); 2) FCM levels are positively related to faecal progesterone metabolite (FPM) and faecal oestradiol metabolite (FEM) levels, as is the case with other mammalian species (Chatdarong et al. 2006, Barja et al. 2008, Liening et al. 2010); 3) older females will have lower FCM levels than younger females as they are more habituated to this type of handling (Rankin et al. 2009); and 4) variations in FCM levels will depend on individual variability based on previous experience and animal personality, among other factors (Fernández-Lázaro et al. 2019, 2023).

Material and Methods

Research subjects and enclosures

Our study involved eight female European mink aged 10 to 60 months, kept in 40-60 m^2 (total area) naturalised enclosures with logs and riparian vegetation along with 5 × 3 × 0.7 m stream water rafts. The enclosures were distributed across four corridors and were accessible to caretakers. Each mink in the study was born and bred in captivity, with their fathers being founding individuals captured from the wild and their mothers being born in captivity. Not all individuals were siblings on the father's side, however, as they came from different breeding centres in Spain and Estonia, this being a requirement of the captive breeding program to maintain genetic standards. The study was conducted at the European mink breeding centre in Casarrubios del Monte, Spain, owned by the Foundation for Research in Ethology and Biodiversity (FIEB). It is the largest European mink breeding centre in Spain, but it also houses animals saved from illegal trafficking and provides enclosures for researchers requiring housing for their study subjects.

Ethical note

The FIEB is a participating centre in the *ex situ* Conservation Programme for European mink, acting as a breeding and research centre promoted by the Spanish government (Ministerio para la Transición Ecológica y Reto Demográfico), and is registered (reference code: ES450410000053) as a zoo centre and animal experimentation centre, covered by the Ministry of Agriculture and Peripheral Services of Castilla la Mancha (Consejería de Agricultura y Servicios Periféricos de Castilla la Mancha). This registration carries implications of housing and handling animals according to animal welfare criteria; consequently, all procedures performed in this study were undertaken following Directive 2010/63/EU of the European Parliament and Council, providing regulations on the protection of animals used for scientific purposes, and Spanish legislation (Royal Decree 53/2013). The experimental protocol did not require ethics committee approval in compliance with these regulations since the hormonal analyses were undertaken on non-invasive faecal samples collected. In addition, the protocol was carried out in compliance with the 'Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE)' guidelines, with the publication and dissemination of results carried out in compliance with 'Animal Research: Reporting of *in vivo* Experiments (ARRIVE)' guidelines.

Experimental methodology

The study took place between late March and mid-May. Typically, FIEB restricts research from late March to early September, making it impossible to carry out procedures during spring and summer. However, females usually come into oestrus around April (Amstislavsky & Ternovskaya 2000), though this period may start earlier (in February) or later (up to May), depending on latitude. Gestation takes 40-42 days, with births usually occurring between June and July (Palazón 2010). Given that the European mink is a highly stress-sensitive animal (Rozhnov & Petrín 2006) with low reproductive success, the risk of compromising reproduction was reduced by minimising caregiver visits and limiting animal manipulation to veterinary issues focused on health and reproduction only. The period between March and May is crucial for detecting oestrus in females and favouring mating by establishing genetically selected couples. Since the European mink is a solitary and territorial animal (Palazón 2010), it

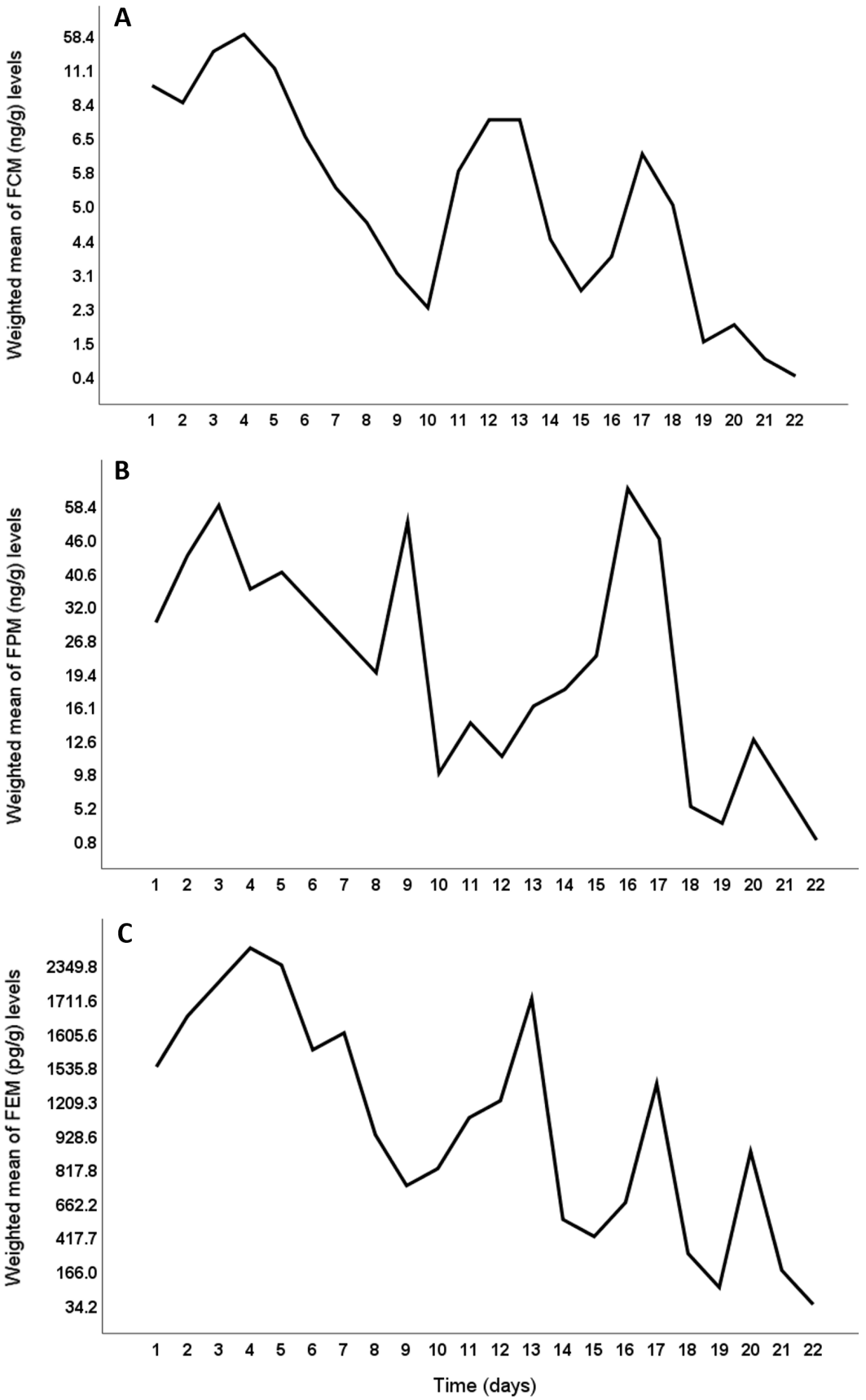

Fig. 1. Variation in hormone levels over time. $\bar{x}[\,]_w$ (ng/g) variation for A) faecal cortisol metabolites (FCM; ρ = −0.791, *P* = 0.0001); B) faecal progesterone metabolites (FPM; ρ = −0.608, *P* = 0.003); C) faecal oestradiol metabolites (FEM; ρ = −0.792, *P* = 0.0001).

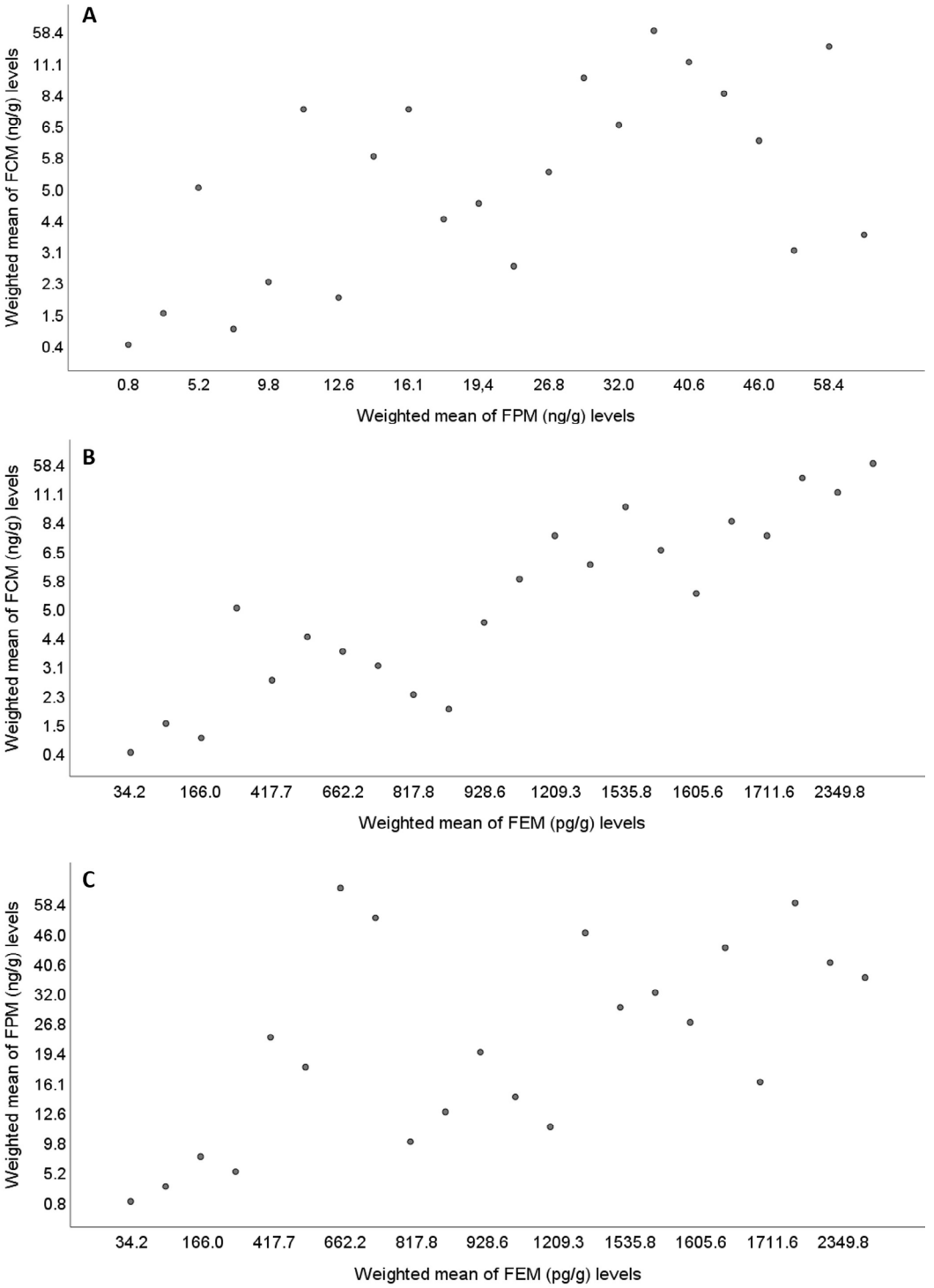

Fig. 2. Relationships between cortisol, progesterone and oestradiol metabolite levels. Correlation of $\bar{x}[\,]_w$ (ng/g and pg/g) between faecal cortisol metabolites (FCM), faecal progesterone metabolites (FPM) and faecal oestradiol metabolites (FEM): A) FCM-FPM ($\rho = 0.566$, $P = 0.006$), B) FCM-FEM ($\rho = 0.899$, $P = 0.0001$), C) FPM-FEM ($\rho = 0.592$, $P = 0.003$).

is housed in individual enclosures in conservation centres to reduce social stress (Blanchard et al. 2001). In our study, heat (oestrus) detection was performed by identifying cornified cells in a vaginal smear collected with a swab and examined under a microscope. Females were considered to have entered oestrus when vaginal smears showed 90% cornification (Amstislavsky et al. 2004). Vaginal cytology was performed over several days, sometimes consecutively and at other times intermittently, until female heat was detected, at which point caregivers brought the couples together in a larger, odour-free facility, which acted as neutral ground to reduce aggression caused by territorial behaviour.

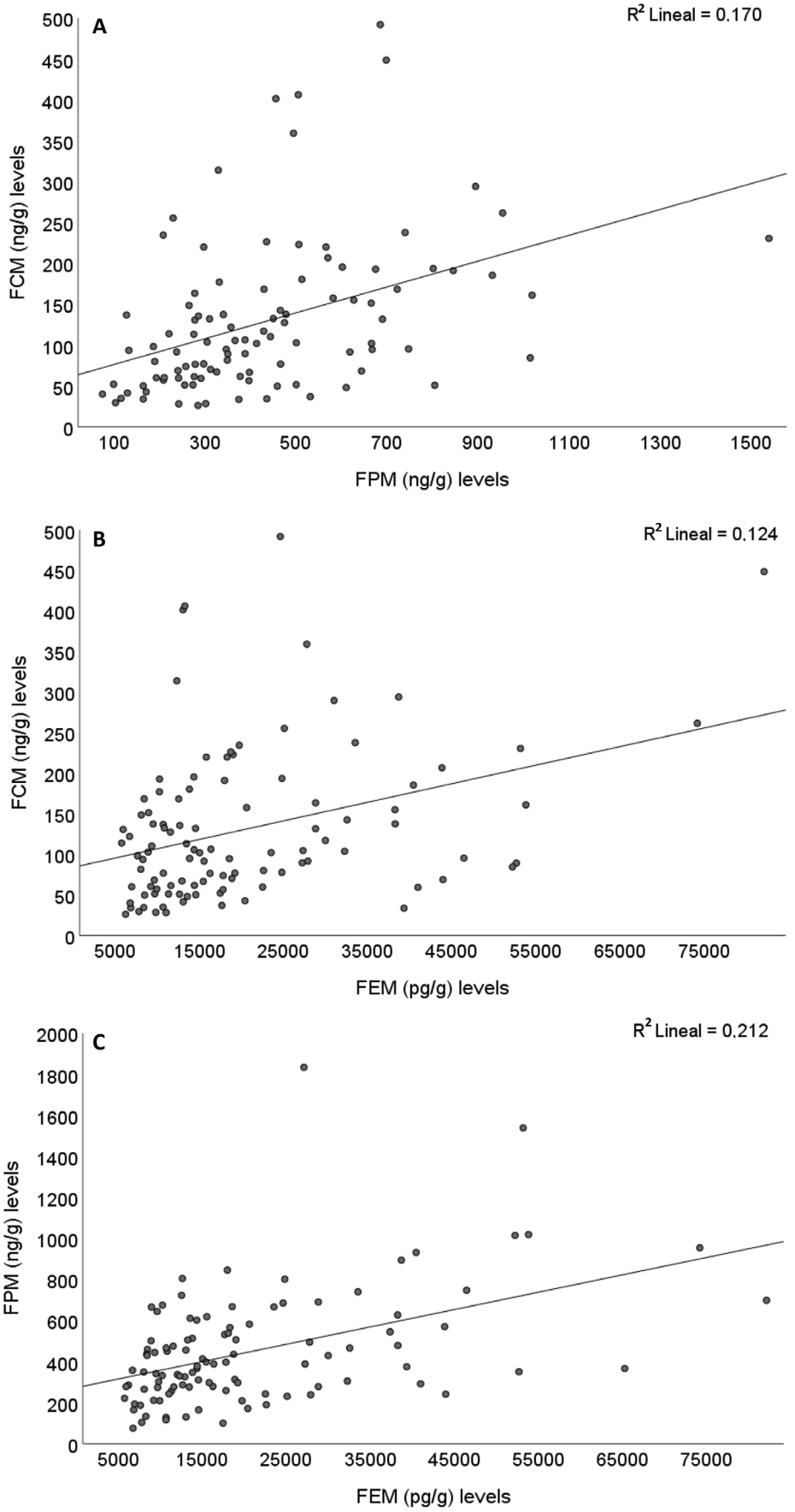

Fig. 3. Correlation between the medians for metabolite levels of faecal cortisol metabolites (FCM), faecal progesterone metabolites (FPM) and faecal oestradiol metabolites (FEM): A) FCM-FPM (ρ = 0.467, *P* = 0.0001), B) FCM-FEM (ρ = 0.325, *P* = 0.001), C) FPM-FEM (ρ = 0.485, *P* = 0.0001). Mass-mass ratios are presented in pg/g for FEM, and in ng/g for FPM and FCM. Note the positive trend line for the three graphs with respect to R^2.

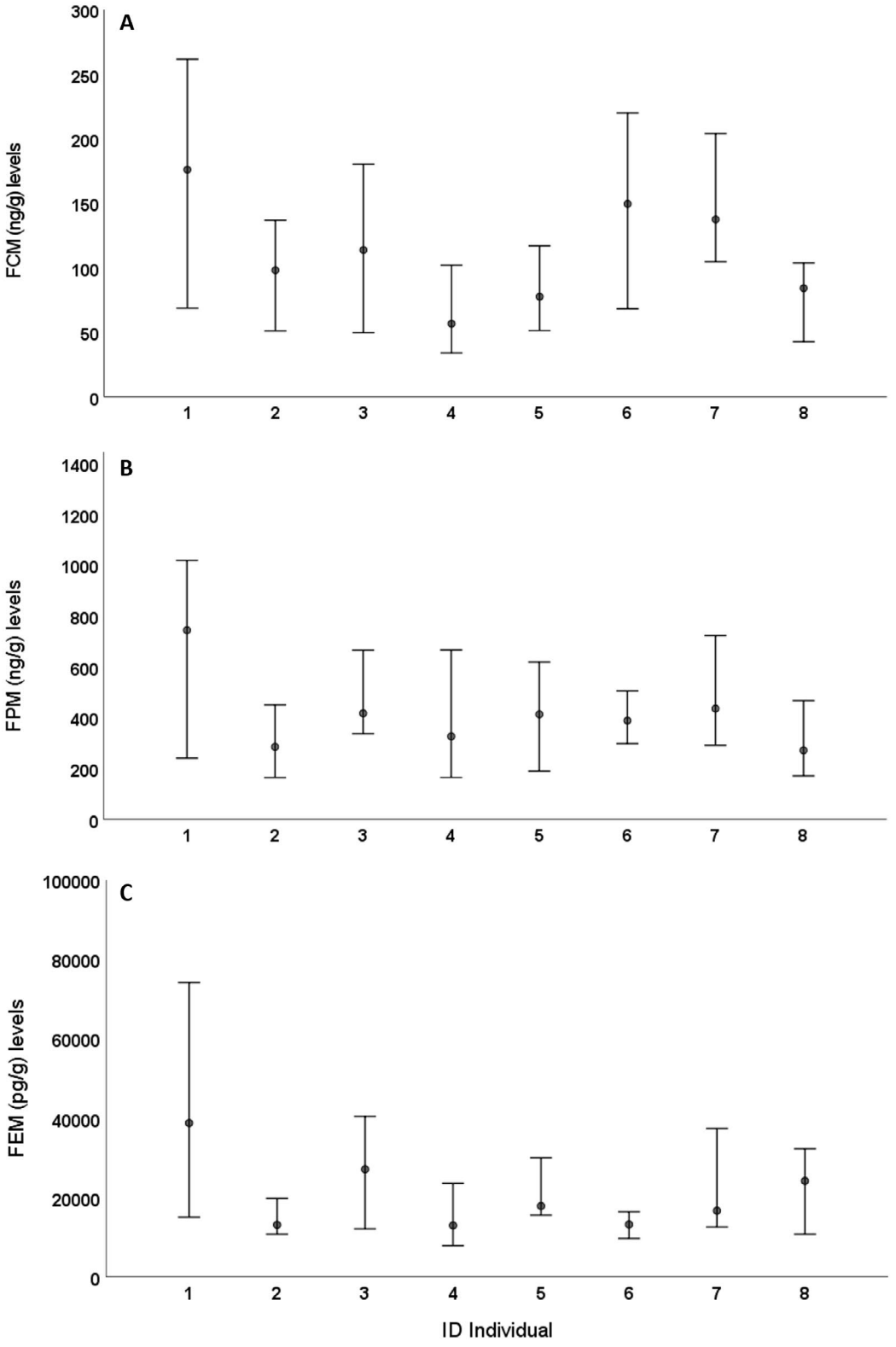

Fig. 4. Median ± CI for individual level A) faecal cortisol metabolites (FCM), B) faecal progesterone metabolites (FPM) and C) faecal oestradiol metabolites (FEM).

If copulation was unsuccessful, the female was subjected to new tests until a new period of heat was detected, the European mink having a polyoestrous cycle, i.e. females may enter oestrus three times if not fertilised (Mead 1989).

Veterinarians and caregivers collected 117 fresh faecal samples from females during the screening days, the samples being collected the day cytology was performed and 24 h and 48 h after cytology to investigate whether repeated testing modified FCM

levels and whether this affected the metabolite levels of two important sex hormones in the reproductive period, i.e. progesterone (involved in gestation) and oestradiol (involved in the ovulatory cycle). Faecal samples were classified as fresh if they showed no signs of dehydration and had a strong odour (Barja et al. 2012, Ortiz-Jiménez et al. 2022). All samples were stored in individually sealed bags and labelled with the date and name of the female. Immediately after collection, the samples were frozen at -20 °C until hormonal analysis.

Extraction and quantification of faecal hormone metabolites

The faecal samples were first dried at 90 °C in an oven for 24 h to eliminate any moisture (Ortiz-Jiménez et al. 2022), and each was homogenised to prevent any potential bias caused by steroids being distributed unevenly throughout the faeces (Barja et al. 2012). We weighed 0.5 g from each sample and added 2 ml of phosphate buffer and 2 ml of methanol. After stirring the solution for 10 s in a manual vortex, the samples were left on an orbital shaker for 16 h. Subsequently, we centrifuged the samples at 2,500 rpm for 15 min and kept the resulting supernatant at -20 °C until quantification.

The concentrations of cortisol, testosterone and progesterone metabolites in the faecal samples were determined using commercial enzyme immunoassays (EIAs; DEMEDITEC Diagnostics, Kiel, Germany), which had previously been validated for measuring faecal steroid metabolites in European mink (Ortiz-Jiménez et al. 2022), i.e. each EIA was specifically validated for quantifying each faecal hormone metabolite (FHM) through parallelism, accuracy and precision tests (Goymann et al. 1999, Young et al. 2004). Parallelism was analysed by comparing the curve produced from a pool of serial faecal extract dilutions with curves constructed from the respective standards provided in each hormone kit. Percentage recoveries were determined by comparing the expected and measured sample values and calculating intra- and inter-assay coefficients (precision) of variation. The lowest analytical detectable level that could be distinguished from the zero calibrator was 3.79 ng/ml for cortisol, 0.045 ng/ml for progesterone and 10.6 pg/ml for oestradiol. Intra-assay coefficients of variation for each hormone were 8.5% for cortisol, 9.2% for progesterone and 8.9% for oestradiol, while inter-assay coefficients of variation were 10.9% for cortisol, 10.8% for progesterone and 10.8% for oestradiol. FCM and FPM levels were expressed in ng/g dry faeces, and FEM levels in pg/g.

The following hormones were evaluated for cross-reactivity (the percentage indicates cross-reactivity at 50% displacement compared with cortisol): pregnenolone < 0.1%, estrone < 0.01%, oestradiol < 0.1%, DHEA < 0.1%, 17-Hydroxyprogesterone 0.8%, prednisolone 54.3%, testosterone < 0.1%, cortisone 76%, corticosterone 2.3%, danazole < 0.1%, androstenedione < 0.1%, prednisone 100%, 11-deoxycortisol 37.5%, estriol 0.4%, dexamethasone < 0.1%, 11-deoxycorticosterone 0.5% and progesterone < 0.1%. In the case of progesterone, the hormones evaluated for cross-reactivity were: progesterone 100%, 17αOH progesterone 0.3%, estriol < 0.1%, oestradiol < 0.1% 17β, testosterone < 0.1%, 11-Desoxycorticosterone 1.1%, DHEA-S < 0.02%, cortisol < 0.02%, corticosterone 0.2%, pregnenolone 0.4%, cortisone < 0.1% and 11-Desoxycortisol 0.1%. The hormones evaluated for cross-reactivity in the case of oestradiol were: oestradiol 17β 100%, androstenedione 0%, androsterone 0%, corticosterone 0%, cortisone 0%, epiandrosterone 0%, 16-Epiestriol 0%, estradiol-3-sulphate 0%, estradiol-17α 0%, estriol 2.3%, estriol-16-glucuronide 0%, estrone 6.9%, estrone-3-sulphate 0%, dehydroepiandrosterone 0%, 11-Deoxycortisol 0%, 21-Deoxycortisol 0%, Dihydrotestosterone 0%, Dehydroepiandrosterone 0%, 20-Dihydroprogesterone 0%, 11-Hydroxyprogesterone 0%, 17α-Hydroxyprogesterone 0.003%, 17α-pregnenolone 0%, 17α-progesterone 0.%, pregnanediol 0%, pregnanetriol 0%, pregnenolone 0%, progesterone 0%, testosterone 0.033% and fulvestrant 3.7%.

The laboratory validation results provided clear evidence that the kits used accurately measured cortisol, progesterone and oestradiol metabolite levels in the faecal samples. The resultant data were statistically assessed using paired t-tests, with the resultant values displayed as mean ± standard error (SE). To assess the suitability of the assay for quantifying FCM, we first exposed five mink (three males, two females) to an external stressor, i.e. recorded human voices generating a noisy stimulus, which resulted in a notable rise in FCM values after the stress-inducing event (control: 97.78 ± 39.65 ng/g; human voices: 232.74 ± 56.84 ng/g; [$t(4) = -3.243$, $P = 0.032$]). FPM concentrations were also evaluated for the six females across two stages of their oestrous cycles. In each case, the keepers supplied faecal samples during the oestrous cycle, taking advantage of limited visits to the handling aisles when they conducted the heat detection tests. FPM levels in non-oestrous females (380.50 ± 34.46 ng/g) were significantly lower than those observed during

oestrous (555.98 ± 64.51 ng/g) [t(5) = –4.628, P = 0.006]). Finally, FEM concentrations were evaluated in the six females across non-breeding and breeding periods. Again, significant differences were observed between the periods, with non-breeding FPM levels being significantly higher (77852.98 ± 27634.82 pg/g) than those during breeding (8764.85 ± 1458.74 pg/g; t(5) = 2.497, P = 0.032).

Data analysis

Non-parametric statistical methods were used throughout, as the variables did not satisfy the assumptions of normality and equality of variance. To assess variation in physiological stress over time, we quantified FHM levels for cortisol (FCM, ng/g), progesterone (FPM, ng/g) and oestradiol (FEM, ϱg/g) according to the duration and frequency of female handling. Since the duration and frequency of handling varied between females, more faecal samples were collected from some individuals than others. Based on this variability, two correction factors were used:

$$[\ ]_w = \frac{[\ ]_i}{t}$$

where $[\ ]_w$ was the weighted hormonal concentration of each sample, $[\ ]_i$ was the hormonal concentration of each sample from female i, and t represented time in days over which faecal samples were collected from each female. This allowed us to obtain the hormonal concentration of k samples for each female.

$$\bar{x}[\ ]_w = \frac{\sum[\ ]_{wi}}{n}$$

where $\bar{x}[\ ]_w$ was the mean weighted hormone concentration, $\sum[\ ]_{wi}$ was the sum of the weighted concentrations from i = 1 to k samples for each female, and n was the number of females (n = 8). In this way, we obtained the weighted mean of FHM of each female over the handling time.

Table 1. Levels of oestradiol metabolites according to vaginal cytology frequency. Median and interquartile range (IQR = Q3-Q1) of faecal cortisol metabolites (FCM; pg/g) in relation to vaginal cytology number (ρ = −0.234, P = 0.013). The number of faecal samples analysed for each group is indicated.

	FEM levels		
Cytology No.	No. faecal samples	Median (pg/g)	IQR
4	4	24182.2	21547.4
5	8	38754.4	59136.0
6	15	13096.3	20998.2
8	46	18673.5	62621.3
10	11	12951.2	21957.2
12	26	13189.0	76166.4

We performed two Spearman's correlation tests to determine the influence of time on variation $\bar{x}[\ ]_w$ of FHM and the relationship between $\bar{x}[\ ]_w$ for pairs of hormones. In addition, we performed a separate Spearman's correlation to determine i) the influence of vaginal cytology frequency (n = 4 – 12) and age (measured in months) on FHM levels and ii) any correlation between the three hormones (FCM, FPM and FEM). Finally, we performed a Kruskal-Wallis test to determine the influence of individual variability on FHM levels, the results being shown as median ± confidence interval (CI).

Results

Variation in $\bar{x}[\ ]_w$ for the three hormones as a function of time was significant in all cases (FCM: ϱ = –0.791, P = 0.0001, n = 22; FPM: ϱ = –0.608, P = 0.003, n = 22; FEM: ϱ = –0.792, P = 0.0001, n = 22; Fig. 1). Likewise, correlations between the $\bar{x}[\ ]_w$ for the three hormones were statistically significant: FCM-FPM: ϱ = 0.566, P = 0.006, n = 22; FCM-FEM: ϱ = 0.899, P = 0.0001, n = 22; FPM-FEM: ϱ = 0.592, P = 0.003, n = 22; Fig. 2).

Table 2. Median and interquartile range (IQR = Q3-Q1) of faecal cortisol metabolite (FCM) and faecal progesterone metabolite (FPM) levels (ng/g) in relation to age (months) of individuals (FCM-age: ρ = 0.378, P = 0.0001; FPM-age: ρ = 0.212, P = 0.023). The number of samples analysed for each group (No. of faecal samples) is indicated.

Age of individuals	No. of faecal samples		Median (ng/g)		IQR	
(months)	FCM	FPM	FCM	FPM	FCM	FPM
10-12	28	29	72.1	312.8	208.4	3028.3
23-25	27	30	91.6	413.4	178.3	1662.6
33-35	32	36	145.9	446.4	414.2	3356.8
58-60	20	20	137.5	457.3	308.3	1399.6
Total	107	115	102.9	414.0	422.5	3356.8

The correlation between FCM and FPM levels was both positive and significant ($\varrho = 0.467$, $P = 0.0001$, n = 105), as was that between FCM and FEM ($\varrho = 0.325$, $P = 0.001$, n = 106) and between FPM and FEM ($\varrho = 0.485$, $P = 0.0001$, n = 112; Fig. 3). Frequency of vaginal cytology was not significantly correlated with FCM and FPM, but was with FEM ($\varrho = –0.234$, $P = 0.013$, n = 114; Table 1). FCM levels were positively correlated with age ($\varrho = 0.378$, $P = 0.0001$, n = 107), as were FPM levels ($\varrho = 0.212$, $P = 0.023$, n = 115; Table 2). Individual variability had a strong and significant influence on FCM levels ($H = 22.609$, df = 7, $P = 0.002$, n = 108), FPM ($H = 15.820$, df = 7, $P = 0.027$, n = 108) and FEM levels ($H = 20.136$, df = 7, $P = 0.005$, n = 110; Fig. 4).

Discussion

Diagnostic tests for oestrus (vaginal cytology) in captive female European mink involve routine handling of individuals, with the potential to trigger changes in the levels of cortisol metabolites that vary over time. During this study, we observed an increase in FCM levels during such tests, reaching a peak on day four, i.e. 72 h after the first cytology was performed. Our results differ from those recorded in other wild mammal species, where faecal cortisol peaks were recorded between 24 and 48 h after induced stress, depending on the species (Wasser et al. 2000, Barja et al. 2012). In our study, however, we need to consider the potential cumulative effects of frequent cytology, with some females not having a break of more than 24 h between cytology tests. This break could explain the delay in reaching peak cortisol levels, since previous response tests to stressors have shown a cumulative effect over time surpassing a typical threshold for test subjects. Unfortunately, our study could not avoid this effect as the conservation centre established the cytology schedule based on the short period that oestrus lasts in females of this species, i.e. from one to ten days (Amstislavsky & Ternovskaya 2000). Such routine cytology tests could have become a stressor, triggering an increase in FCM; however, we observed habituation to veterinary handling over the period following the point at which maximum cortisol concentration was reached, i.e. four days after the start of testing. This cortisol peak may have been triggered by an acute response to novel handling, which only occurs during the three-month breeding season, with the youngest females experiencing such handling for the first time. The additional two peaks observed likely represent acute episodes experienced by females who received 24-72 h rest between cytology tests. This will have occurred when the cytology routine was interrupted by a female being moved to a neutral enclosure to allow copulation due to oestrus, the vaginal cytology routine restarting after the break. Note that female European mink can enter heat up to three times if they fail to copulate (Amstislavsky & Ternovskaya 2000).

At the conservation centre where this research took place, efforts were made to minimise unnecessary contact with the animals. Furthermore, this was especially true during the breeding season, as studies have shown that European mink are highly sensitive to stress at that time (Rozhnov & Petrín 2006). Despite avoiding routine interaction throughout the year, the handling of females necessarily increased during the three months when cytology examinations were conducted. This also meant a decrease in familiar handling practices and introducing a type of handling the females had not previously encountered or only briefly experienced each year. Though it appears that the females were able to gradually adapt to this more intensive handling over the three-month testing period, it may be beneficial to acclimatise the females throughout the year to this type of handling, especially in the case of younger females facing it for the first time or those with limited prior experience with this potential stressor. Despite the FIEB centre's preference for limiting regular contact with the animals to maintain their wild nature, it may prove beneficial to implement positive reinforcement training (Heidenreich 2007) for those females not intended for reintroduction and instead kept at the centre for breeding purposes. Numerous studies have demonstrated that mammals, including certain carnivores (Broder et al. 2008), are capable of voluntarily cooperating in medical and breeding procedures in zoo and research settings following such positive reinforcement training (Laule 2003, McKinley et al. 2003, Laule & Whittaker 2007, Coleman et al. 2008).

In this study, maximum FPM peaks occurred during the habituation days prior to handling (Rankin et al. 2009), coinciding with the lowest FCM levels. These FPM peaks coincided with supposed entry into oestrus, days during which there was no copulation, and days when gestation began. In these cases, FPM concentrations likely increased to inhibit the production of FSH, which causes follicular maturation and the production of LH, responsible for ovulation (Filicori et al. 2002, Murray & Orr 2020). Furthermore, the highest FEM peaks coincided with those of FCM but were opposite to those of FPM. If progesterone levels were lower, FSH inhibition would not occur, and the concentration of oestrogens and oestradiol

would increase (Wiele et al. 1970). Note that our results showed a positive correlation between FCM and FPM when using weighted means and medians. This is consistent with other mammalian studies, especially those studying cortisol and progesterone interaction (Chatdarong et al. 2006) and those comparing all three hormones (Barja et al. 2008, Liening et al. 2010). For example, a study on the European marten (*Martes martes*) showed positive correlations between FCM levels and levels of FPM, testosterone, and oestradiol (Barja et al. 2011).

The number of cytology examinations was positively correlated with FEM levels but not with FPM or FCM levels. While this correlation does not confirm causality, it would appear that the total number of vaginal cytology examinations did not induce a continuous increase in FCM levels over the study period and, consequently, did not have an adverse effect, despite being a potential stressor during the reproductive season for an animal considered highly sensitive to stress (Rozhnov & Petrín 2006). In addition, older females showed higher FCM levels than young females, a result that contrasts with other studies, which tend to show younger females having higher FCM levels (Azevedo et al. 2019) or no significant differences between age groups (Stevenson et al. 2018). Some studies, however, have suggested that animals exhibit increased levels of glucocorticoids as they age, with ageing reducing the functionality of the endocrine system and affecting glucocorticoid secretion (Thompson et al. 2020). Studies with other mammals have also shown no differences in faecal progesterone (Burgess et al. 2012) and fat progesterone as a function of age in non-pregnant females (Atkinson et al. 2020). These conflicting conclusions point to the need for more accurate empirical data addressing deficiencies in how age influences the physiological stress response.

While variations in FPM and FEM levels are most likely attributable to differences in individual female menstrual cycles, variations in individual FCM levels can be attributed to a wide range of factors, such as gender, age, life experience and quality of life, which modulate an individual's behaviour over their lifetime (Fernández-Lázaro et al. 2019, 2023, Kirschbaum et al. 1992). These experience-based behaviours are essential for each individual and are responsible for shaping the individual's personality, i.e. sets of correlated behaviours expressed in different situations (Carere & Eens 2005). It has been suggested that animals with proactive personalities are likely to be more successful in a stable environment than those with reactive personalities, while the more cautious style of reactive animals may be more successful in a changing environment; thus, there is no one-size-fits-all personality for all situations (Cockrem 2005). Individual variability could be caused by habituation after several repeated tests or through variations in neuronal plasticity (Noer et al. 2015, 2016). For example, Fernández-Lázaro et al. (2019) have suggested that more active and aggressive individuals may have higher levels of FCM. Consequently, we consider it important to consider the significance of mink personality when designing management criteria, especially for breeding females, as different personalities may be more or less vulnerable to stressors and diseases (Carere et al. 2010). Regarding the management of animals intended for reintroduction, personality may also be crucial in selecting those best suited for survival. For example, individuals with proactive or cautious traits may be chosen after evaluating the area into which they will be reintroduced. Indeed, recent studies indicate that individual variation within a species can buffer against strong fluctuations in the species' natural habitat (Koolhaas & Van Reenen 2016).

In conclusion, repeated vaginal cytology does not appear to constitute a major stressor inducing increased FCM levels that could compromise reproductive success or alter behaviour during copulation. Furthermore, female age and individual variability were the main factors influencing cortisol and sex hormone metabolite levels. The results obtained in this study provide a first approximation for the elaboration of European mink management strategies during the reproductive season, especially for those individuals most susceptible to stress agents such as veterinary handling, in this case, adult females. Females older than one year are often prioritised for mating as some are already likely to have experience with males and will be sexually mature, while it is likely to be a new experience for younger females. With a view to future research, it would be interesting to convince conservation centres to standardise vaginal cytology methodology by testing stable groups of females receiving the same number of vaginal cytology tests and then increasing the sample size. We also consider it important to pay attention to the personality of each female since studies suggest that the most fearful individuals will be those exhibiting increased FCM levels, which could affect reproduction if remaining high over the long term. As such, we recommend tailoring management to each female to minimise the stressor's effect as much as possible. In the case

of females destined solely for breeding (with no possibility of reintroduction), it could also be helpful to establish positive reinforcement training programs to facilitate habituation to this type of handling.

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Author Contributions

I. Barja and L. Ortiz-Jiménez conceived and designed the study. Material preparation and data collection was performed by L. Ortiz-Jiménez. L. Ortiz-Jiménez, I. Vivas and I. Barja carried out the statistical analysis. The first draft of the manuscript was written by L. Ortiz-Jiménez. I. Barja and I. Vivas commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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