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Measuring kea parrot *(Nestor notabilis)* stress and reproductive hormone metabolites by non-invasive faecal sampling

Diploma Thesis

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Submitted by

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Declaration of Authenticity

I hereby declare that this diploma thesis was entirely written on my own. I did not use any sources or aid other than the ones cited in my work. To my best knowledge and belief, this thesis does not contain any material previously published and I have not published or handed in my work at any other place.

Larissa Reiter

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Table of Contents

1. Introduction

1.1 The kea parrot

The kea parrot (*Nestor notabilis*), a noteworthy yet endangered species (1) holds an important place among New Zealand´s avian fauna as “the world´s only alpine parrot” (2). Distinguished by its vibrant plumage, cognitive capability, and insatiable curiosity, the kea parrot beckons attention from both scientific researchers and nature enthusiasts. The following paragraphs give but a brief insight into the kea parrot´s evolution, ecology, and behaviour to better understand the projects goals and why the kea is such a unique study subject. For a more detailed description see for example (3).

1.1.1 Taxonomy

The kea belongs to Strigopidae, a small family of parrots native to New Zealand, marking a unique avian lineage (4). This family encompasses two living genera: Strigops, represented by the Kakapo (*Strigops habroptilus*), the sole species of this genus, and Nestor, which includes the kea (*Nestor notabilis*), the kaka (*Nestor meridionalis*), and the Norfolk kaka (*Nestor productus*), the last species considered extinct (5, 1).

1.1.2 Morphological features

Possessing distinguishing morphological features, the kea is well adapted for its diverse and challenging territory across the South Island of New Zealand (3). It measures approximately 48 cm in length and has an olive-green plumage with bright orange underwings. The kea's beak is robust and slightly curved, well-adapted for versatile tasks such as manipulating objects and extracting various food sources. While showing minimal sexual dimorphism, the kea's females are slightly smaller in size, and their beaks are shorter compared to males (6). Juveniles, upon developing their full plumage, stand out beside their adult relatives with a bright yellow colouring around the eyes, cere, the area around the nostrils and the lower bill (3).

1.1.3 Behavioural characteristics and cognitive abilities

Kea parrots, renowned for their social dynamics, exhibit intriguing behaviours both in and out of the breeding season. Outside of the breeding period, kea form communal foraging flocks, comprising breeding pairs, fledglings, and unrelated individuals of varying ages (3). This social structure facilitates cooperative foraging and interaction among the diverse members of the flock.

Come breeding season, a notable shift occurs in their behaviour. Kea become territorial, with breeding pairs selecting a ground-level nest cavity in a secluded location. These pairs fiercely defend an approximate 10-meter radius around their nesting site (7). This territoriality is a critical aspect of their breeding strategy, ensuring a secure environment for incubation and rearing of offspring.

Nesting duties are distinctly divided between the male and female. The female takes on the responsibility of incubation and nestling care, devoting three weeks to the incubation period and 13 weeks to the nestling phase (8). Meanwhile, the male diligently provides nourishment for the family throughout these critical stages. Once fledglings leave the nest, families reunite with other kea individuals, forming larger foraging groups once again.

After fledging, kea show a prolonged period of dependency on their parents (8). Male kea reach sexual maturity only in their fourth or fifth year (9). Most authors propose a distribution into four age classes (8): fledglings, juveniles, subadults and adults. Fledglings are all birds in their first summer after leaving the nest, juveniles are birds in their second summer and subadults are birds in their third or fourth summer. The latter only show an incomplete yellow ring around the eyes and their cere and beak are already dark. Upon reaching maturity, adult kea parrots lose all indications of yellow markings (8).

Beyond their remarkable social strategies, the kea parrots are acknowledged for their cognitive abilities, displaying problem-solving skills reminiscent of corvids and exhibiting tool-use behaviours . This cognitive capability, coupled with a natural inclination for social interactions, renders kea parrots highly intriguing subjects for behavioural studies.

1.1.4 Conservation aspects

The kea is listed as an endangered species by the IUCN red list (1) with a decreasing population of about 4.000 mature individuals. Among the most important causes for the species´ decline these days are anthropogenic factors such as deforestation and the spread of invasive species posing a threat to kea´s health and their nests. Although kea parrots gained full legal protection in the early 1970s, it is reported that an unidentified number of birds is still killed by farmers each year. A detailed assessment of threats is available at (1).

1.1.5 Endocrinological interest in the Kea

In the context of avian endocrinology, the kea parrot is underexplored. In contrast to its better studied parrot counterparts (10–12), the kea, with its phylogenetic distinctiveness, fascinating social structures, and cognitive capability, offers a unique possibility to get new insights from avian behaviour and reproductive endocrinology.

1.2 Steroid hormones

1.2.1 Sex steroid hormones

Sex steroid hormones play a crucial role in reproductive processes influencing behavioural and physiological changes connected to breeding. They are synthesised in the gonads, which are the testicles in males and the ovaries in females. Key hormones addressed in this study are androgens such as testosterone, oestrogens and progesterone. These hormones are metabolised in the liver and are excreted via faeces in the form of metabolites which vary between species (13).

1.2.2 Glucocorticoids

Glucocorticoids are a group of steroid hormones synthesised in the cortex of the adrenal glands. The primary glucocorticoid in birds is corticosterone (14). These hormones are necessary for various important physiological tasks including metabolism, immune response, inflammation regulation and most of all their involvement in the body´s stress response (15). The production and release of glucocorticoids is regulated by the hypothalamic-pituitary-adrenal (HPA) axis, which defines a complex interplay between the organs for which it is named. Stressful situations, for instance, result in higher glucocorticoid synthesis and output, initiated by increased adrenocorticotropic hormone (ACTH), released by the pituitary gland (16). The liver

represents the most crucial organ of metabolism and species-depending levels of metabolites are secreted via urine and faeces (15).

1.3 Non-invasive sampling method

The conventional method of quantifying steroid hormones requires the drawing of blood samples. This process however implicates certain disadvantages and risks that should be kept in mind, as it is highly invasive and stress-associated, especially for undomesticated animals, and might even pose dangers when handling with wild or zoo animals (17). This fact alone might for instance result in higher stress levels and correspondingly false high glucocorticoid levels in blood due to the preceding stressor making an accurate analysis of the data impossible.

Over the last decades, more and more research has been dedicated to a non-invasive sampling method, which has the benefit that no direct handling of an animal is necessary (17). Among the possible sampling materials, faeces exhibit the most promising results as samples are comparatively easy to collect and to process. Important to know is that faecal samples, compared to blood samples, which show an almost instantaneous steroid level, display a delay of changing hormone metabolite levels due to the intestinal passage time (18) and as steroids are almost fully metabolised by the liver and gastrointestinal tract, they are only present as metabolites in faeces (19). Furthermore, one sample represents a pooled value of a few hours depending on the frequency of defecation. In order to use faecal samples accordingly, the method has to be validated for each species and each steroid as its metabolism and excretion might differ strongly (19). The need for validation holds one of the key elements of this study, which leads us to the aims of the diploma thesis.

1.4 Study aims

Its unique characteristics and worrying conservation status explain why the kea parrot serves as a relevant study subject. An important step in research would be to address the notable gap in the field of endocrinology concerning this species. As a consequence, this thesis aims to start to fill this gap with a contribution to understanding kea´s endocrinology. To achieve this, the study uses a non-invasive method of faecal sampling for quantifying key reproductive and stress hormone metabolites. The hormones of interest include oestrogens, progesterone, testosterone, and glucocorticoids such as corticosterone in birds.

My first step involves the validation of an enzyme immunoassay (EIA) for the different metabolites. A validation is necessary because of the variability in the level and metabolism of hormones across different species (20, 21). Furthermore, the various available EIAs lead to the need to rule out the most suitable assay for accurate and reliable hormone measurements in kea parrots. This process ensures the applicability and precision of the EIA for this species.

After EIAs have been successfully validated, we will establish baseline hormone levels in kea parrots, both within and outside the breeding season, similar to a work done by (10) in which he and his colleagues examined annual fluctuations of urofaecal androgen and progestogen metabolites. An understanding of these baseline levels is essential for interpreting future hormonal studies and improving our knowledge in reproductive and stress physiology of the kea parrot.

The following sections of this thesis will detail the methods employed for the validation process and the establishment of baseline hormone metabolite levels. Through this research, we aspire to contribute valuable data to the field of avian endocrinology, enhance our comprehension of kea parrot physiology, and pave the way for future studies on reproductive and stress hormones in this species, utilizing faecal sampling as a non-invasive and therefore ethically justifiable method.

1.5 Study hypotheses

Considering observed seasonal variations in kea parrot social behaviours, my study posits hypotheses focused on reproductive hormones and glucocorticoids:

1. **Reproductive Hormones:**
    - **H1a:** Reproductive hormones (oestrogens, progesterone, and androgens) will exhibit higher levels in sexually mature individuals compared to juveniles.
    - **H1b:** During the breeding season, the reproductive hormones mentioned under H1a will show elevated levels in both males and females compared to the non-breeding season.
2. **Glucocorticoids:**
    - **H2:** Glucocorticoid metabolite levels will be higher in both sexes during the breeding season, reflecting increased physiological demands associated with reproductive activities.

These hypotheses align with the overall goal of validating a method for measuring reproductive hormones and glucocorticoids in kea parrot faecal samples. Anticipated findings will contribute to a nuanced understanding of endocrine dynamics, shedding light on hormonal changes linked to distinctive social behaviours during different seasons.

In conclusion, this exploration into kea parrot behavioural and reproductive endocrinology underscores the broader significance of studying unique species. The research not only advances scientific understanding in avian biology but also holds practical implications for conservation and biodiversity preservation.

2. Materials and methods

2.1 Subjects and housing

The study was a collaboration between the KeaLab at Haidlhof research station (HHR) near Bad Vöslau, Austria, under the management of the Comparative Cognition Unit of the Messerli Research Institute of the University of Veterinary Medicine Vienna Austria, and the Tiergarten Wels, Austria. The reason for the collaboration pergwas a lack of juvenile individuals at HHR, which were needed for a consistent distribution throughout age groups and sexes. More information about the number of individuals and the necessity of having juveniles in the study are stated under 2.3.2.

The group at HHR consisted of eleven out of 29 captive kea parrots *(Nestor notabilis)*. Detailed information about the composition of the group is listed in *Tab. 1*. They are all housed together in a large outdoor aviary (52x10x4 m, LxWxH), equipped with hanging branches, ponds, sleeping shelters, feeding tables, and a variety of environmental enrichment, which is regularly renewed. Food is distributed three times daily, and consists of fruits and vegetables, seeds, and a protein (cooked meat or eggs) once daily. Fresh water is available *ad libitum*.

At Tiergarten Wels, six out of twelve captive kea parrots participated in the study. Details about the group's composition are once again listed under *Tab. 1*. They are housed in a large outdoor aviary (47.4 m^2, 4.15 m high at least) with an additional outdoor shelter (7.1 m^2) and with access to two indoor compartments (4.7 m^2 each) that were connected by a smaller room (2.6 m^2). A smaller outdoor aviary (15.1 m^2; 2.95 m high at least) outside the visitor´s view was connected to the main aviary and indoor rooms. The smaller aviary could be separated from the remaining enclosure. The equipment is similar to the one at HHR, enrichment is changed regularly, and food is distributed 3 times daily consisting of fruits, vegetables, seeds and a protein source once daily. Fresh water is available *ad libitum.*

Tab. 1. Details about each individual participating in the study. Every bird aged 2 years or younger is defined as juvenile, all other birds have shown sexual behaviour and are defined as adults.

	Name	ID	Year (Age in years)	Sex
Tiergarten Wels				
	Lily	-	2021 (2)	f
	Ryan	-	2021 (2)	m
	Gabe	-	2021 (2)	m
	Zeta	-	2022 (1)	f
	Matilda	-	2022 (1)	f
	Silver	-	2022 (1)	f
HHR				
	Lilly	Ly	2007 (16)	f
	Ponga	Po	2022 (1)	m
	Alpi	Al	2022 (1)	m
	Jean-Luc	Je	2015 (8)	m
	Kermit	Ke	2004 (19)	m
	Anu	An	2007 (16)	m
	Pick	Pi	2004 (19)	m
	Willy	Wy	2007 (16)	f
	Plume	Pl	2007 (16)	f
	Coco	Co	2007 (16)	f
	Diana	Di	2017 (6)	f

2.2 Ethical note

The study was approved by the ethics and animal welfare committee of the University of Veterinary Medicine Vienna, Austria, ETK-191/12/2022, in accordance with good scientific practice guidelines and national legislation.

The housing conditions comply with the Austrian Federal Act on the Protection of Animals (Animal Protection Act -§ 24 Abs.1 Z 1 and 2: § 25 Abs. 3 – TSchG, BGBI. I Nr. 118/2004 Art. 2). As the study was strictly non-invasive, according to the Austrian Animal Experiments Act (TVG 2012), it was not classified as an animal experiment. The subjects participated voluntarily in the study: they either were brought individually to the testing aviary after voluntarily entering an adequate transport cage or were separated together with other individuals. Further details concerning animal handling are stated under 2.3.1. If a subject showed a lack of motivation or any other discomfort, the sample collection for this day was skipped and retried on another day.

2.3 Data collection

2.3.1 Sample collection

The sample collection procedure began with preparing the aviary adequately by covering the compartment floor with a clean, transparent, 3 mm thick polyethylene tarp to avoid any contamination of the faecal samples with sand or other unwanted material. Once a subject dropped a sample, the experimenter collected it with a clean spatula made from stainless steel making sure to collect only the faecal part of the urofaecal sample and put it in a 5 ml plastic sample tube. The tube was then labelled and immediately put into the freezer to be stored at -20 °C (19) until further processing. Samples were always collected within the same period of the day between 12:30 and 15:30, beginning 30 minutes after the mid-day meal.

Fig. 1. Sample collection departments at HHR.

At HHR, two different compartments *(Fig. 1)* outside the main aviary were used for the sample collection to ensure a smooth workflow. Every subject was separated individually to better assign the sample to the individual. The birds were brought from the main aviary to the collection compartment using a transport cage made from stainless steel with which they have been familiarized in every-day training for prior projects projects and husbandry. All participants were trained to enter the transport cage on their own using positive reinforcement. If a subject refused to enter the cage voluntarily, that subject was dropped for the day. Subjects were never forced to enter the cage involuntarily.

At Tiergarten Wels, the six subjects included in the study were separated into the smaller outdoor aviary *(Fig. 2)* altogether, as they were neither used to being separated alone nor being transported in a cage. The experimenter had to watch the birds closely to assign the faecal sample to the correct individual. A sample was only collected if it could be matched with certainty to an individual.

Fig. 2. Sample collection compartment at Tiergarten Wels (15.1 m2; 2.95 m high at least).

Once all samples from one phase of the experiment were collected, they were transported to the laboratory as quick as possible in a cool box with freezer packs to assure that the samples stayed frozen. At the laboratory, they were put into a freezer (-20 °C).

2.3.2 Steroid extraction

All following steps were performed under supervision of Dr. Sabine Macho-Maschler at the laboratory belonging to the Unit of Physiology, Pathophysiology, and Experimental Endocrinology from the University of Veterinary Medicine Vienna, Austria.

The steroid extraction from the faecal sample marked the first step to allow further analysis (19). The samples were defrosted at room temperature and for every sample a portion of 0.5 g wet faeces, thoroughly blended beforehand, was weighed and put into a new glass tube (15 ml). The portion was then suspended with 5 ml of 60 % methanol solvent. Sometimes, the portion of faeces was less than 0.5 g. In this case, the amount of solvent was also reduced to the extent

that the suspension always had the same proportion of solvent (example: 0.4 g faeces with 4 ml solvent). The tubes were then shaken (RapidVap, Labconco Corporation, USA) for 30 min at 24 °C and centrifuged (Allegra^{TM} X-12R Centrifuge, Beckman Coulter, USA) at 3.750 RPM, 21 °C, for 10 min. Out of the supernatant 700 µl were pipetted into small Eppendorf tubes (1.5 ml). The extracts were then frozen again until further analysing.

2.3.4 Enzyme immunoassay validation

In order to use an EIA to determine steroid metabolites, the EIA must first be validated for the species, as metabolism and excretion of steroids show large variation between species (21, 20). To validate EIAs for reproductive hormone metabolites, the results of the tested EIAs were compared between sexually mature and immature individuals (22) and the assay, which showed the largest difference between the two groups was considered to be best suited for use in the species.

For the EIA validation phase, eight samples were collected per individual. Each sample was collected on a different day as described under 2.3.1. All adult subjects (four males and five females) for the validation came from HHR. For the juveniles, four females and two males came from Tiergarten Wels and two males from HHR. Thus we obtained a balanced set of adults/juveniles and males/females respectively.

2.3.4.1. Tested enzyme immunoassays (EIAs)

To measure androgen metabolites, a testosterone EIA (detecting 17ß-hydroxyandrogen metabolites) and an epiandrosterone assay (measuring 17-oxoandrogen metabolites) were utilized. Details of the EIAs including cross-reactions are described in (23) and (24), respectively. Furthermore, a progesterone EIA (5α-P4; (25) and a total oestrogen EIA (24) were also used. A cortisone EIA (16) measuring FCMs with a 3,11-dione structure, has been found suitable for non-invasive measurement of adrenocortical activity in kea (26) (unpublished data, Amelia Wein 2021).

2.3.3 Performing the EIA

All EIAs were performed on 96 well microtiter plates. All variable solutions (standards, pools and sample extracts) were placed into a pipetting robot (Flexible JANUS® G3 Automated Workstations, PerkinElmer, USA) together with the microtiter plates, previously washed (BioTek 405 LS Washer, Agilent, USA)) to transfer the plates. After this step, 100 µl biotinylated label and antibody were added to all wells with a 96 well pipetting tool (Liquidator™ Mettler Toledo, Germany). The plates were then incubated and shaken (Titramax 1000, Heidolph, Germany) overnight at 4 °C.

The next day, the plates were washed (BioTek 405 LS Washer, Agilent, USA) and 250 µl of the enzyme streptavidin horseradish peroxidase was added to each well. After another washing step, a total of 250 µl substrate (tetramethylbenzidine and H_2O_2) was added. The plates were shaken (Titramax 1000, Heidolph, Germany) for 45 min at 4 °C between each step. After the last waiting period, the solutions changed colours to blue, depending on the amount of enzyme. The enzymatic reaction was then stopped with 25% sulphuric acid, which turned the solution yellow. The last step was to read the plates at 450 nm using a photometer (BioTek ELx808 Absorbance Microplate Reader, Agilent, USA) and to calculate the results using BioTek Gen5 Software (Agilent, USA).

2.3.5 Determining baselines of reproductive hormone and glucocorticoid metabolites

Once the appropriate EIAs for the different hormone metabolites were validated, we were able to determine baseline levels of these hormone metabolites in kea parrots. For this phase, faecal samples of the adult birds were collected three times per week both during the breeding season from February to April and outside of the breeding season from June to August, up to a total of 17 samples per subject per season. There was no sample collection during May in order to have a clear gap between the seasons.

2.4 Statistical analysis and expected results

2.4.1 Choosing the EIA

To validate the best suited EIA for reproductive hormone metabolites, the median values from sexually mature and immature birds were compared to each other (22). The EIA which showed the largest difference was considered the adequate test to use for this species. A student´s t-test as well as a linear mixed effects model was used to statistically justify the choice of tests.

2.4.2 Calculating baselines

Baseline hormone metabolite levels for individual subjects were determined using the median concentrations derived from all collected samples within and outside of the breeding season. The choice of the median (18) over the mean aimed to enhance robustness, particularly in the presence of potential outliers. This method was used for determining baseline levels of reproductive hormone metabolites as well as glucocorticoid metabolites.

3. Results

3.1 EIA validation

In the validation process, the initial eight samples obtained from each individual were utilized, resulting in a total of 136 samples.

3.1.1 Testosterone EIA validation

Fig. 3 illustrates varying levels (ng/g) of testosterone metabolites categorized by age class and sex for the validation samples. Each data point on the graph represents the median of eight samples from one individual. The x-axis delineates age classes, distinguishing between adults (> 2 years of age) and juveniles (< 2 years of age) indicated by different colours. The average testosterone metabolites levels were higher in juveniles compared to adults for both sexes. Notably, male juveniles exhibited considerable variation in their individual means. Adult males consistently showed higher testosterone metabolites levels than adult females.

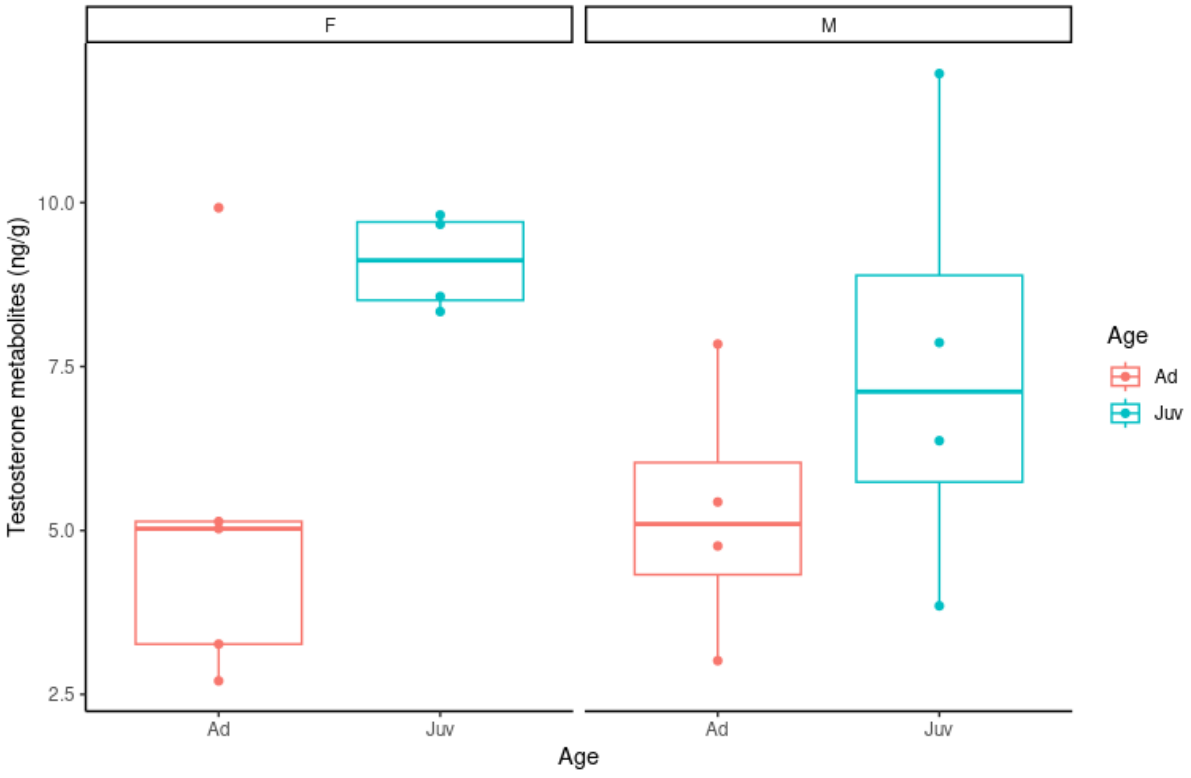

Fig. 3. Boxplot showing testosterone metabolite levels (ng/g) of different age groups and sexes for the validation samples. On the x-axis "Ad" stands for adults, coloured in red, and "Juv" for juveniles, coloured in blue. "F" are females and "M" males.

The results from a Welch two-sample t-test revealed no significant difference between age groups ($p = 0.4739$) and between sexes ($p = 0.6337$). Similarly, when employing a linear mixed effects model on the results before logarithmic transformation, no significant effects of age groups or sex were detected.

3.1.2 Epiandrosterone EIA validation

Fig. 4 shows the variations in epiandrosterone metabolite levels (ng/g faeces) on the y-axis categorized by age group and sex for the validation samples. Each data point on the graph represents the median of the first eight samples of one individual. The x-axis delineates age classes, distinguishing between adults (> 2 years of age) and juveniles (< 2 years of age) indicated by different colours. The average epiandrosterone metabolite levels were higher in adult females compared to adult males. Juvenile females exhibited slightly higher levels than their adult counterparts. Juvenile males showed lower levels than adult males.

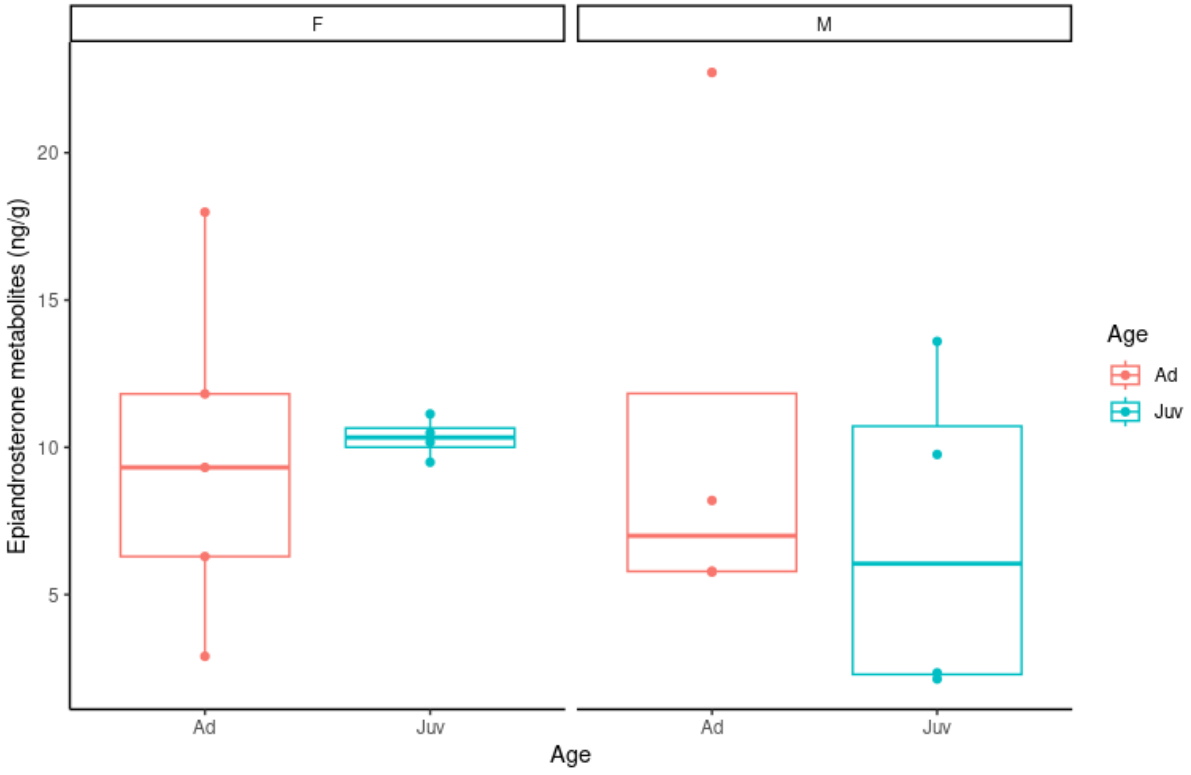

Fig. 4. Boxplot showing epiandrosterone metabolite levels (ng/g) of different age groups and sexes for the validation samples. "Ad" stands for adults, coloured in red, and "Juv" for juveniles, coloured in blue. "F" are females and "M" males.

A Welch two sample t-test showed no significant difference between age groups ($p = 0.7524$) and between sexes ($p = 0.3117$). A linear mixed model performed on logarithmized results showed no significance in either age groups or sexes.

3.1.3 Oestrogen EIA validation

The investigation into oestrogen metabolites in kea parrot faeces faced difficulties due to the nature of the data. The assessment focused on one plate, and the results primarily indicated either non-detectable levels or very low concentrations across the tested samples.

For the selected plate with 38 samples ($n = 38$), measurements for oestrogen metabolites were generally minimal, with a count of 29 out of 38 of non-measurable results, and a maximum of 0.97 ng/g of faeces for the remaining samples. Given the lack of consistency across age groups and sexes, further detailed statistical investigations were not undertaken.

3.1.4 Progesterone EIA validation

The exploration of progesterone metabolites in kea parrot faeces showed inherent inconsistencies in the data. The assessment centred on a single plate, revealing a spectrum of concentrations without clear trends linked to specific age groups or sexes.

On the tested plate with 38 samples ($n = 38$), the range of progesterone metabolite levels varied widely, spanning from 1.1 to 98.6 ng/g of faeces. However, these measurements lacked a discernible pattern or consistency when correlated with age groups or sexes. Given the absence of clear associations and the variance in concentrations, no detailed statistical analysis was followed.

3.2 Calculating baselines

3.2.1 Epiandrosterone baselines

In *Fig. 5*, there was no visible tendency of epiandrosterone metabolite baseline concerning the comparison between males and females. However, most individuals showed higher variabilities during the breeding season.

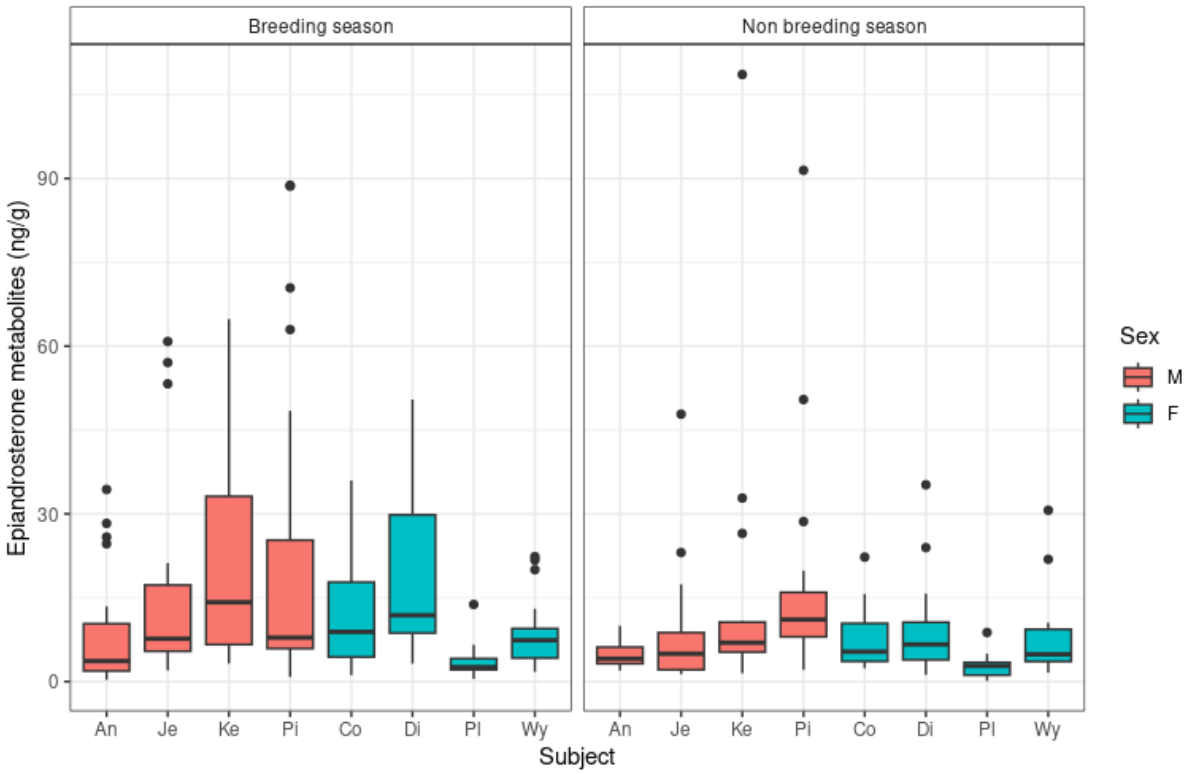

Fig. 5. Boxplot showing baseline epiandrosterone metabolite levels (ng/g) of every adult individual within and outside the breeding season. "M" stands for males, coloured in red, and "F" stand for females, coloured in blue. A detailed list of subjects with their ID´s can be found under Tab. 1.

Observing the average epiandrosterone metabolite levels over the months of the sample collection *(Fig. 6)*, there was a general trend for males to show higher levels (red trendline) compared to females (blue trendline). Additionally, both trendlines decrease over time and the male´s trendline shows a slightly steeper decrease than females.

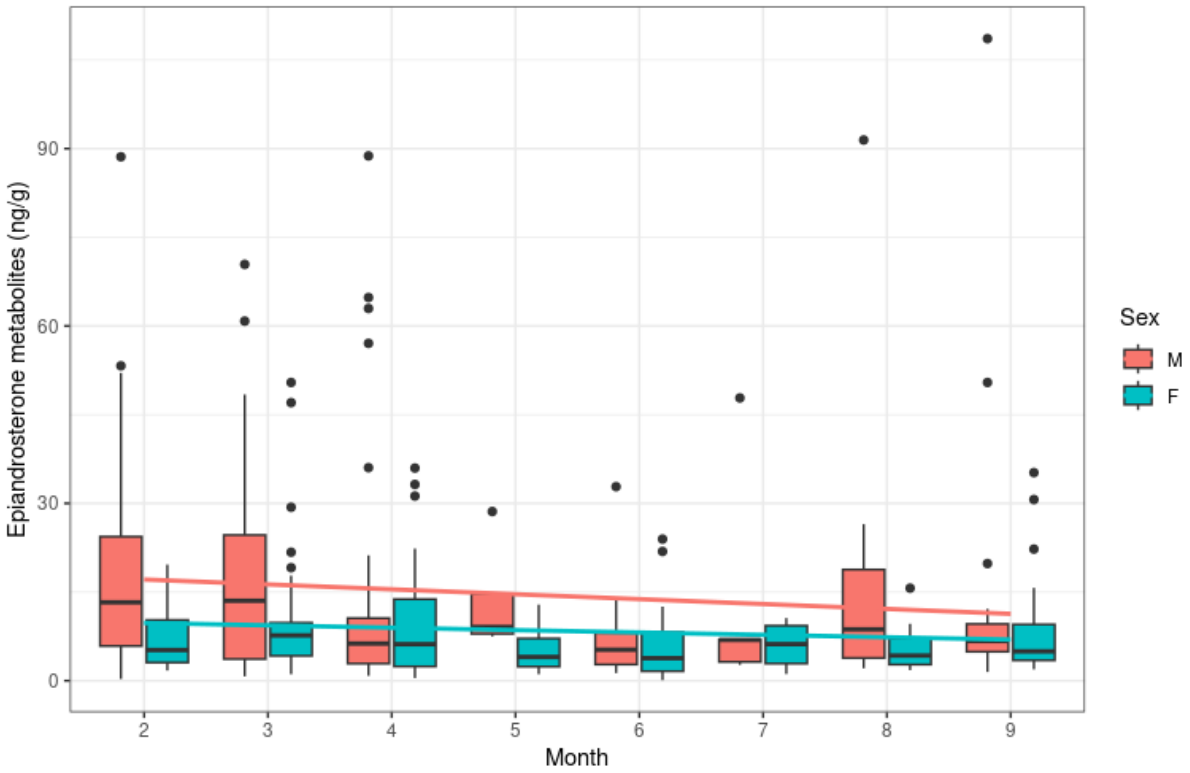

Fig. 6. Boxplot showing baseline epiandrosterone metabolite levels (ng/g) of one sex over months of the sample collection phase. "M" stands for males, coloured in red, and "F" stand for females, coloured in blue.

3.2.2 Glucocorticoid baselines

Considering every subject´s individual baseline within and outside the breeding season, there was a general tendency toward higher median levels and a larger variability outside of the breeding season for males as well as females *(Fig. 7)*. There was no visible bias indicating a distinction between sexes.

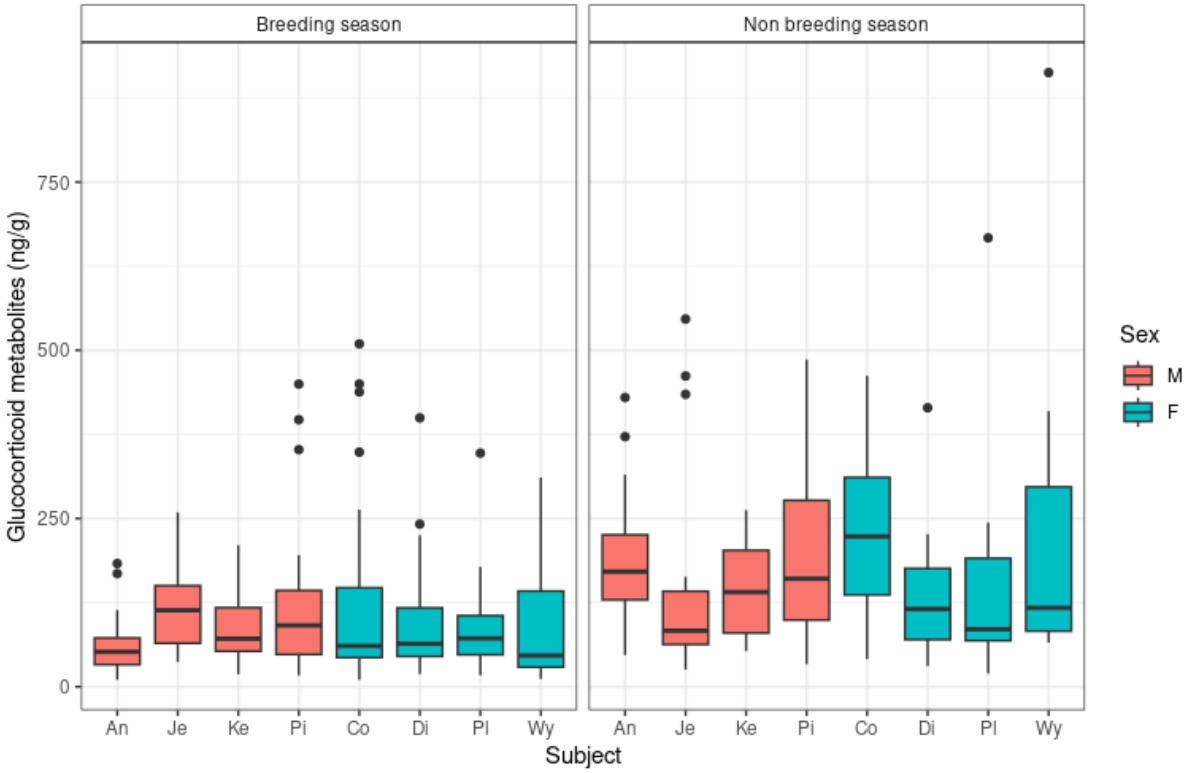

Fig. 7. Boxplot showing baseline glucocorticoid metabolite levels (ng/g) of every adult individual within and outside the breeding season. "M" stands for males, coloured in red, and "F" stand for females, coloured in blue. A detailed list of subjects with their ID´s can be found under Tab. 1.

By examining the monthly changes of the average glucocorticoid metabolites levels *(Fig. 8)*, the tendency towards higher levels during the non-breeding season became clear. The general trendline rose over time similarly for both sexes. The months of August and September seemed to exhibit the highest values along with the largest variability. Furthermore, the trendlines for both sexes overlap strongly, making it almost impossible to see the male line (red colour).

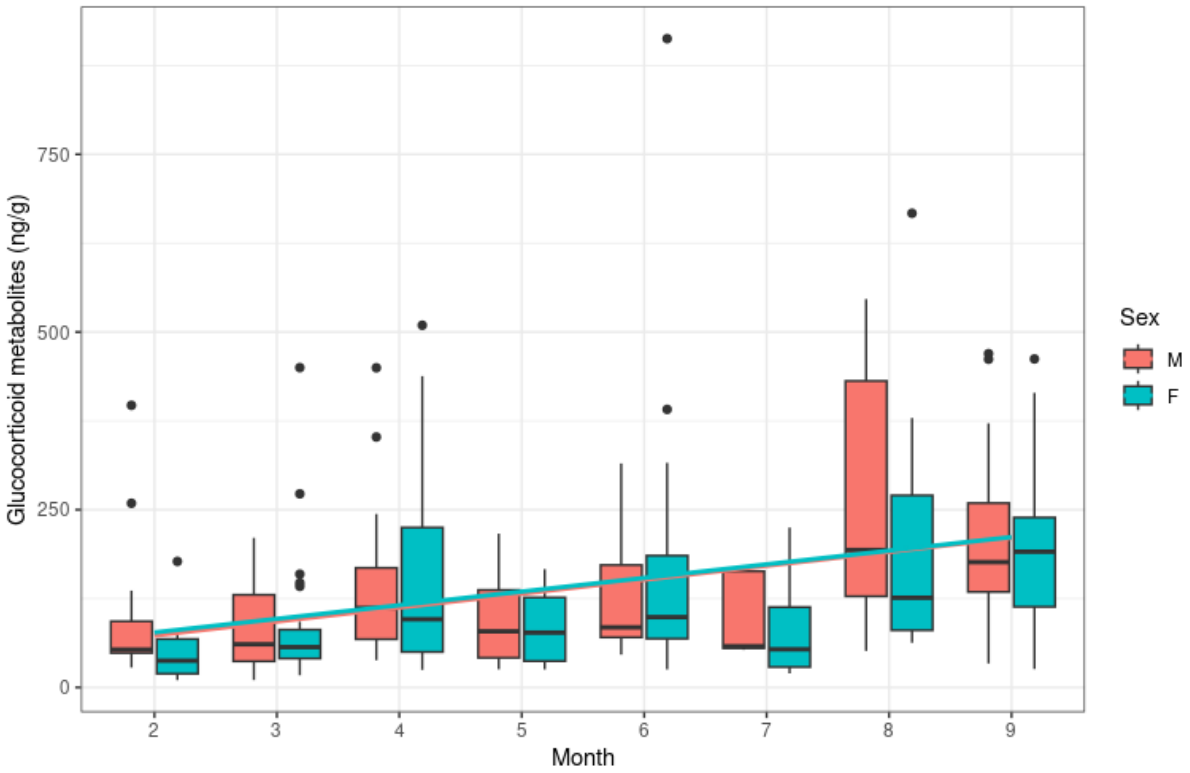

Fig. 8. Boxplot showing average baseline glucocorticoid metabolite levels (ng/g) of one sex over the months of the sample collection phase. "M" stands for males, coloured in red, and "F" stand for females, coloured in blue.

3.3 Other findings

3.3.1 Dependency of glucocorticoid metabolite levels based on locations

As there were two locations involved in the sample collection, we wanted to assess whether there was a difference between glucocorticoid metabolite levels depending on the subjects´ housing. The means of glucocorticoid metabolite levels were as follows. For juvenile males (n = 2) at Tiergarten Wels the mean was 250 ng/g faeces, as for juvenile females (n = 4) the mean was 200 ng/g faeces. At HHR, the mean of juvenile males (n = 2) was 68 ng/g faeces. There were no samples collected from juvenile females at this location. For this data set, the sample results from both the validation phase were used (first eight sample per individual).

Glucocorticoid metabolite levels *(Fig. 9)* were substantially higher in juveniles housed in Wels, represented by the colour blue, than in the same age group at HHR (individuals “Al” and “Po” were juveniles).

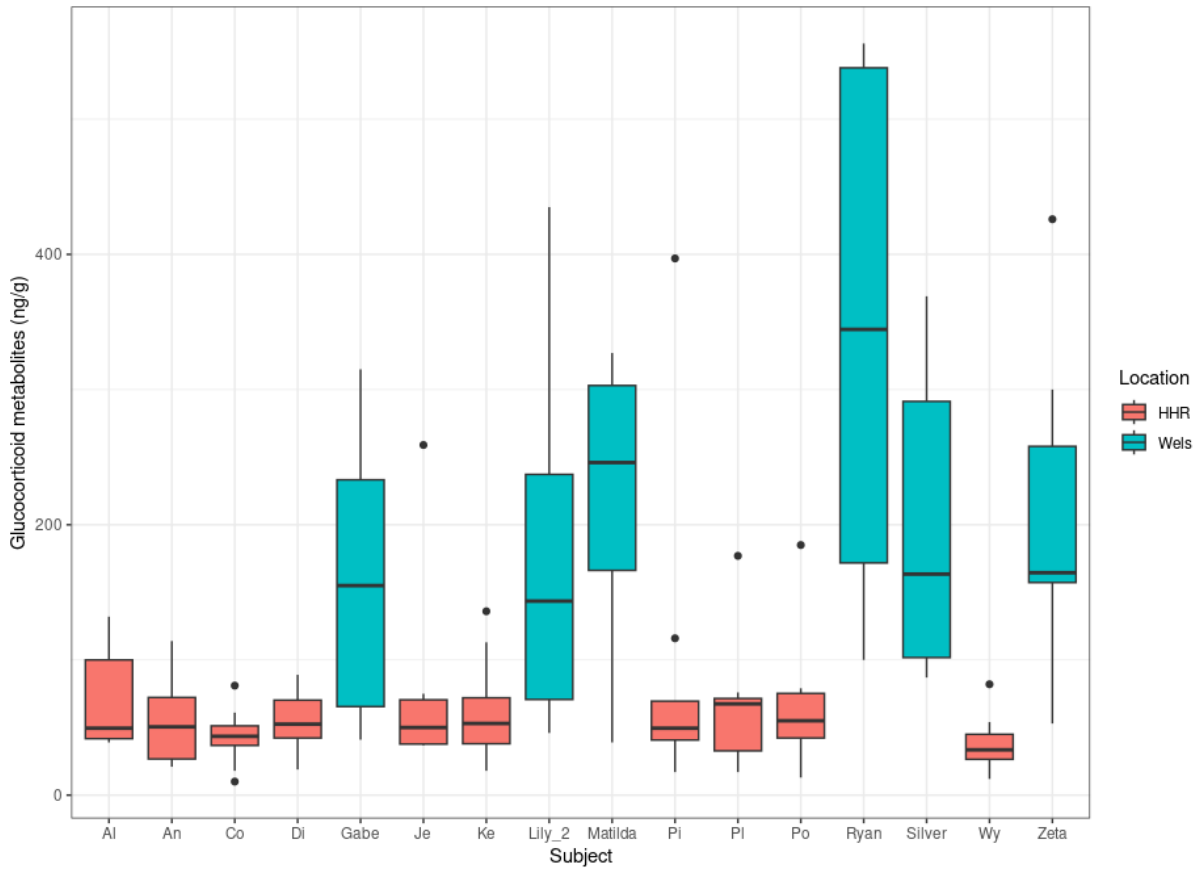

Fig. 9. Boxplot showing glucocorticoid metabolite levels (ng/g) of all individuals based on different locations shown by different colours; red for HHR and blue for Wels. "Al" and "Po" are juveniles at HHR. A detailed list of subjects with their ID´s can be found under Tab. 1.

3.3.2 Glucocorticoid metabolite levels based on age groups and sexes

Similar to the boxplots used for the EIA validation detecting testosterone and epiandrosterone metabolites, we wanted check whether there were differences in the different categories of age groups and sexes *(Fig. 10)*. Both female and male juveniles had higher levels than their adult counterpart, and individual male juveniles showed a higher variability due to the fact that two of them came from Wels and two came from HHR and visually highly different levels in both have been stated under 3.3.1.

A Welch two sample t-test showed no significant difference between age groups ($p = 0.06408$) and between sexes ($p = 0.9823$).

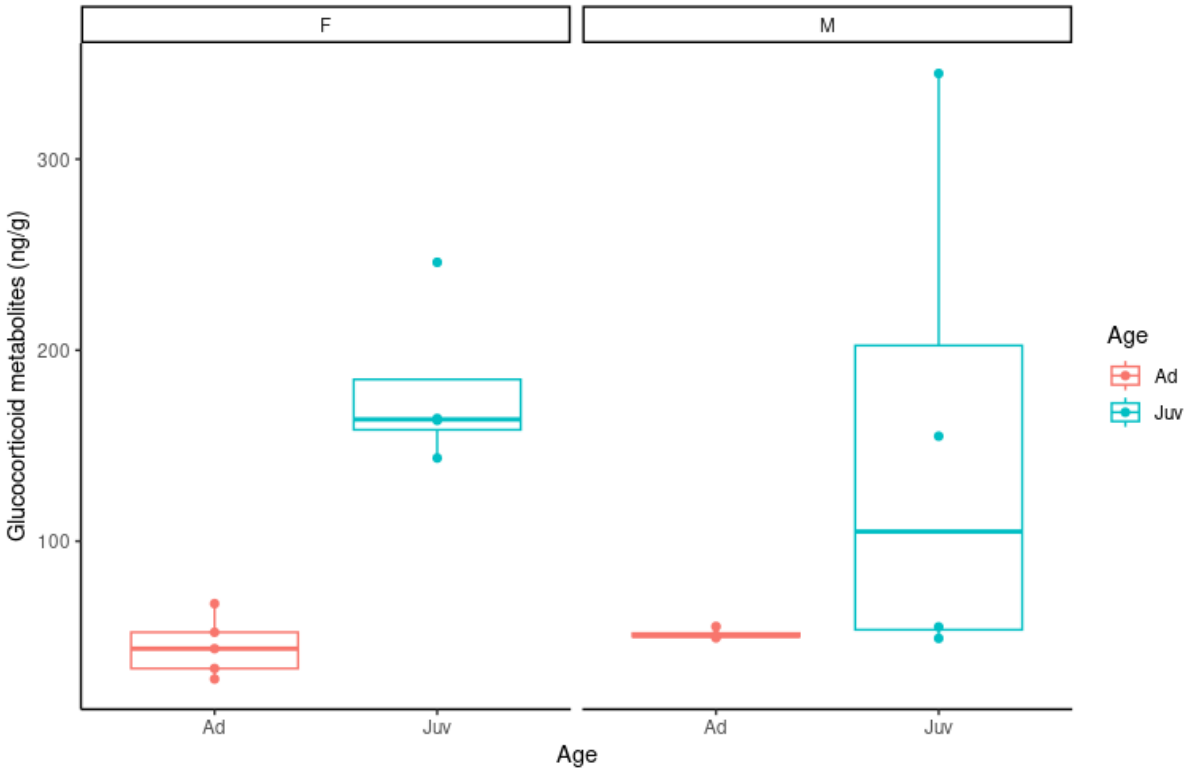

Fig. 10. Boxplot showing glucocorticoid metabolite levels (ng/g) of different age groups and sexes. One point on the graph represents the median level of one individual. "Ad" stands for adults, coloured in red, and "Juv" for juveniles, coloured in blue. "F" are females and "M" are males.

4. Discussion

This study aimed to 1) validate suitable EIAs for reproductive hormone metabolites and 2) to calculate baseline levels of those hormones, as well as for glucocorticoid metabolites. The research hypotheses are repeated here:

1. Reproductive Hormones:
    - **H1a:** Reproductive hormones (oestrogens, progesterone, and androgens) will exhibit higher levels in sexually mature individuals compared to juveniles.
    - **H1b:** During the breeding season, the reproductive hormones mentioned under H1a will show elevated levels in both males and females compared to the non-breeding season.
2. Glucocorticoids:
    - **H2:** Glucocorticoid metabolite levels will be higher in both sexes during the breeding season, reflecting increased physiological demands associated with reproductive activities.

4.1 EIA Validation

The validation process was a challenge. The testosterone EIA detecting 17β-hydroxyandrogen metabolites showed no satisfying results. In contrast to H1a, and against any biological doctrine (14), our results exhibited higher values in juveniles that in their adult counterparts. The epiandrosterone EIA measuring 17-oxoandrogen metabolites showed somewhat more promising results, presenting higher levels in adult males than in juvenile males. However, juvenile females expressed slightly higher amounts compared to adult females, which again seems questionable, and puts into question the validity of the test. Furthermore, both, the progesterone EIA measuring 20-oxo-pregnanes and the total oestrogen EIA, provided only inconsistent results, which were not applicable for statistical analysis.

Similarly to this study´s findings, other authors such as (10), who established urofaecal steroid profiles of Blue-fronted parrots *(Amazona aestiva)*, reported challenges in oestrogen assay validation as well, suggesting the complexity of this task and the need for species-specific adjustments. Our selected EIA might have lacked the necessary sensitivity to detect oestrogens present in low concentrations in faecal samples.

Out of the two tested EIAs detecting androgens, the epiandrosterone EIA seemed to be more suitable than the testosterone EIA. However, the applicability of this test to measure androgen metabolites in kea parrots should be evaluated critically, as the results are not entirely convincing, as female juveniles showed higher levels than female adults (27). In the case of progesterone and oestrogens, no EIAs could be validated in this study.

There are two possible ways to overcome insufficient validation of an EIA. In a study published by (28) for example, the author uses a method of enzymatic deconjugation using beta-glucuronidase / arylsulphatase, enzymes secreted by the snail *helix pomatia.* This method (hydrolysis) is used to make conjugated metabolites accessible for detection. However, the process is linked to a considerable additional workload and the outcome is not clear, which is why this method was not applied for this project from the beginning. However, this method may be worth trying, due to the unclear results found with the current methodology.

A second possible way to overcome the difficulties in validation would be to try other EIAs. A good test to continue the investigation on androgen metabolites, for instance, would be one of the two newly introduced androgen metabolite EIAs developed by (Möstl et al. 2023)5β-dihydrotestosterone EIA. The use of enzymatic deconjugations and/or to try other available EIAs could be applied for androgens as well as progesterone and oestrogens. These tests may be more sensitive to androgen metabolites in kea parrots than those already tried.

There are a few considerations to propose concerning the study´s results. First and foremost, there was only a limited number of study subjects, and there were no egg-laying females within the cohort. Egg-laying females would possibly exhibit extreme levels of reproductive hormones, but they had to be excluded from this study because they are too territorial and aggressive to work with directly, which excluded sample collection using the current methodology. Especially androgen levels might critically rise during the egg-laying and incubation period. Further studies using different collection techniques could allow egg-laying females to be included. Moreover, the higher testosterone and epiandrosterone levels in juveniles compared to adults might be due to the possibility that the EIA does not only detect steroids produced in the gonads, but also detect steroids originating from the adrenal cortex. This was the reason why we decided to analyse glucocorticoid metabolite levels based on the

different categories as well *(Fig. 10)*. As levels of the juveniles were simultaneously high, we can presume that the steroids (or their precursors) measured with the testosterone and the epiandrosterone EIA may originate from the adrenal cortex.

To conclude this part of the discussion, this study was not able to identify suitable EIAs to quantify reproductive hormone metabolites, which underlines the necessity to validate a test and to use a supported technique to do so. Further investigations are crucial to advance in this matter.

4.2 Baselines

This study established baselines for epiandrosterone metabolites, if one assumes that the EIA is suitable for kea parrots, as well as baselines for glucocorticoid metabolites.

Concerning the epiandrosterone baselines, our two hypotheses (H1a) that reproductive hormones will exhibit higher levels in adults compared to juveniles, and (H1b) that they will be higher during the breeding season in both males and females compared to the non-breeding season would align with the results. However, as we are not able to fully recommend the usage of the test for this species, these results must be taken with caution, and more research is needed in this field.

Our hypothesis H2 established on glucocorticoid metabolite levels was that they will be higher in both sexes during the breeding season. This was not the case. Over the months of sample collection, the trend rises, being highest in August and September. However, the kea´s breeding season extends over the months of February to April, which leaves August and September being the months farthest away possible from breeding season. As glucocorticoid release is not only linked to reproductive activities, but also for example to environmental changes, we assume that this might be linked to the heatwaves during these months, as kea parrots are not used to these high temperatures. They showed signs of heat stress - hunched posture, laying flat on the ground, ruffled feathers, and a lack of motivation to work with the experimenter, compared to the months before - during this period. Another paper published in 2022 on faecal glucocorticoid metabolites associated with illness, sex, age, and season in a kea population at the Cincinnati Zoo by (29) also discussed this hypothesis. Their results did not show higher

concentrations during the summer months, but they utilized a method, which was not physiologically/biologically validated (18). One fact to underline this hypothesis is, that the trendlines for both sexes *(Fig. 8)* overlapped almost perfectly, which implies that neither sex nor reproductive state were responsible for the rise during the summer months, but a different, most likely environmental factor.

In a nutshell, the most valuable insights into the baselines of reproductive and stress hormone metabolites might be that kea parrots indeed showed signs of heat stress based on their higher glucocorticoid levels.

4.3 Other findings

Working with two different groups, I also investigated glucocorticoid metabolite levels based on the subjects´ location. The results show significantly higher levels at Tiergarten Wels compared to the juveniles at HHR. There are a lot of different variables that could influence these higher levels, from different collection methods to environmental aspects such as housing conditions or group dynamics leading to biological factors such as higher stress in general or even illness. A few months after the sample collection at Tiergarten Wels, some birds in the group were diagnosed with aspergillosis after one of them sadly died (unpublished data). This might be an explanation for the higher glucocorticoid levels preceding the illness.

In another paper elevated glucocorticoid metabolite concentrations were not observed in the kea prior to onset of illness (29). However, the authors of the study discuss, that “sample collections were opportunistic” and that the results could have been influenced by the variation of the individual and the method used (29).

This finding might give a new insight into glucocorticoid levels during an illness in kea parrots and to further investigate this matter, it would be intriguing to revaluate the kea´s levels at Tiergarten Wels after they have recovered from their disease.

In conclusion, our exploration marks a first step into the kea parrot´s behavioural and reproductive endocrinology. We gained new and interesting insights into the kea´s stress response, leading to elevated glucocorticoid metabolite levels induced by environmental changes and illness. As the study used a non-invasive method of faecal sampling, this knowledge could be used to elaborate specific markers or a specific method to measure rises in stress levels and to evaluate them accordingly. This might be especially helpful in captive birds to ensure their well-being. However, concerning reproductive hormone metabolites, the study could not establish suitable EIAs for kea parrots and more scientific effort has to be put into this matter.

5. Summary

This study aimed to unravel endocrinological dynamics of kea parrots *(Nestor notabilis)* using a non-invasive method of faecal sampling. The first step was to validate enzyme immunoassays (EIAs) for measuring metabolites of reproductive hormones - androgens, oestrogens, and progesterone. Additionally, baseline levels of the assays, along with glucocorticoid metabolites validated in an unpublished study, were established within and outside of the breeding season.

Two groups of captive kea parrots participated in the study, consisting of adults and juveniles, necessary for EIA validation, as the test showing the largest difference between adults and juveniles was rated the most suitable for the species.

Although the study was not able to validate an EIA for any of the tested reproductive hormone metabolites, glucocorticoid metabolite baselines showed higher levels during the summer months, indicating heat-stress. Furthermore, the two groups, originating from two different locations, showed different glucocorticoid metabolite levels being significantly higher at Tiergarten Wels compared to Haidlhof Research Station. Months after sample collection, the birds at Tiergarten Wels were diagnosed with Aspergillosis, which led to the theory that glucocorticoid levels rise prior to an illness and to the display of symptoms.

The results showed that it can be challenging to validate EIAs for a species. Furthermore, the study provided evidence that glucocorticoid metabolites might provide a suitable marker of environmental stressors and health issues in kea.

6. Zusammenfassung

Ziel dieser Studie war es, endokrinologische Gegebenheiten von Kea-Papageien *(Nestor notabilis)* mithilfe einer nicht-invasiven Methode der Kotprobenahme zu entschlüsseln. Der erste Schritt war die Validierung von Enzymimmunoassays (EIAs) für Metaboliten von Reproduktionshormonen - Androgene, Östrogene und Progesteron. Außerdem wurden Referenzwerte der validierten Hormonassays sowie der in einer unveröffentlichten Studie validierten Glukokortikoid-Metaboliten ermittelt. Dies sowohl während als auch außerhalb der Brutsaison.

An der Studie nahmen zwei Gruppen von in Gefangenschaft lebenden Kea-Papageien teil, bestehend aus Erwachsenen und Jungtieren, was für die Validierung des EIA erforderlich war, da der Test, der den größten Unterschied zwischen Erwachsenen und Jungtieren aufwies, für die Art am besten geeignet war.

Obwohl die Studie nicht in der Lage war, einen EIA für einen der getesteten Metaboliten der Fortpflanzungshormone zu validieren, zeigten die Glukokortikoid-Metaboliten in den Sommermonaten höhere Werte, was auf Hitzestress hindeutet. Außerdem wiesen die beiden Gruppen, die von zwei verschiedenen Standorten stammten, unterschiedliche Glukokortikoid-Metabolitenwerte auf, wobei sie im Tiergarten Wels signifikant höher waren als in der Forschungsstation Haidlhof. Monate nach der Probenahme wurde bei den Vögeln im Tiergarten Wels eine Aspergillose diagnostiziert, was zu der Annahme führte, dass die Glukokortikoidspiegel vor einer Erkrankung und vor dem Auftreten von Symptomen ansteigen.

Die Ergebnisse zeigten, dass es herausfordernd sein kann einen EIA für eine Tierart zu validieren. Darüber hinaus zeigte die Studie, dass Glukokortikoid-Metaboliten ein geeigneter Marker für Umweltstressoren und Gesundheitsprobleme bei Kea sein könnten.

7. List of Abbreviations

ACTH …….. Adrenocorticotropic hormone

EIA ……..… Enzyme immunoassay

HHR ……..... Haidlhof research station

HPA axis …….…….. Hypothalamic-pituitary-adrenal axis

8. List of Tables and Figures

9. References

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