Ex-situ conservation and breeding management of Southern cassowaries *(Casuarius casuarius)*

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Ex-situ conservation and breeding management of Southern cassowaries *(Casuarius casuarius)*

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Abstract

Despite a long captive history, breeding success of Southern cassowaries *(Casuarius casuarius)* in zoological organisations has been sporadic. Very little is known about their courtship and breeding behaviours, in particularly the part that vocalisations play in courtship display. In addition, as birds can be aggressive towards conspecifics, bringing birds together for the purpose of breeding (mixing) must be timed correctly. Therefore, understanding courtship and reproductive biology in this species is essential to improving management and breeding in zoos. This study examined three aspects of courtship and breeding in Southern cassowaries – courtship precursor behaviour, vocalisations, and hormonal profiles – across multiple birds and institutions. Instances of courtship behaviours and vocalisations were extracted from video footage and audio recordings, respectively. Hormone levels were monitored through non-invasive faecal sampling. I found that both precursor behaviours and vocalisations increased prior to mixing and varied seasonally, increasing in the spring and summer months before dropping in the winter. Rates at which both behaviours and vocalisations occurred differed widely between zoological organisations. Hormone levels showed seasonal changes, increasing between April and October and dropping again in the winter. However, changes in relation to mixing were not observed. There was no clear relationship between behaviour changes and hormone concentrations in the birds studied. Although mixes were successful, with no separation due to aggression, fertile eggs were not produced during the study period. Despite this, observations of Southern cassowary behaviours and vocalisations could still be helpful in determining the correct time for mixing as, anecdotally, when used to time mixes, aggression among conspecifics was limited. Hormone profiling of these birds may also be beneficial as a husbandry tool for identifying potential reproductive problems. Implementing monitoring programmes could help to improve reproductive success and management of this complex and potentially dangerous species in captivity.

Introduction

Birds are a wide ranging and diverse group, varying significantly in their biology. This diversity is mirrored in their mating systems. As a result, ex situ conservation breeding programmes can be extremely challenging to establish and maintain (Collar & Butchart 2014), especially where available information is limited. To date, focus has often been concentrated on developing programmes for passerine and psittacine species (Wilkinson, 2000, Wahle, et al, 2024). Because of this, programmes for some of the more unusual taxa that are not as commonly kept in zoological organisations are less well developed. Southern cassowaries (*Casuarius casuarius*) are one such species. Despite a long history of keeping cassowaries in captivity, breeding has been sporadic (Rothschild, 1900). Very little is known about their breeding behaviour and our understanding of courtship is limited, particularly the role of vocalisations. Information is mostly anecdotal, with insufficient applicable data available to help improve breeding success in captivity (Biggs, 2013).

Cassowaries belong to the superorder Palaeognathae, which includes ratites such as ostriches, emus, rheas, kiwis, as well as tinamous (Phillips, et al, 2010). Generally, ratites have promiscuous mating systems with communal nests and male-only parental care encompassing nest building, incubation, and chick rearing (Valdez, 2022). This is an unusual breeding strategy in birds and is thought to be an ancestral condition (Burley & Johnson, 2002). Southern cassowaries are a solitary species, only coming into close contact in the breeding season. They are also sequentially polyandrous, with females forming sequential pair bonds with multiple males across the breeding season (Biggs 2013). Males invest up to nine months of parental care in small clutches of two to four chicks each year. This combination of male only parental care and polyandry is rare among birds (Jenni 1974) and arguably the least understood of mating systems (Owens, 2002). As a result, ex situ breeding programmes for polyandrous species, particularly large, dangerous birds, such as Southern cassowaries, can be difficult.

Southern cassowaries are challenging to manage in captivity. As birds are solitary and territorial, they must be kept separate for most of the year, but enclosures should be connected to allow the birds to be brought together for the purpose of breeding (mixing). Cassowaries can be dangerous to both conspecifics and keepers, capable of causing serious harm and even death (Biggs, 2013). Mixing is also very challenging, as bringing the birds together for breeding relies on accurate timing. When the timing is wrong, birds may become aggressive towards one another and need to be separated to avoid injury (Biggs, 2013). To achieve successful breeding, the birds should be brought together at the right time to avoid aggression and should remain together until eggs are produced or until the end of the breeding season. To date, the decision to bring birds together for breeding was based on anecdotal information from keepers and curators or based on the time of year when breeding behaviour is expected. Systematic records of these mixings are sparse, and breeding success has been varied. To improve ex situ breeding management, a better understanding of courtship and breeding in this species is required.

Courtship behaviours play an important role in sexual selection across bird species, as well as promoting breeding coordination between the male and female (Fusani, 2008). The expression of these behaviours in Southern cassowary may indicate that the birds are in breeding condition and are therefore ready to mix. Very little is known about courtship in Southern cassowaries either in the wild or in captivity. Anecdotally, it is reported that females' food intake decreases as their vocalisations increase and tolerance to conspecifics and keepers increases prior to the breeding season (Biggs, 2013). No specific changes in males around the breeding season have been documented, except for an increase in boldness, making them more confident in their behaviour and in their environment (Biggs, 2013). As Southern cassowary courtship is yet to be documented in detail, similarities from comparable species courtship displays must be made. In emus *(Dromaius novaehollandiae)*, the closest living relative of cassowaries (Naish & Perron, 2014), courtship involves circling and head posturing in both sexes. Females may also become aggressive if the male approaches too fast in his courtship dance (Patodkar, et al, 2009). Breeding cycles, and therefore expression of courtship behaviours, are often seasonal in birds, and may be influenced by photoperiod, rainfall, food availability and diet (Ball & Ketterson, 2008, Wikelski, et al, 2000). Facilitating the right conditions for courtship to occur is another challenging aspect of ex sit breeding programmes.

Vocal displays are also an important part of courtship in many species. In some species, vocalisations are an integral part of mate selection, and can indicate quality (Nowicki & Searcy, 2004). Southern cassowaries have been reported to use infrasonic vocalisations, but little is known about the function of the calls, or whether they are part of courtship and breeding (Mack & Jones, 2003). As with other behaviours, vocalisations can be influenced by the seasons, with frequency of vocalising changing throughout the season (Ehnes, et al, 2018). Vocal behaviour in zoological organisations may also be influenced by factors absent in the wild. For example, the accuracy and effectiveness of vocalisations in zoo-housed birds may be influenced by anthropogenic contributions to the soundscape (Lewis, et al, 2024). This therefore poses another challenging aspect to ex situ breeding programmes.

Hormonal changes often underlie courtship behaviour and breeding (Earl, et al 2022) and, as a result, tend to fluctuate across the year. Seasonal changes in hormone concentrations have been documented across a wide range of bird taxa (Brown, et al, 2016, Valdez, 2022, Crofoot, et al, 2003 and Fusani 2008), revealing common patterns across species. Among males, an increase in testosterone drives courtship displays and breeding behaviour (Fusani, 2008). In females, oestrogen levels increase as they come into breeding condition, and spikes in progesterone are produced when eggs are laid (Pereira, et al, 2018). Assessing hormone levels for birds in zoological organisations allows us to monitor changes in hormone levels to identify cycles and assess breeding potential in birds that display abnormal behaviour. These techniques can also identify potential issues, such as a lack of reproductive hormone cycling, which could explain unsuccessful breeding attempts (Schoech, et al, 1991). As there can be marked variations in reproductive hormones, even between individuals, the ability to assess endocrine activity, easily, and safely has improved understanding of factors affecting reproduction (Schwarzenberger & Brown, 2013).

In this study, I will document courtship in Southern cassowaries to improve ex-situ breeding success. Firstly, I will record the occurrence of social behaviours associated with courtship from behaviour observations to establish if patterns in the rate at which these behaviours are displayed exist. Secondly, I will record vocalisations and look at the role they play within courtship. Finally, from faecal samples I will extract hormone profiles to establish seasonal changes in both male and female cassowaries in connection to breeding, and how changes impact courtship behaviour. As these aspects of courtship have not been examined in this species before, I aim to gain insight into behaviour, vocalisation, and endocrinology in this little researched species to determine the role they play in breeding success and potential implications for captive management.

Methods

Behaviour and vocalisations

Subjects and housing

I studied Southern cassowaries in Vogelpark Avifauna, The Netherlands (AF), Birdland, UK (BL), and Chester Zoo, UK (CZ). (Table 1).The three pairs varied in age and breeding history. The AF female hatched in June 2012 and the male in May 2015. This pair have produced chicks in 2017 and in 2021. However, during the study period (2022), the birds laid eggs that did not hatch, and necropsy of the eggs showed no signs of embryo development. At BL, the female and the male hatched in June 2011 and this pair are yet to produce chicks. During the study period (2022), the pair produced eggs which showed no signs of fertility and were broken during incubation. The CZ female hatched in October 2000 and the male in July 2006. The pair are yet to produce chicks but produced eggs in two of the study years (2019 & 2020) which showed no signs of fertility on necropsy.

Table 1: Dates that audio and video recordings were collected at each zoological organisation.

Location	SAMPLE	JANUARY	FEBRUARY	MARCH	APRIL	MAY	JUNE	JULY	AUGUST	SEPTEMBER	OCTOBER	NOVEMBER	DECEMBER
Avifauna	Audio	NA	NA	NA	NA	17/05/2022 - 31/05/2022	01/06/2022 - 20/06/2022	NA	NA	NA	NA	NA	NA
	Video	NA	NA	NA	13/04/2022 - 30/04/2022	01/05/2022 - 31/05/2022	01/06/2022	NA	NA	NA	NA	NA	NA
Birdland	Audio	NA	16/02/2022 - 28/02/2022	01/03/2022 - 31/02/2022	01/04/2022 - 30/04/2022	NA	17/06/2022 - 30/06/2022	01/07/2022 - 31/07/2022	01/08/2022 - 29/08/2022	NA	NA	NA	NA
	Video	NA	NA	NA	NA	NA	16/06/2022 - 30/06/2022	01/07/2022 - 31/07/2022	01/08/2022 - 16/08/2022	NA	NA	NA	NA
Chester	Audio	01/01/2020 - 06/01/2020	NA	26/03/2019 - 31/03/2019	19/04/2018 - 26/04/2018 01/04/2019 - 09/04/2019 09/04/2020 - 15/04/2020	01/05/2020 - 06/05/2020	09/06/2018 - 24/06/2018	02/07/2018 - 21/07/2018 13/07/2019 - 27/07/2019	23/08/2019 - 29/08/2019	13/09/2019 - 16/09/2019	05/10/2019 - 10/10/2019	NA	31/12/2019
	Video	01/01 - 31/01 2018, 2019,2020	01/02 - 28/02 2018, 2019, 01/02 - 29/02/2020	01/03 - 31/03 2018, 2019,2020	01/04 - 30/04 2018, 2019,2020,	01/05 - 31/05 2018, 2019,2020,	01/06 - 30/06 2018, 2019,2020,	01/07 - 31/07 2018, 2019,2020	01/08 - 31/08 2018, 2019,2020	01/09 - 30/09 2018, 2019,2020	01/10 - 31/10 2018, 2019,2020	01/11 - 30/11 2018, 2019,2020	01/12 - 31/12 2018, 2019,2020

In all three zoological organisations, male and female Southern cassowaries are kept in separate enclosures for most of the year to limit physical attacks (Appendix A, Figure A1,

Figure A2 and Figure A3). Cassowaries are territorial and can be very aggressive outside the breeding season causing severe injury and even death to conspecifics (Biggs 2013). Enclosures are separated by fencing, but birds have visual access to one another in certain areas to allow interaction. Enclosures are connected by gated systems that are opened to facilitate bringing the birds together (mixing) for breeding purposes. Mix date was determined by the holding zoological organisation in each year and varied between organisations (Table 2).

Table 2: Mixing and separation dates for Southern cassowary pairs at different zoological organisations for the years studied. The duration of time the birds were studied for each year is also included.

Organisation	Mixed	Separated	Time Studied
Chester Zoo	25/06/18	27/07/18	01/01/18 – 31/12/18
Chester Zoo	24/04/19	24/07/19	01/01/19 – 31/12/19
Chester Zoo	06/05/20	30/07/20	01/01/20 – 31/12/20
Birdland	02/05/22	16/08/22	16/06/22 – 16/08/22
Avifauna	18/04/22	01/06/22	13/04/22 – 01/06/22

Data collection

Video Data

I collected video footage during 2018, 2019 and 2020 for the Chester Zoo (CZ) birds using CCTV footage of cameras already installed in the building. Remote cameras were sent to Avifauna (AF) in 2022 and set up by the staff there for one breeding season. The cameras were then returned so I could review the footage collected during this season. At Birdland (BL), I set up the remote cameras in the enclosure for one season in 2022. I then collected the cameras and analysed the data recorded. However, because this video data was collected after the birds were mixed, it did not include precursor behaviours and was therefore not included in this study. Video footage at BL was conducted between 16 June - 16 August 2022 and at AF between 13 April – 01 June 2022 (Table 1). At both zoological organisations recordings were made with Swann enforcer swdvk-846854mqb-eu DVR CCTV system, using three and four cameras in BL and AF respectively. At BL three cameras were used, two filming the outside enclosures and one filming the indoors. All three had infrared provisions so night filming could occur. AF had four cameras filming the outside enclosures. Again, these had infrared capabilities, so night filming was possible.

Data collection at CZ was ongoing from 01 January 2018 to 31 December 2020 (Table 1). Networked CCTV cameras were used which included four cameras filming the enclosure with two indoors and two outside. The indoor enclosure is also fitted with an infrared lighting system for recording at night. Camera position and enclosure designs can be seen in Appendix A Figure A1, Figure A2 and Figure A3. In all cases, video data did not include audio recordings, and these were collected separately.

An ethogram was constructed based on key behaviours deemed relevant to courtship, which were identified from observations of the CZ birds prior to the start of data collection in 2018. These behaviours all relate to interactions between the cassowaries. Since cassowaries are a strictly solitary species outside the breeding season social behaviour was interpreted as precursor to courtship. Opportunistic observations prior to the start of this study suggested that these social behaviours increased towards the start of the breeding season.

Five main precursor behaviours were identified as follows:

Behavioural sequence 1: Male initiates interactions without response from the female, ('M Interested in F').

This behaviour describes the actions of the male towards the female but without the reciprocation of the female (Figure 1). The female must be present in the enclosure and in the field of vision of the male. No other stimuli are present other than the female. For an event to be included in the analysis, the birds needed to demonstrate the following sequence of behaviours:

1. The male approaches within 1m of the divider between enclosures with his body orientated perpendicular to the divider.
2. The female is more than 1m away from the divider and does not change behaviour.
3. The male lifts his head above the solid section of the divider (if present), stretching his neck to its full length.

4. The male remains at the divider with his head orientated towards the female. He may be stationary or pace slowly parallel to the divider. The female remains more than 1m from the divider, with no observable response to his presence.
5. The event ends when the male moves more than 2m away from the divider for more than 15 seconds or when the female moves out of the field of vision of the male.

Behavioural sequence 2: Female initiates interactions without response from the male, ('F Interested in M').

This sequence is identical to sequence 1 but with the sex roles reversed (Figure 1).

Behavioural sequence 3: The male and the female interact, ('Mutual Interest').

This behaviour describes the actions of the birds towards each other when they are both at the divider between enclosures with visual contact with one another (Figure 1). For an event to be included in the analysis the birds needed to demonstrate the following sequence of behaviours:

1. Both birds are opposite one another and within 1m of the divider with their bodies orientated perpendicular to the divider.
2. Both lift their heads above the solid part of the divider (if present), stretching their necks to their full length.
3. They remain at the divider and opposite each other keeping within the field of one another's vision, each focusing on the other.
4. They may be stationary or pace slowly, parallel to the divider either together or separately. During this, both must remain within 2m of the divider.
5. The event ends when either the male or the female or both birds move more than 2m away from the divider for more than 15 seconds or either bird goes out of visual contact of the other.

Behavioural sequence 4: Female showing 'submissive behaviour' towards the male, ('Female Submissive').

This behaviour describes the actions of the birds towards each other when they are both at the divider between enclosures and are in the field of view of one another (Figure 1). Submissive behaviour in male and female Southern cassowaries have been recorded by Biggs (2013) and is identified as a subtle lowering of the beak whilst the body is in a sternally recumbent position, and the head and neck are in the normal rooting position. For an event to be counted, the birds must demonstrate the following sequence of behaviours:

1. Both birds are opposite one another and within 1m of the divider between enclosures with their bodies orientated perpendicular to the divider.
2. The female keeps her body in a normal standing state whilst she points her beak down in a curved position, parallel to the neck and in turn can move it either to the right or left when on a ventral plane to the male.
3. When either left or right plane is facing the male, the female tends to dip the head and turn it away from the male, but this is not vital for this behaviour to be included in the analysis as it is the dipping motion of the head that it important.
4. The female can also lean her body forward during this event but will remain stationary during the behaviour.
5. The event ends when the female returns to normal posture for at least 15 seconds.

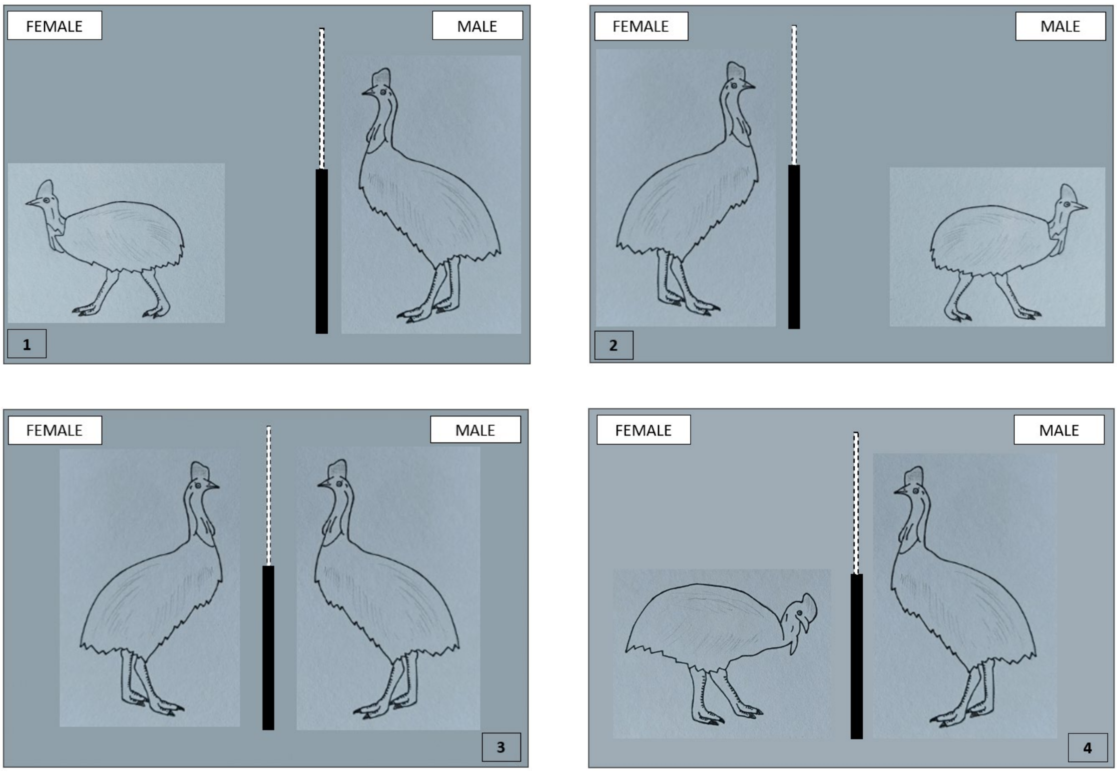

Figure 1: Visual representation of precursor behaviours 1-4.

Behaviour sequence 5: Vocal Posture

When vocalising, cassowaries produce a sequence of distinct behaviours (Figure 2). The association between vocalisations and this sequence was confirmed by comparing video data to audio recordings (see below). However, in some cases, the posture associated did not have a corresponding sound event in the audio recording when the different records were aligned. It is, therefore, possible that birds display vocal posture without producing sound. I counted the number of vocal posture displays produced by each individual from CCTV footage. However, as it cannot be confirmed that all instances of vocal posture are associated with vocalisations, vocal posture was quantified separately from vocalisations counted from audio recordings.

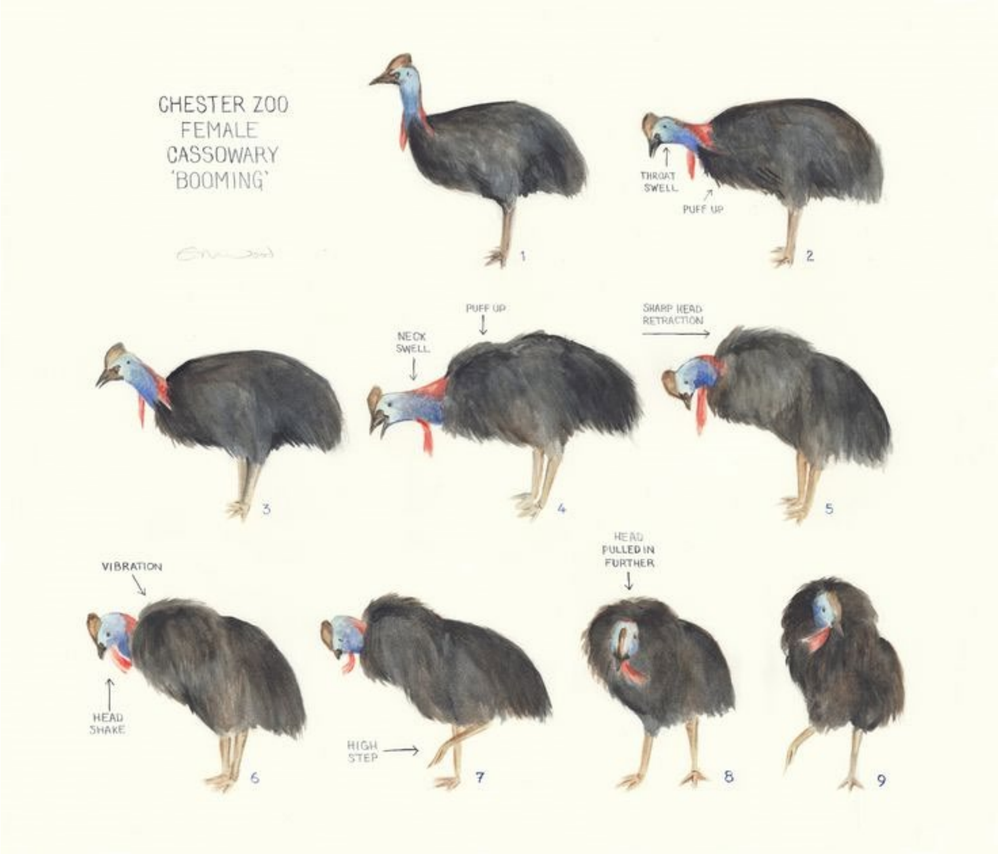

Figure 2: Sequence of distinct behaviours displayed by Southern cassowary during vocal posture/vocalisation, also referred to as booming (Art by Emma Wood).

Precursor behaviours were observed premix and in times of separation whereas vocal posture was observed throughout the year. It should be noted that precursor behaviours were defined by the presence of a barrier. Once mixed, as with vocalisations, courtship behaviour was continued to be observe between the pair including submission and mating. However, these behaviours are not comparable to those during premix because of the absence of a barrier. The purpose of this study is to establish courtship behaviours that indicate this otherwise solitary species is receptive to meeting another cassowary for the purpose of breeding. Therefore, incidences of courtship and mating when the birds were mixed were not included in this study. Instances of precursor behaviour and male and female vocal posture were counted from CCTV footage. These are measured as discrete events. I calculated mean daily behaviour within each week by dividing the total instances of behaviour produced during the week by the number of days (24-hour periods in all three zoological organisations) where data were collected. I then examined this data in two ways. Firstly, occurrence rates were examined centred to the mixing date (i.e., week of mix set to 0). As the mixing date varies each year based on the birds' behaviour, staffing, or previous mixing experience, centring the data to the mixing date allows for observations in consistency of managerial decisions to mix the birds across years (for CZ) and across zoological organisations (CZ, AF & BL). Secondly, the data was examined across the calendar year to assess if cassowaries change behaviour seasonally. I compared rates of vocal posture between the male and female at CZ using a paired Wilcoxon test, as data were not normally distributed, pairing data with weeks. I removed 49 weeks where neither individual exhibited vocal posture, to avoid zero-inflation of data. Data were not compared for AF and BL due to a lack of recorded instances of vocal posture.

Acoustic data

Acoustic data was collected sporadically for the same three years at CZ and for one season in 2022 for the AF and BL birds.

Acoustic data were collected at CZ, BL, and AF, with the amount and timing of data collection varying between zoological organisations (Table 1). Sound recordings were made using Wildlife Acoustics SM4 recorders (Wildlife Acoustics Inc., Maynard, MA, USA) placed within the enclosures as central as possible (Appendix A, Figure A1, Figure A2 and Figure A3). The audio recorders were sensitive to frequencies as low as 20Hz, making them suitable for detecting the infrasonic vocalizations of cassowaries.' At CZ the recorder was placed in the same position each year. Recordings were made with a sampling frequency of 24kHz, 16-bit depth and were saved as Wav files. Recorders were set to record daily between 05:00h and 10:00h.

Three call types were identified, two produced by the female and one by the male (Figure 3). Sex-specific calls were identified by comparison of audio recordings with CCTV data. In CCTV footage, it is possible to identify individual birds (i.e., male or female). Instances of vocal posture from each individual were identified in CCTV footage and related to the corresponding time in the audio recording. This allowed me to assign vocalisations to each sex, describe each call type, and produce a set of reference calls.

Female birds produce two different types of calls referred to as a ‘pulsed call’ and a ‘long call’. Female pulsed calls consist of one to six short elements, each with an inverted-u shaped frequency modulation, with a maximum frequency of ~250 Hz. Long calls consist of one to three long duration calls in sequence, again with a maximum frequency of ~250 Hz. Males produced a single, pulsed, call. These consisted of one to six short elements, with a maximum frequency of ~350 Hz. These elements are associated with a short “cough” at the beginning of the pulse, which can reach a frequency of up to ~650 Hz. Spectrograms of these vocalisations can be seen in Figure 3.

Recordings were manually examined in Kaleidoscope and instances of each call type (based on reference calls) were counted. The mean daily rate for each week was calculated, with a single average calculated across call types for each individual bird. Data were examined both in relation to mix date (mix-centred) and seasonally across calendar years. I compared rates of vocalisations between male and female at CZ using paired Wilcoxon tests, pairing data with weeks at each location. Comparisons between zoological organisations were not conducted due to large discrepancies in amount and timing of data collection.

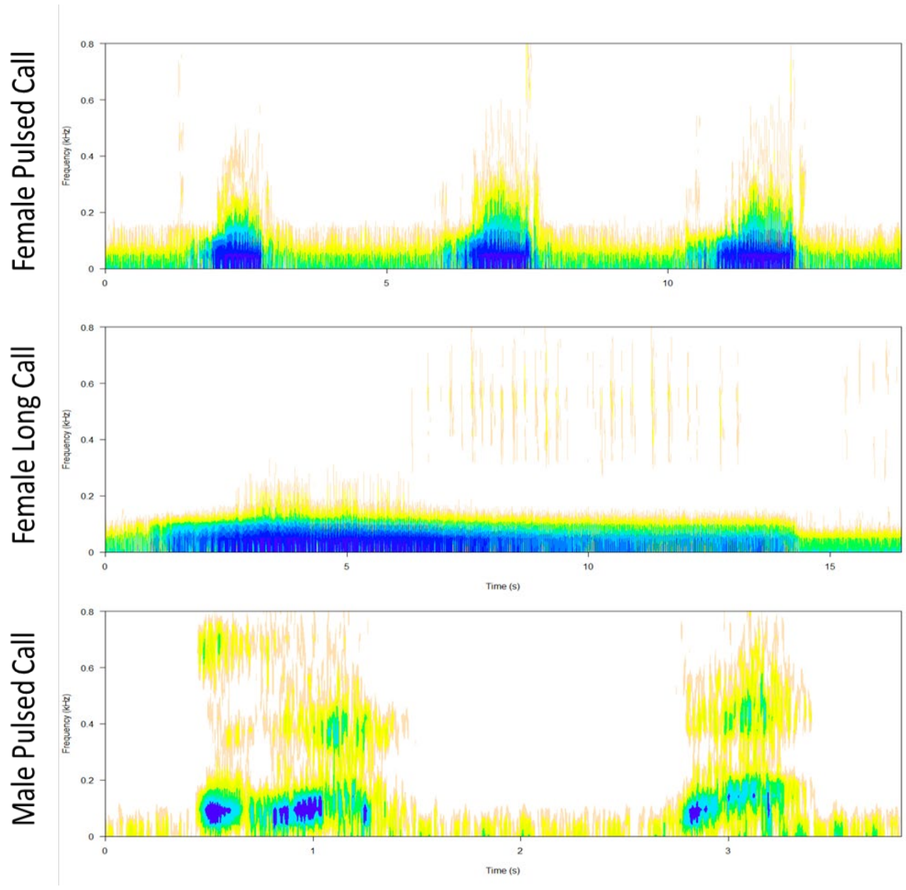

Figure 3: Spectrograms of male and female vocalisations used as reference calls.

Endocrine Data

Subjects

I studied the hormone profiles of six Southern cassowaries housed across three zoological organisations within Europe. This included the same birds from Chester Zoo, UK (CZ), a pair from Zoo Frankfurt, Germany and a pair from Zoo Aquarium de Madrid, Spain. The female at Frankfurt hatched in June 2003 and the male in July 1993. This pair have produced eggs every year since 2007. Fertility was seen in 2014 where egg necropsy showed advanced embryo development and chicks were produced in 2016. In recent years no fertility was observed on egg necropsy. The Madrid female hatched in July 1996 and the male in May 1995. This pair have produced eggs in 2001 only but with no fertility on egg necropsy.

Sample Collection

Duration of sample collection varied between zoological organisations. In CZ, samples were collected from January 2018 to December 2020, in Frankfurt, samples were collected throughout 2022 and the start of 2023, and in Madrid samples were collected between March and October 2022 (Appendix D).

Faecal samples were collected by keepers using the Cassowary Faecal Collection Protocol (Appendix B). Faecal matter was sampled at regular intervals, with ~3-4 samples collected per week. Suitable faecal matter was uncontaminated and as fresh as possible to avoid hormone degradation. Fist sized samples were collected and stored at -20 °C before shipping to Chester Zoo Endocrine Diagnostic Laboratory following the protocols, (Appendix C). For the samples to be included in analyses, each needed to be identified to the individual level, either by direct or CCTV observation or by default due to separation of the birds. The exact number of samples included varied between zoological organisations due to identification issues, cross-contamination, or a lack of suitable samples available for collection. Further details on sample size are available in Appendix D.

Extraction and Processing

Thawed samples were extracted using a dry weight shaking extraction similar to that described by Brown et al. (2016) and Edwards et al. (2013) with some modifications. Briefly, faecal samples were thawed, homogenised and lyophilised. The dry samples were then sifted to remove any undigested material. 0.10g (+/- 0.02g) of the sifted faecal material was added to 5ml of 70% methanol before shaking on a multitube vortex (1250RPM) for 30 mins. The samples were then centrifuged for 20 minutes at 1500*g* and the supernatant dried under air in a water bath at 50^{o}C. Finally, the dried samples were re-suspended in 1ml of 100% methanol and stored at -20^{o}C until analysis.

Enzyme immunoassay

Faecal progestagen and oestrogen metabolites in females and androgen metabolites in males were measured using a double antibody enzyme immunoassay (EIA) protocol as previously described by Edwards et al 2019 and Glaeser et al 2020, incorporating an anti-mouse (progestagen) or anti-rabbit (oestrogen and androgen) immunoglobulin (A008 and A009, Arbor Assays Inc., Ann Arbor, USA). This protocol was modified for the specific hormones of interest by incorporation of antisera (50µl monoclonal anti-mouse progesterone CL245 diluted 1:100,000, polyclonal anti-rabbit oestradiol-17β R0008 diluted 1:750,000, or polyclonal anti-rabbit testosterone R156/7 diluted 1:600,000, C.J. Munro, University of California, Davis); corresponding horseradish peroxidase (HRP) conjugated label (50 µl, 1:200,000, 1:200,000, and 1;200,000, respectively, C.J. Munro, University of California, Davis) and standards (50µl, 15.6 – 4,000 pg/ml, 78-20,000 pg/ml, and 46.8 – 12,000 pg/ml, respectively, Sigma-Aldrich, UK). Cassowary faecal extracts were diluted 1:5-1:100, 1:5-1:100 and 1:5-1:60, respectively for the three assays, and run in duplicate (50µl) on respective EIAs. Antibody cross-reactivities have been reported elsewhere (CL425 (Walker et al 2008), R0008 (Amaral et al 2013) and R156/7 (de Catanzaro et al 2003).

The immunoassays were validated biochemically for measuring progestogen and oestrogen metabolites in female Southern cassowary faecal extracts, and androgen metabolites in male Southern cassowary faecal extracts through parallelism and matrix interference assessment, and subsequent regression analyses (Table 3). To pass this validation, serial dilutions of faecal extract yielded displacement curves parallel to the standard curve and there was no evidence of matrix interference, as addition of appropriately diluted faecal extracts to assay standards did not alter the amount observed. Inter-assay CVs for high and low concentration synthetic and mid-range biological controls were 3.8%, 11.6% and 9.6% for the progesterone assay, 7.7%, 11.2% and 10.8% for the oestradiol 17-β assay, and 5.5%, 14.0% and 13.2% for the testosterone assay. All standards, samples and controls were run in duplicate, with duplicate CV's maintained below 10%.

Table 3: Biochemical validation of the three enzyme immunoassays for use with female and male faecal extracts. Parallelism assesses whether the assay quantifies the endogenous metabolites present in the sample proportionately to the synthetic standard and is assessed by regression of serial dilutions of the faecal extract relative to serial dilutions of the assay standard; significant regression indicates the sample binding is significantly predicted by standard binding. Matrix interference assessment assesses potential interference caused by substances contained within the biological sample and

is assessed by regression of expected vs. observed concentrations following addition of appropriately diluted faecal extracts to assay standards; significant regression indicates observed concentrations following subtraction of background concentrations in the sample are significantly predicted by expected concentrations.

Assay	Parallelism	Matrix interference
Progesterone CL425	$R^2 = 0.996$, $y = 0.982x + 5.803$, $F_{1,7} = 1590.582$, $P < 0.001$	$R^2 = 0.999$, $y = 0.873x + 0.131$, $F_{1,6} = 9197.0445$, $P < 0.001$
Oestradiol 17-β R0008	$R^2 = 0.986$, $y = 1.148x - 4.972$, $F_{1,7} = 509.082$, $P < 0.001$	$R^2 = 0.994$, $y = 0.900x + 5.536$, $F_{1,6} = 956.383$, $P < 0.001$
Testosterone R156/7	$R^2 = 0.974$, $y = 0.904x + 8.765$, $F_{1,7} = 257.487$, $P < 0.001$	R^2 Δ Σ] ∘

Data analysis

Baseline levels for each individual, year and metabolite (progestogen, oestrogen, or androgen) combination were calculated using a previously described iterative process (Brown et al. 1996). In this method, samples greater than the overall mean plus 2.0 standard deviations (SD) are removed, and the process is repeated until no more samples can be removed in this way. The iterative process was performed in R ver. 4.2.1 (R Core Team 2022) using the 'hormLong' package (Fanson & Fanson, 2014).

Concentrations were considered elevated when the first of at least two consecutive samples exceeded the baseline (i.e., the mean + 2SD), and no longer elevated at the first of at least two consecutive samples at baseline levels. As metabolite concentrations from faecal matter can be variable (Wang, et al, 2016) single points above or at baseline were not considered sufficient to signify the start or end of a peak and were assigned to the same category as the surrounding points.

Data were examined seasonally giving a profile of yearly hormonal changes for each individual. Data were not mix-centred when examining hormonal profiles. Hormonal profiles are likely to be affected by a range of factors, in particular egg and chick production. Therefore, it was not appropriate to combine years with different levels of breeding success for analysis. Separation of the birds may also be an important factor for hormonal change. Separation dates varied between years and zoological organisations, so could not be included where years were combined.

For the CZ birds, hormone, behaviour, and vocalisation data were collected simultaneously and were compared to establish if there was a relationship between metabolite concentrations and behaviour patterns. The weekly mean and standard deviations were calculated for each hormone. This allowed a direct comparison with the weekly mean rates of precursor behaviour sequences and number of vocalisations. To examine the correlation between hormone levels and the frequency of behaviours in the CZ birds, I conducted a series of Spearman's rank correlations comparing sex specific behaviour and hormone combinations. For the female, I correlated estradiol and progesterone levels with each of vocal posture, F interested in M, female submissive, and mutual interest. For the male, I correlated testosterone with each of vocal posture, M interested in F, and mutual interest. Spearman's rank correlations were used as data were not all normally distributed. Due to the number of tests conducted (n=11), I used a Bonferroni correction to reduce the likelihood of false positives, with a new significance level of $\alpha=0.0045$.

Results

Behaviour and Vocalisation

Behavioural sequences – Video data

At CZ, all four precursor behavioural sequences and male and female vocal posture increase in the weeks prior to the birds being mixed. There are some fluctuations as the season progresses, but all behaviours show an increased rate during this time. The four precursor behaviours were observed premix and in times of separation. Vocal posture was observed throughout the year and decreases once the birds were mixed. When the birds were separated, the four precursor behaviours and the male and female vocal posture increase once again (Figure 4).

Figure 4: Average Mix Centred Behaviour Data for Southern cassowaries. M = male and F = female. The red dotted line represents the week of mix as week 0. Greyed out sections show periods of time where data collection did not occur. For Chester Zoo (CZ), data collected in 2018, 2019 and 2020 was averaged whereas for Avifauna (AF) data were only collected in 2022. The data were recorded weekly.

At AF, a similar pattern is seen, with all precursor behaviours increasing before mixing (Figure 4). Although data collection was limited for AF, the rates of precursor behaviour are higher compared to CZ. In particular, ‘mutual interest’ was observed at a rate of 15 times per day in the week prior to mixing at AF (2022), whereas the daily average rate at CZ in the week prior to mixing was only 0.75 times per day (Figure 4). Data was not included from BL as video footage from before the birds were mixed was not available and therefore no precursor behaviours were recorded.

Patterns of vocal posture rates were similar for CZ and AF. Although there is limited data to examine rate changes pre-mix for the AF pair, rates of both male and female vocal posture reduced following mixing. The rates then increased again later in the mixing period. On the week of mixing, vocal posture was less often observed at AF with the maximum rate 0.28/day for the male, 0.28/day for the female in comparison to CZ where the maximum rate was 1.0/day for the male and 1.6/day for the female (Figure 4).

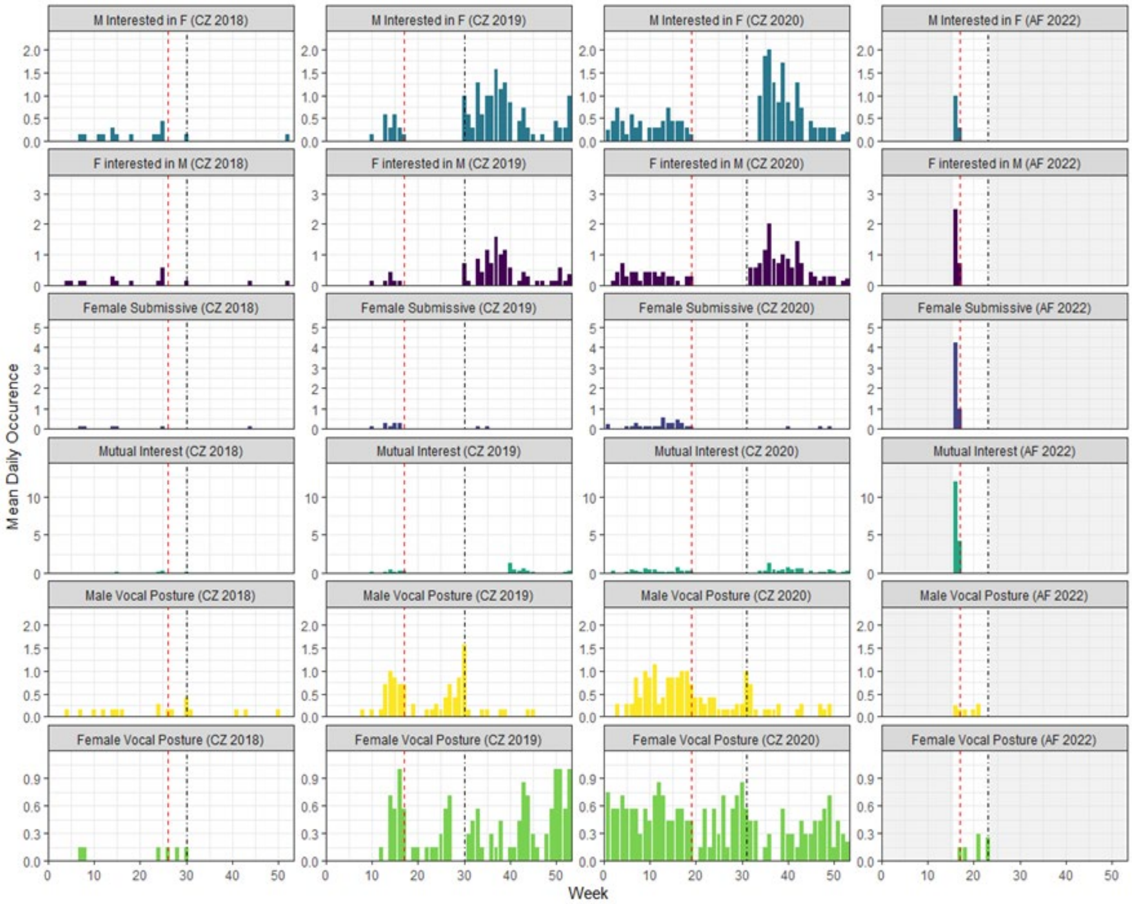

Figure 5: Seasonal behaviour patterns for Southern cassowary in Chester Zoo and Avifauna. The red dotted line represents the week of mix, and the black dotted line represents the week of separation. Greyed out sections show periods of time where data collection did not occur. Each panel shows the rate/day of behaviour for each year.

At CZ, across all year's, the four precursor behaviours show the same trend where the rate increases in the run up to the mix (Figure 5). Although behaviours are observed in all years studied, they are sporadic in 2018 and early 2019. Rates increased in 2019 and 2020, which are the years eggs were produced. In 2019 and 2020 separate interest shown by the CZ birds increases pointedly after separation in comparison to 2018. Male interest in female and female interest in male showed a maximum rate of 0.15/day in 2018 for both behaviours. In 2019 and 2020 the maximum rate of male interested in female and female interested in male was 1.7/day and 2.0/day respectively This corresponds with the years in which the male was sitting on eggs. Mutual interest does not show the same trend with 2019 and 2020 showing a maximum rate of 1.25/day in both years. Female submissive behaviour at CZ is also lower than separate interest rates with a maximum rate of 0.15/day for both 2019 and 2020 (Figure 5).

Vocal posture in both the male and the female were observed when the birds are separate and mixed. At CZ, male vocal posture rate steadily increases prior to mixing over the three years. In 2018 the maximum male vocal posture rate is 0.28/day, in 2019 is 1.0/day and in 2020 is 1.23/day. Female vocal posture rate shows a similar trend prior to mix with a slight dip in 2020. In 2018 at CZ, the maximum female vocal posture rate is 0.14/day, in 2019 is 1.23/day and in 2020 is 0.87/day prior to mix, showing that in 2019 and 2020 vocal posture rate was higher for both the male and the female. During the mix, in 2018 the vocal posture rate for both birds is pointedly lower than in 2019 and 2020. The maximum male vocal posture rate during mix for 2018 is 0.40/day, for 2019 is 1.60/day and for 2020 is 1.0/day. The maximum female vocal posture rate during the mix for 2018 is 0.14/day, for 2019 is 0.75/day and for 2020 is 0.87/day (Figure 5). Although the female showed a higher mean rate of vocal posture compared to the male, this did not reach significance (paired Wilcoxon Test: v=1979.5, p=0.059)

When comparing these results to the birds at AF, similar patterns of behavioural change can be observed. In general, the rates of behaviours, although a much smaller sample size, are higher for the AF birds then they are for the CZ birds, especially mutual interest, and submissive behaviour from the female towards the male prior to mix (Figure 5). For CZ, the maximum rate for mutual interest behaviour in 2018 for the week prior to mix is 0.29/day. In 2019 this rate increases to 0.40/day and in 2020 was a maximum of 0.70/day prior to mix. At AF, the maximum rate of mutual interest is 12.0/day, a pointed increase from that seen in the CZ birds. Vocal posture for the male increases notably just prior to separation in 2019 and 2020 at CZ and in 2022 at AF. Eggs were present in the enclosure in these time periods. Female vocal posture rate for CZ also increases steadily towards the end of the mix in 2019 and 2020. However, this is not seen in 2018. The CZ female laid her first clutch of eggs between 05/07/2019 and 18/07/2019 and her second between 17/07/2020 and 31/07/2020. Similarly, the AF female vocal posture also increased at the end of the mixing period as she laid and completed her clutch of eggs between 16/05/2022 and 26/05/2022.

Vocalisations – Audio Recordings

In general, female birds vocalised more than males. At BL, the female had a significantly higher vocalisation rate than the male (paired Wilcoxon test; v=366.5, p<0.001). At AF, the female's mean call rate was higher than the male's, although the difference did not reach significance (paired Wilcoxon test; v=38, p=0.075). At CZ, there was no significant difference in vocalisation rate between the male and female across all years studied (paired Wilcoxon test: v=119.5, p=0.833). However, this may be due to the birds displaying different call rates across this period. In 2018 the male was observed to call more than the female. However, in 2019 and 2020 the female's mean call rate was higher than that of the male. The CZ female vocalisation rate increases towards the mixing date with a maximum call rate of 5.5/day, two weeks before mixing. The CZ male vocalisation rate also shows the same trend as the female but peaks at 3.8/day, four weeks prior to mix. When the birds are together, the vocalisation call rate for both the CZ male and female birds drops dramatically, stopping completely for long periods of time. During the mix period, both birds show a maximum vocalisation rate of 1.5/day for one week only throughout this time. (Figure 6).

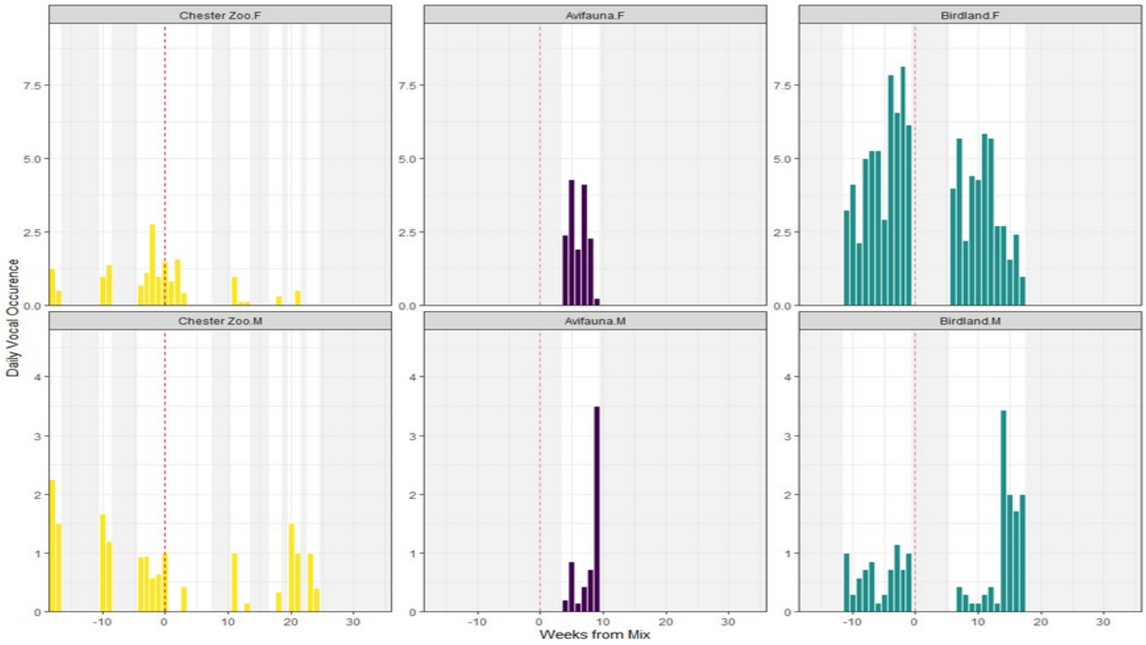

Figure 6: Average Mix Centred Audio Recordings. M = male bird and F = female bird. The red dotted line represents the day of mix and is therefore day 0. Greyed out sections show periods of time where data collection did not occur. For Chester Zoo (CZ), data collected from 2018 to 2020 has been averaged whereas for Avifauna (AF) and Birdland

(BL) only one year of data was collected in 2022. The data is recorded weekly. The scale on the y-axis differs for female and male figures to improve resolution for the latter.

When the CZ data is compared to the BL audio recordings prior to mixing, the BL female shows a similar patter to the CZ female. The BL female vocalisation rate shows a steady increase towards the mixing date, peaking at a maximum of 8.1/day, three weeks before mixing. Although the BL female's pattern of vocalisation is comparable to the CZ female, the vocalisation rate is substantially higher for the BL female. Post mix, the BL female shows a much higher vocalisation rate compared to the CZ female. Like the CZ female, after the break in data collection, the BL female shows a notable decrease in vocalisation rate compared to pre-mix rates with a maximum vocalisation rate 5.5/day. This again is a markedly higher rate than that of the CZ female whose maximum vocalisation rate post mix is just 1.5/day (Figure 6).

The BL male does not show the same increase as the females prior to mix but, like that of the CZ male, shows a more level vocalisation rate in the run up to the mix. It can also be seen that the male's vocalisation rate at both BL and CZ start to increase at the end of the mixing period. The CZ male has a maximum vocalisation rate of 1.5/day after mix and the BL male has a maximum vocalisation rate of 3.4/day after mix.

Although there is less data from the AF pair, with no data recorded prior to mixing, the AF female shows a very similar pattern and vocalisation rate to that of the BL female post mix. The AF female has a maximum vocalisation rate of 4.4/day post mix and the BL female has a maximum call rate of 5.8/day post mix. This vocalisation rate is sustained before decreasing once again as the season progresses. The AF male shows a very similar pattern to that of the male at both CZ and BL post mix where the males vocalisation rate rises with increasing time post mix. Again, the male at AF shows a comparable vocalisation rate to that of the male at BL with the maximum vocalisation rate of 3.5/day and the BL male having a maximum vocalisation rate of 3.4/day.

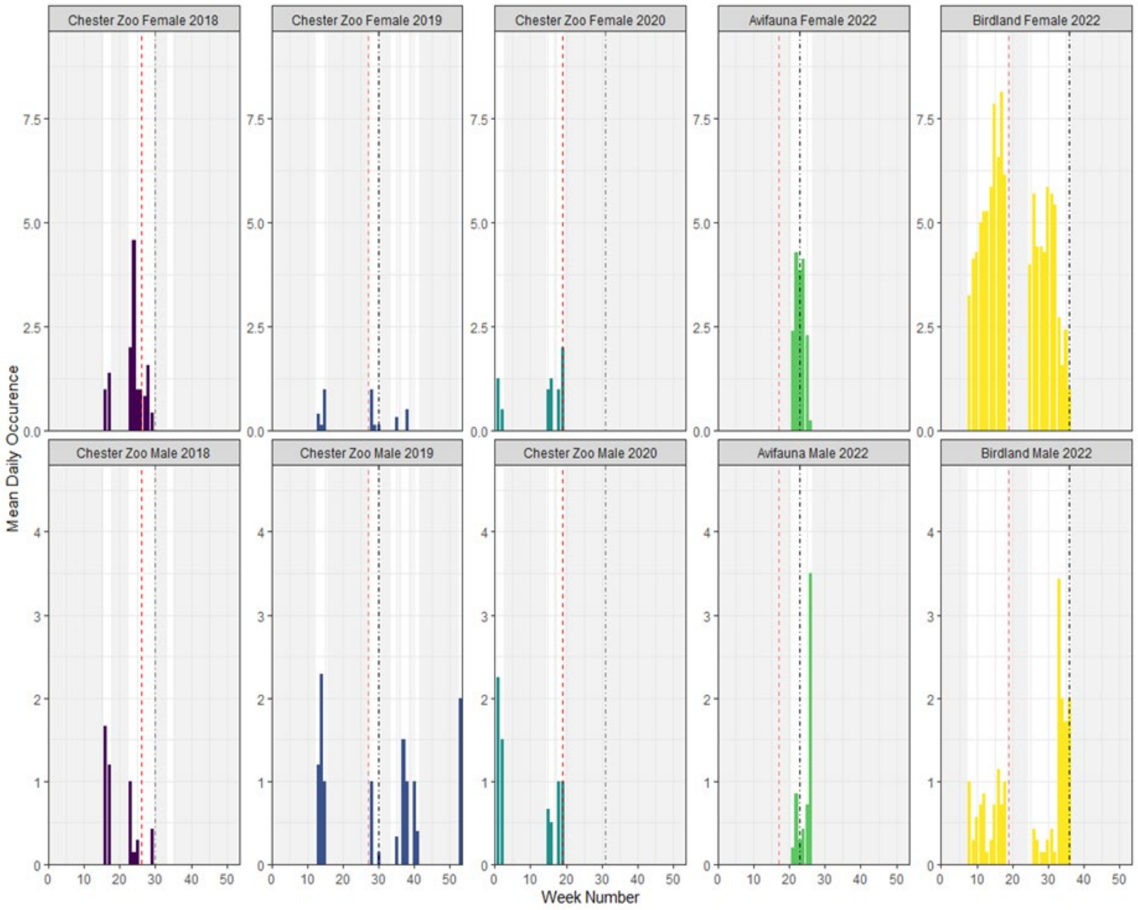

Figure 7: Seasonal Audio Recordings. The red dotted line represents the day of mix, and the black dotted line represents the day of separation. Greyed out sections show periods of time where data collection did not occur. Each panel shows the rate/day of behaviour for each year. The scale on the y-axis differs for female and male figures to improve resolution for the latter.

Several differences between years and organizations were observed in the data (Figure 7). For the CZ birds, there is a difference in the vocalisation rates for the male. In 2018 although sound recorders were placed in the enclosure, there were no vocalisations recorded once the birds were separated. However, in 2019 there is an increase in the number of vocalisations from the CZ male with a maximum vocalisation rate of 1.5/day post separation. For the AF male, were post separation audio recorders were captured, there is also a sharp increase in the vocalisation rate with the male showing a maximum of 3.5/day. Although no audio recordings were collected in 2020 at CZ after the birds were separated, the vocal postures for the male increase during, and just after separation (Figure 5). As for precursor behaviours, this corresponds with the presence of eggs for CZ and AF. This is also the case for the BL male, where the female laid between the 07/08/2022 and the 15/08/2022.

Endocrinology

Across males, testosterone was elevated above baseline following mixing, with peaks reported in the males in Madrid and Frankfurt, and for two of the three years studied for the male at CZ. At CZ elevation occurred just prior to the mix in 2019 and at Frankfurt in 2022. For all three zoological organisations levels remained continually elevated for a minimum of 28 days. Although the CZ male's testosterone was elevated following mixing in 2020, this was not sustained, with testosterone levels dropping below the baseline during the mix period. In two out of the three zoological organisations studied, testosterone levels remain elevated above baseline for a period after separation. In 2019 and 2020 at CZ and at Frankfurt in 2022 this elevation lasted for a minimum of 39 days. At CZ, in 2018 a drop in testosterone occurs directly on separation and for Madrid this decrease occurs in the middle of the mixing period (Figure 8).

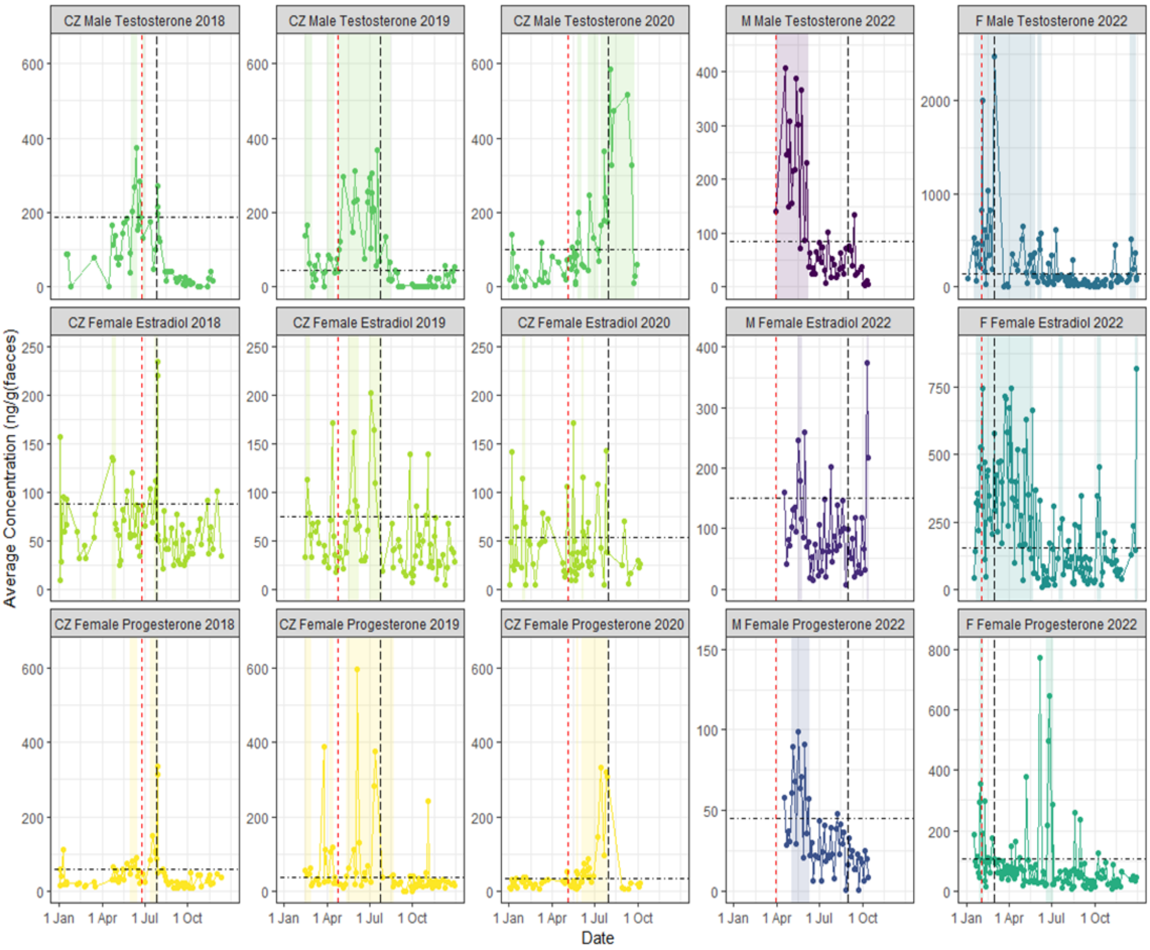

Figure 8: Yearly hormone profiles for 2018, 2019 and 2020 at Chester Zoo (CZ), and 2022 for Madrid Zoo (M) and Frankfurt Zoo (F). The vertical red dotted line shows the mixing

dates and the vertical black dotted line the separation dates. The horizontal dotted line shows the baseline hormone level for each bird. Coloured sections on each figure shows periods of time the birds' hormones exhibited two points or more of elevation above the baseline. The Y axis differs between organisations for individual resolution purposes.

Across all individuals and years, testosterone levels were elevated above baseline within the month of June. This appears to be independent of mixing status, as birds were mixed in two of the zoological organisation:year combinations and separate in three cases.

Testosterone concentrations differed in magnitude among individuals. In the CZ male, baseline levels across the three years ranged from 45.78 to 187.50ng/g(faeces). Peak levels increased across the years studied, from 375.38ng/g in 2018 to 583.88ng/g in 2020. Testosterone levels in the male at Madrid are similar in magnitude to those of the CZ male, with a baseline of 83.88ng/g and a peak of 407.18ng/g. However, whilst the male at Frankfurt however has a similar baseline to other individuals, 139.54ng/g, peak concentration reaches 1460.90ng/g, x2.5 higher than any other male studied. This pair have bred in the past and, although fertility was not seen in eggs produced in 2022, in subsequent years both have proven fertility (Figure 8).

No clear patterns in estradiol levels were apparent across individuals and years. However, the data suggests that peaks in estradiol levels occur around mixing. For the CZ female (2019 and 2020) and the Madrid female, peaks in estradiol levels occur while birds are mixed. In the Frankfurt female, estradiol levels are raised just prior to the mix date and remain elevated for the duration of the mix, a total of 28 days, but continues after separation for a further 80 days. This is similar to patterns seen in the Frankfurt male's testosterone levels. In most cases, females' estradiol levels reduce below baseline prior to or shortly after separation and remain at base levels. However, the Frankfurt female has two additional periods of raised estradiol concentration later in the year.

Estradiol concentrations differed in magnitude among individuals. For the CZ female, baseline levels range from 53.60 – 87.56ng/g, with peak concentrations ranging from 171.91 to 234.51ng/g. Estradiol levels are similar in the Madrid female, with a baseline of 149.96ng/g and a peak of 373.34ng/g after separation. However, although the Frankfurt female had a comparable baseline of 155.35ng/g, her peak concentration was markedly higher at 821.22 after separation.

Across individuals and years, progesterone concentration was elevated above baseline at some point during the mixing period. In the CZ female in 2018 and 2019 and the Frankfurt female, peaks in progesterone were also observed prior to mixing. In most cases, progesterone concentrations decreased to base levels prior to or at separation and did not peak again for the rest of the year. In the CZ female in 2019, progesterone levels remained raised for 46 days following separation, but did not peak again following the return to baseline. However, the Frankfurt female had a second peak in progesterone concentrations in July, during the separation period, which coincided with the laying of a second clutch of unfertilized eggs.

Individuals differed in their magnitude of progesterone concentration. In the CZ female, baseline levels ranged from 34.54 to 60.07ng/g, with peak levels ranging from 332.42ng/g to 597.44ng/g. The Madrid female had a similar baseline level at 45.45ng/g but lower peak concentration at 90.81ng/g. As for estradiol, the Frankfurt female had higher progesterone levels, with a baseline at 105.95ng/g, with peak concentration at 770.59ng/g, x1.2 higher than any other female studied (Figure 8).

Relationships between behavioural data and hormonal profiles (CZ)

Precursor behaviours

There is no clear relationship between testosterone and precursor behaviours. Average testosterone concentration was not significantly correlated with instances of any precursor behaviour (Spearman's rank correlation; M interested in F: rho=-0.026, p=0.818; mutual interest: rho=-0.006, p=0.954). Prior to mixing, peaks in testosterone do not coincide with any increase in precursor behaviours. Although there are peaks in testosterone while birds are mixed, precursor behaviours do not occur during mixing. Following separation, patterns between testosterone and behaviour changes are not consistent. A peak in testosterone concentration in the male at CZ correlates with an increased rate of 'Male Interest in Female' behaviour in 2020. However, a corresponding peak in testosterone is not apparent in 2019, despite a similar increase in the behaviour. Similarly, whilst increased 'Mutual Interest' following separation is associated with increased testosterone levels in 2020, increased rates on ‘Mutual Interest’ following separation in 2019 do not have a corresponding peak in testosterone concentration.

Figure 9: Yearly, seasonal hormone and behaviour profiles for 2018, 2019 and 2020 at CZ. Grey sections indicate the rates of behaviours observed in these years. Each behaviour is separated into male or female events and correlated with their respective hormone profiles for that year. The exception is mutual interest where both birds’ hormone profiles are shown alongside the behaviour data. The vertical red dotted line shows the mixing dates and the vertical black dotted line the separation dates.

Across female hormones, there is also no consistent relationship with precursor behaviour rates during the years studied. Neither female submissive behaviour nor mutual interest correlated with either estradiol (female submissive: Spearman’s rank correlation: rho= -0.060, p=0.577; mutual interest: Spearman’s rank correlation rho=-0.207, p=0.050) or progesterone levels (female submissive: Spearman’s rank correlation: rho 0.020, p=0.849; mutual interest: Spearman’s rank correlation rho=-0.210, p=0.048). F interested in M was not significantly correlated with estradiol (Spearman's rank correlation rho= -0.284, p=0.007) but was negatively correlated with progesterone levels (Spearman's rank correlation rho=-0.317, p=0.002) concentrations. Peaks in progesterone levels primarily occur within the mixing period. Outside of the mixing period, peaks in progesterone levels are sparse and short in duration, and elevations above baseline do not relate with any changes in the behaviour rates. As with progesterone, peaks during the mixing period cannot be associated with precursor behaviours. There are progesterone and estradiol peaks during the mixing period when 'Mutual Interest' behaviour cannot be exhibited. In 2019 and 2020, large peaks in rates for 'Female Interest in Male' and, to a lesser extent, 'Mutual Interest' are seen but do not correspond with any notable changes in either progesterone or estradiol levels (Figure 9).

Vocal posture

Male vocal posture shows peaks in 2019 and 2020 on separation which are matched with elevations in testosterone levels during this time (Figure 10). In 2019 this peak six days before separation is 368.05ng/g and in 2020 364.25ng/g five days before separation. This does however appear to be the only relationship associated with testosterone levels and rates of male vocal posture. There was no significant correlation between average testosterone concentration and male vocal posture across the years studied (Spearman's rank correlation; rho = 0.183, p=0.096). Females displayed vocal posture more consistently throughout the season compared to males (Figure 10). In 2019 and 2020 female vocal posture rate decreases after the mix then steadily increases towards separation. This is congruent with peaks in progesterone which also occur during this time. However, vocal posture behaviour in the female is consistent over the year. Although vocal behaviour does correspond with periods of elevations in progesterone, it is also seen when progesterone does not peak and increases in vocal posture behaviour occurs much later then the hormone elevation. Elevations in estradiol levels do not show associations with female vocal posture. There was no significant correlation between estradiol or progesterone concentrations and female vocal posture rates (Spearman's rank correlation: estradiol: rho = -0.202 p=0.056; progesterone rho= -0.196, p=0.066).

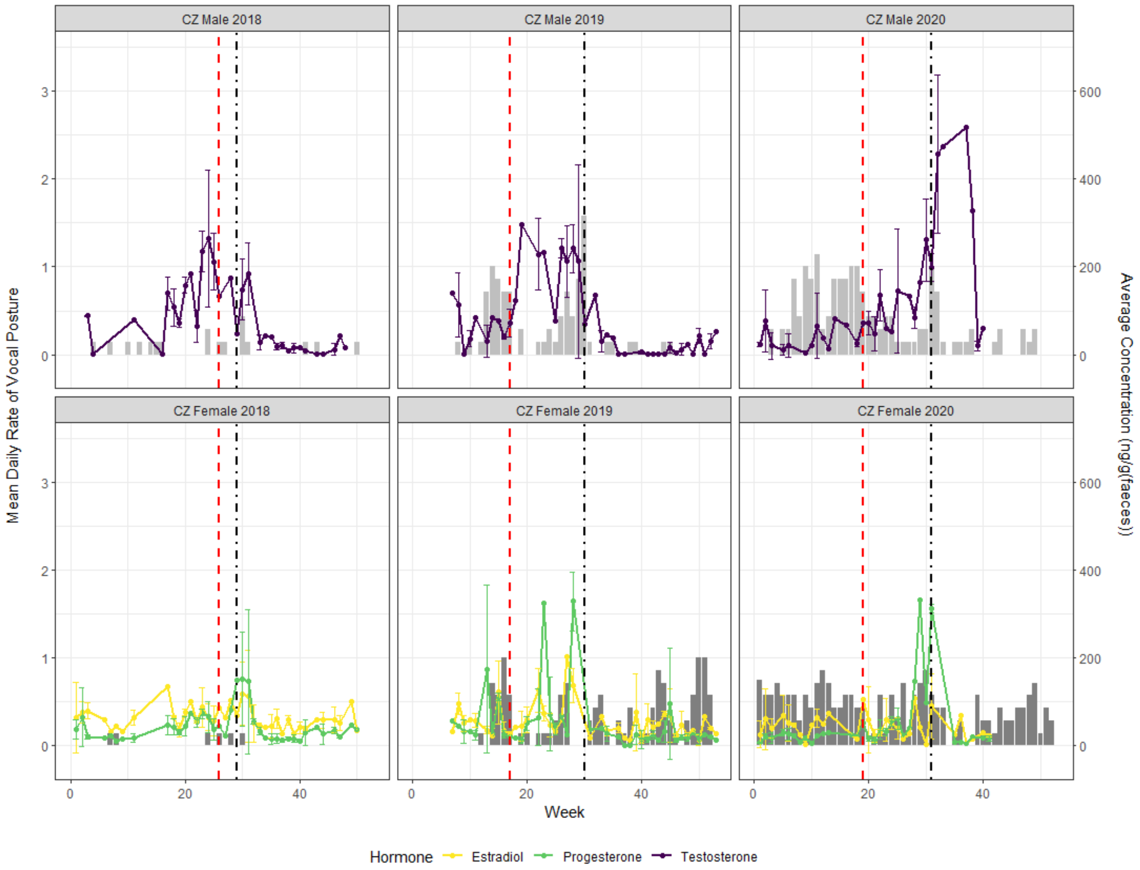

Figure 10: Yearly hormone and vocal posture profiles for 2018, 2019 and 2020 at CZ. Grey sections indicate the rates of vocal postures observed in these years. These are separated into male and female vocal postures and related to their respective hormone profiles for that year. The vertical red dotted line shows the mixing dates and the vertical black dotted line the separation dates.

Vocalisations

Acoustic data were collected intermittently throughout all three years, resulting in an incomplete data set. As a result, vocalisations were not recorded during important events such as mixes and therefore a comparison between hormonal data and vocalisations cannot be done. The subsequent data would in turn not provide useful data.

Discussion

In general, frequency of courtship precursor behaviours and vocalisations in Southern cassowaries increased prior to mixing. However, the rate at which behaviours occurred differed between individuals and zoological organisations. As well as their relationship to mixing date, these behaviours also showed seasonal changes across the year, increasing in the spring and summer, and dropping again in the winter months. Similarly, hormone profiles changed seasonally, with peaks above baseline occurring between April and October before dropping below baseline in the winter. Some consistent patterns were observed between years and zoological organisations, although changes related to mixing date were less apparent. There was no clear relationship between endocrinology profiles and behaviour changes in the CZ birds as peaks in hormone levels were not associated with specific behavioural changes.

As cassowary are a solitary species (Moore 2007), changes in social and communicative behaviours are considered to be important for courtship. Increases in these behaviours prior to mixing, as seen across zoological organisations, could therefore indicate increased readiness to mate or at least signal increased social tolerance. In the Southern cassowaries studied, all interaction behaviours increased premix in both the male and the female. Very little is documented about the changes in behaviour connected to courtship in this species either captive or wild. However, these findings are comparable to other ratite species. In Common ostrich (*Struthio camelus*) courtship includes an increase in female posturing towards males and increased male aggression to form small territories (Mukhtar, et al, 2017). In Great tinamou *(Tinamus major),* it was presumed that males courted females. However, a recent study by Guo et al (2024) showed that females predominantly court the males with a complex courtship display. Although ostriches are not solitary like cassowaries, Great tinamou show similar lifestyles, and these reported behaviour changes throughout the breeding season are similar to those observed in Southern cassowaries. As well as observing behaviour changes to establish a mixing date, precursor behaviours also showed some seasonal fluctuations. Precursor behaviours began to increase in early spring. After dropping during the mix, they then slowly increased as the season progressed before dipping again in the winter months. This suggests that external factors, such as photoperiod or food availability may also play a role in mediating courtship behaviours. Whilst some zoological organisation may alter dietary and light provision throughout the year, it is not a requirement in husbandry and more research is needed on the benefits this may provide. However, these examples would suggest that the changes I have observed in Southern cassowaries are related to courtship display and seasonal influences.

Patterns for vocalisations are similar to those for precursor behaviours. I found that female vocalisation increased prior to the breeding season, especially for the young female at BL. In all zoological organisations studied, the females' vocalisations rate dropped after mix while the male vocalisation rate increased. This was also reflected in vocal posture where data was collected. Southern cassowaries have been identified to communicate in infrasound (Mack & Jones 2003). However, how this relates to social interaction is not well understood. As both vocalisation and vocal posture increased prior to mix alongside precursor behaviours suggests that the birds are more interested in communications and social interaction during the breeding season. This is also seen in Greater rhea *(Rhea americana)* where adult males are relatively silent outside of the breeding season, but vocalise over great distances during it (Pérez-Granados & Schuchmann, 2020). This is similar to Southern cassowaries although the sexes are reversed, as it is the females who have been observed to vocalise more premix. Increases in male vocalisation and vocal posture were particularly noticeable when males were in contact with eggs and was seen across all three zoological organisations studied. Females also steadily increased their rate of vocalisation and vocal posture after eggs were laid or on separation from the males, but this decreased once again as the season progressed.

Cassowaries are sex role reversed compared to most other bird species. Although some other ratite species show male-only parental care (Brennan, 2012), they do not demonstrate complete role reversal in other aspects of courtship. However, in Great tinamou, role reversal is not only seen in parental care but also in courtship display (Guo, et al, 2024) potentially due to the male post-mating reproductive investment outweighing the total female reproductive investment (Andersson and Iwasa 1996). Because males are the primary parental caregiving sex, they select females based on courtship displays and are choosy when selecting a mate. Given that Southern cassowaries are polyandrous, and demonstrate male only parental care, it is possible the same is true as behaviours, particularly vocalisations and vocal posture suggest that females vocalise to attract males. This is supported by patterns in behaviour observed post separation. Increases in female vocalisation rate post separation may signal their readiness to mate again with other males. On the other hand, increases in male vocalisations in the presence of eggs could be for reestablishment of territories to allow incubation and chick rearing and therefore signals to the females to leave. However, some evidence also suggests that wild male cassowaries may attract more than one female to their nest, meaning this species could be both polyandrous and polygamous (Moore, 2007), further explaining this change in vocalisation rate in both males and females.

Faecal hormone analysis is a non-invasive technique for assessing an animal's reproductive status, which can be beneficial to ex situ conservation (Edwards, et.al 2014). In the Southern cassowaries studied there did not appear to be any correlations in hormone concentrations relating to the mixing dates, with variable hormone levels between years and zoological organisation. However, there does appear to be some relationships between the time of year and hormonal changes, with elevations in testosterone, estradiol and progesterone observed between the months of April and July in all three zoological organisations. Since this is the first endocrine study on Southern Cassowaries the data cannot be compared to other studies. Seasonal changes in hormones are reported in a range of other species (Schoech, et al, 1991, Earl, et al, 2022 and Brown, et al, 2016), with peaks in estrogen, progesterone and testosterone associated with the onset of the breeding season due to stimulation from increased daylight hours (Leska & Dusza, 2007). In temperate species, long photoperiods are required to achieve full gonadal size in males, with long photoperiods and supplementary factors such as food availability required for follicle development in females (Ball & Ketterson, 2008). Whilst these changes are well understood in temperate species, mechanisms that drive the same behaviour in tropical species is less well understood. However, studies in Spotted antbirds *(Hyophylax naevioides)* show that tropical seasons can be as predictable as those in northern temperate zones, with birds showing clear shutdowns in reproductive capacity and behaviour from October to February (Wikelski, et al, 2000). In the male Southern cassowaries studied, testosterone levels varied seasonally, with all birds showing peaks during the spring and summer months. Male courtship behaviour is strongly influenced by testosterone (Fusani, 2008). Therefore, it is possible that, as in other species, courtship occurs during the spring months in cassowaries. Peaks may also correlate with specific breeding events. In Whooping cranes *(Grus americana)* (Brown. et al 2016), egg laying females had significantly higher mean estrogen, but lower mean progesterone compared to non-egg laying females. In the female cassowary at Frankfurt, peaks in estrogen levels are associated with the two clutches of eggs she produced. However, my findings also show that this female had substantially higher levels of progesterone in comparison to others observed, including non-egg laying females. In contrast, the CZ female had low levels of estrogen in both 2019 and 2020 where she produced eggs. In 2019 estrogen levels peaked above baseline in the month she laid but this same pattern did not occur in 2020. She also had low levels of progesterone compared to the Frankfurt female. These differences could be due to several factors. As validation testing was done prior to this study, it would suggest that the assays performed appropriately, and that hormone levels observed are correct. The female at CZ has had an abnormal background, not producing eggs until she was 19 years of age. This is extremely late as young females usually start laying between four and five years of age (Biggs, 2013). Therefore, the CZ female may be compromised on a physiological level, showing unusual hormone profiles.

In the CZ birds, there was no clear relationship between precursor behaviours and the endocrine profiles of both birds. However, for the female there was a negative correlation between 'Female interest in Male' behaviours and progesterone. It is possible that this result is influenced by the presence of eggs in the enclosure driving these behaviours as opposed being related to courtship. Given that Southern cassowaries are usually solitary and would not be in close proximity to a male guarding/incubating egg, observing the male do this may have been of interest to the female and caused this increase in behaviours. It should also be noted that autocorrelation between weeks was not accounted for in the statistical analysis, which may have resulted in overinterpretation of the data. Breeding attempts at CZ did not result in aggression and the birds remained together until the unfertile eggs were laid. There are several reasons why this may be the case. First, the birds may be compromised physiologically (i.e. not producing enough hormones or producing them atypically) and therefore do not show typical seasonal hormonal fluctuations or behaviour. Second, management decisions based on precursor behaviours for the mixing date may not be accurate, resulting in mixes being inappropriately timed. Alternatively, changes in behaviour may be showing that pairs are behaviourally ready but are not physiologically in breeding condition. In all cases, steps can be taken to induce breeding condition and synchronisation of the pair. As photoperiod is an important factor in inducing breeding condition in many species (Hau, et al, 1998), amending the light levels where birds are shut into indoor housing overnight during the winter months could help to promote hormonal changes in the birds and naturally bring them into breeding condition. It was reported that cassowaries favoured red or pink fruits and higher protein content food items prior to the breeding season suggesting that female cassowaries, like other birds require certain food items to promote follicle production (Biggs, 2013). Therefore, dietary changes can be introduced to husbandry routines to help with seasonality. Although Southern cassowaries have been kept in captivity for many years, diet variations in relation to breeding are still not well understood.

Across all aspects of Southern cassowary biology studied, there were large differences in magnitude between individuals. Levels of mutual interest were highest in AF birds, with pre-mix levels approximately 10 times higher than those in the CZ birds. This is the youngest pair and have bred successfully in the past. The Frankfurt pair are also a successful breeding pair and showed the highest hormone concentrations in this study but are comparatively older (19 and 29 years). It is possible that pair compatibility affects levels of behaviour or hormones observed. Both precursor behaviour and hormone levels are lower in birds which have not had breeding success with their current partner.

Results of this study suggest that pair compatibility could be important for success and may be indicated by both behaviour and endocrinology. Mate choice in female birds is well studied and certain secondary sexually selected traits have evolved in males to facilitate females to choose the best mate. In passerines, song is often used in mate choice, and may indicate male quality (Andersson, 2005, Riters, et al 2000 and Gil & Gahr 2002). Morphological features may also play a role. Peahens *(Pavo cristatus)* choose males which not only have the largest tails but also the ones who have the largest number of eye spots (Nowicki & Searcy, 2004). As Southern cassowaries are polyandrous, male rather than female mate choice could be the key in establishing suitable pair bonds and increasing breeding success, as is the case in Great tinamous (Guo 2024) and other polyandrous species such as jacanas (Fresneau, et al, 2021) and phalaropes (Schamel, et al, 2004). Given that other polyandrous ratites exhibit male mate choice and based on my findings in this study where female have exhibited role reversal in behaviours such as vocalisation patterns, Southern cassowary may also have male mate choice. Therefore, changes in husbandry may need to be implemented to allow this to occur. In particular, changing existing social structures of one female to one or two males to multi-female single male groups may be beneficial. In the wild, females would naturally move away from males outside of the breeding season and during incubation and chick rearing. This is not possible during current captive management, and understanding the impact of reduced movement and its effect on the natural cycles of breeding behaviour would also be a fertile research area.

Management of Southern cassowaries is challenging, as birds pose a significant threat to conspecifics. Mixings require staffing levels to be adequate so that intervention can occur should the birds show elevated levels of aggression. Traditionally, mixes have been timed on keeper and curator experience, using anecdotal behaviour to time the mix, or simply using the time of year when breeding behaviour is expected. In both cases details of the timing is sparse and success of mixes is extremely varied. This study has shown that behavioural observations may be helpful for informing and timing mixes in this species. Although these observations are yet to produce a mix where fertile eggs are produced, anecdotally, using behaviours to time mixing has been successful in reducing aggression. The behaviours chosen for this study therefore might be helpful when looking for readiness to mix. However, as little information is available on how wild Southern cassowaries interact, we do not know how the behaviours observed in captivity relate to those exhibited in wild birds. Given that behaviours change throughout the different stages in the breeding cycle, and keepers have reported decreased aggression when using this method, I recommend that zoological organisations use behavioural observations and monitoring of vocalisations as a tool for mixing their birds. Alongside behaviour observations, hormonal profiling would be extremely useful for understanding hormonal cycling in relation to breeding condition in this species, as considerable individual differences were seen in the birds studied. Male mate choice may also be an important aspect to consider in future management to improve captive breeding success in Southern cassowaries.

Some limitations experienced during this study include the overrepresentations of the birds at CZ. Three years of consistent data verse one year for the other two zoological organisations studied. Acoustic data for all three zoological collections was acquired sporadically. Again, at CZ data was collected opportunistically over 9 months of the year in comparison to both BL which had 6 months of almost solid records and AF which only had 5 weeks of continued recordings. In addition to this, cameras could only cover a certain percentage of the enclosure and therefore could have led to behaviours being missed in all three zoological collections. Further study of this species would benefit from a broader range of zoological organisations with different breeding success participating in all forms of data collections.

This study has therefore created many further research opportunities to better understand not only the breeding behaviour of Southern cassowary but also their biology. Vocalisations of this species have not been described. Expansion of this aspect of my thesis would allow for greater knowledge in this area and create the potential to use this as a tool in breeding management. A full behaviour repertoire of the species throughout the year in multiple organisations would help further develop courtship behaviour understanding, alongside hormone profiling. Having greater knowledge in these areas could help develop the husbandry guidelines for this species in the future. It could also allow for zoological collections holding this species to have more confidence in the behaviours they are witnessing and use these as a tool for mixing these potentially dangerous birds together at the correct time. This would then have a positive impact on the welfare of the species alongside increasing its breeding success.

Overall, I found that precursor behaviours and vocalisations increased prior to mixing, and behaviours, vocalisations, and hormones changed seasonally. Across all parameters measured, there was large variation in magnitude between individuals. My results suggest that compatibility of individuals may be important in determining breeding success. Based on these findings, I recommend that holders observe birds throughout the year. Where possible, hormone profiles will help to determine cycling and pair synchronisation. Larger scale changes in management, including altering group composition to multi-female single male groups to allow male mate choice may also be beneficial. Further research is needed to determine factors impacting seasonal changes in behaviour and hormones and to determine the role that vocalisations play in their courtship. Increasing the number of hormonal profiles of Southern cassowaries in all breeding situations will give a much better understanding of how hormones change and how hormone levels vary between successful and unsuccessful pairs. This could include the role that testosterone plays in female courtship behaviour in sex role reversal. This may in turn become a useful tool for future mixings in combination with behaviour and vocalisation observations.

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Appendix

Appendix A

Avifauna

The southern cassowary enclosure consists of two outdoor paddocks that have a series of well-established trees and are approximately 385m², surrounded by 2.5m high chain link fence. The paddocks both have separate indoor housing which are approximately 7.5m². (Figure 1). The enclosures are linked by 3 sliding gates. There is no additional lighting or heating in the indoor areas.

Birdland

The southern cassowary enclosure consists of two outdoor paddocks that are both approximately 385m², each with a shallow pond. The public can only view the birds from one side of the enclosure. The paddocks have a series of well-established trees and are mainly grassed, surrounded by a 2.5m high chain link fence. The paddocks lead into an indoor enclosure which has two areas both measuring 7.5m², (Figure 2). The enclosure can be linked using a central sliding door in the indoor house and two external sliding gates in the outside paddocks. There is no additional heating or lighting, just windows in the side of the shed.

Chester Zoo

The southern cassowary enclosure at Chester Zoo consists of two large outdoor paddocks both measuring approximately 360m². The 'Females' side includes a shallow moat accessible to the birds which is approximately 1.5m at its deepest point. The Male's side also has a water body. The public can view the birds from two areas of the enclosure. Both outside paddocks are well planted and surrounded by a chain link fence which is approximately 2.5m high. Both paddocks have a 25m² holding pen attached to the outside paddocks which links to the indoor area consisting of two 30m² areas. Each paddock is connected to the other with sliding doors (Figure 3). The house is heated to a maximum of 18°C with overhead heaters and has lighting on a natural cycle, replicating the season changes in hours of day light.

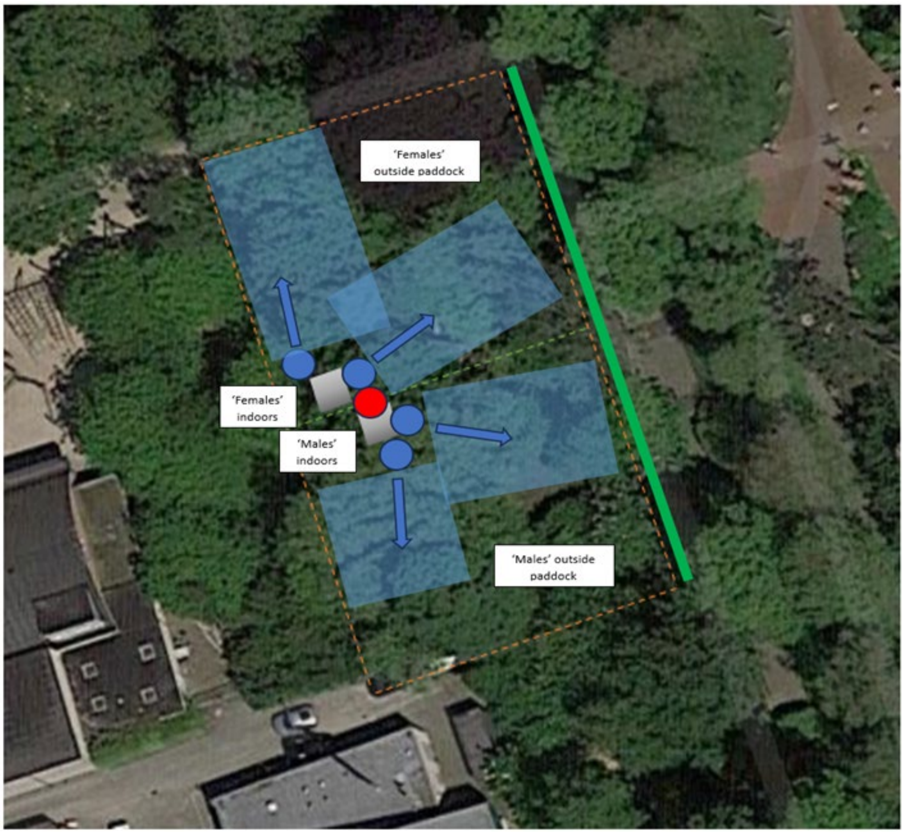

Figure A1: Networked CCTV coverage of the southern cassowary enclosure – Avifauna. Blue circles indicate the locations of the cameras and the arrows the direction in which they are pointing. The light blue areas indicate the range that can be seen on each camera. Red circles indicate the locations of the sound recorders. Green line shows the public viewing areas. Orange dashed line shows the enclosure boundaries.

Figure A2: Networked CCTV coverage of the southern cassowary enclosure – Birdland. Blue circles indicate the locations of the cameras and the arrows the direction in which they are pointing. The light blue areas indicate the range that can be seen on each camera. Red circle indicate the locations of the sound recorders. Green line shows the public viewing areas. Orange dashed line shows the enclosure boundaries.

Figure A3: Networked CCTV coverage of the Southern cassowary enclosure – Chester Zoo. Blue circles indicate the locations of the cameras and the arrows the direction in which they are pointing. The light blue areas indicate the range that can be seen on each camera. Red circle indicate the locations of the sound recorder. The green line shows the public viewing areas. The orange dashed line shows the enclosure boundaries.

Appendix B

Cassowary Faecal Collection Protocol

Proper Identification

- The most important requirement for any sample collection protocol is that you know **which animal the sample came from**
- The best approach is to collect when birds are separated overnight
- If the birds are mixed, you must observe the animal defecating and collect the sample as soon as possible

Frequency of Collection

- The second most important requirement is to collect samples at a regular frequency, for Southern cassowary 3-4 samples should be collected every other day (i.e. Monday/Wednesday/Friday/Sunday OR Tuesday/Thursday/Saturday)

Contamination

- The faeces are not contaminated with another individuals sample
- Try to collect as soon as possible, no longer than 8 hours. Samples are best collected either during morning cleans so samples are from the previous night, or evening cleans when samples will be from that day (hormone concentrations in samples left exposed to the environment for extended periods will increase the risk of incorrect values)

Collection

- Once you have properly identified the sample, collect sample into zip-lock bags
- Do not collect the entire faecal sample (as 'pockets' of hormone concentrations can be found in the faecal sample)
- Sample size equal to a handful/fist size is adequate
- Label the bag using a waterproof permanent marker (*i.e. Sharpie pen*) with:

  House name/Local ID
  Sex
  Species
  Date (day/month/year)

Storage

- Store sample ASAP in a freezer at -20°C (hormone concentrations will degrade if samples are left out too long)

Postage

- When your freezer is close to full please contact us and we will arrange for the samples to be delivered to us

For more information please contact:
John O'Hanlon, Laboratory Technician, Chester Zoo, U.K.
Phone: +44 1244 389471, Email: endocrine@chesterzoo.org

Appendix C

CHESTER ZOO ENDOCRINE LABORATORY

SHIPPING OF BIOLOGICAL SAMPLES

Use an Overnight Courier Service to Deliver the Frozen Samples

Shipments outside of the UK

- Couriers should be reputable and experienced in transporting material of this nature (e.g. Fed-Ex,DHL)
- Shipments from EU member states, Switzerland, Norway, Iceland, Liechtenstein, the Isle of Man and the Channel islands **MUST** include a copy of facilitation letter to help prevent any problems with entry to the UK.
- If relevant, arrangements must be made by the shipper to ensure that the operators are able to carry consignments containing dry ice.
- If the specimens are being consigned by air, it is essential that a courier is employed and that they are consigned as freight

Shipments within the UK

- Couriers should be reputable and experienced in transporting material of this nature (e.g. Fed-Ex, DHL).

Please email tracking number of package to endocrine@chesterzoo.org before shipping

How to Package the Material:

Diagnostic specimens must be package in accordance with Packing Instruction 650

- Samples must be placed in a primary leakproof receptacle *(i.e. plastic tubes or ziplock bags)*
- Samples then must be placed in a second leakprook container *(i.e. a Styrofoam container)* with refrigerant *(ice packs or dry ice)*
- Absorbent material must then be placed between primary and secondary receptacles *(not required for solid samples)*
- The secondary container must then be placed in a rigid outer packaging *(cardboard box)*

Between the secondary container and the outer packaging place a letter with the following 3 forms:

- Sample Submission Form
- Non-Hazardous Declaration Form
- Record of Consignor, Transporter and Receiver Form
- Facilitation letter (If samples are coming from outside the UK)

(Please email a copy of the above forms and tracking number to endocrine@chesterzoo.org)

Specimens Packed in Dry Ice

If dry ice is used as a refrigerant, Packing Instruction 904 must also be applied.

- The packaging must be designed and constructed to permit the release of carbon dioxide gas to prevent the possibility of a build up of pressure that could rupture the packaging.
- The net weight of the dry ice must be marked on the outer packaging together with 'UN 1845' (ID number for dry ice) and thr the Class 9 (miscellaneous dangerous goods) warning diamond.

For all shipments the out packaging must include the following:

- Consignor and consignee addresses.
- Name address and telephone number of a responsible person to be included on the package.
- **'BIOLOGICAL SUBSTANCE, CATEGORY B, UN3373 '** and warning diamond.
- Non-Hazardous Declaration form for diagnostic specimens
- Facilitation letter (If samples are coming from outside the UK)

Address samples to:
Rebecca Mogey/John O'Hanlon
Chester Zoo, Endocrinology, Science Centre, Caughall Road, Upton-by-Chester, Chester, CH2 1LH, UK
Please contact us (e-mail or by phone) to let us know what date the samples will be arriving/tracking number of package to insure someone will be on hand to receive the samples.

For more information please contact:
Rebecca Mogey Laboratory Coordinator/ John O'Hanlon Laboratory Technician
North of England Zoological Society, Chester Zoo,
UK Phone: +44(0)1244 650295/389471
Email: endocrine@chesterzoo.org

Appendix D

Number of faecal samples collected from each zoological organisation.

Bird ID	Organisation	Year	JANUARY	FEBRUARY	MARCH	APRIL	MAY	JUNE	JULY	AUGUST	SEPTEMBER	OCTOBER	NOVEMBER	DECEMBER	TOTAL
CZ_F	Chester	2018	7	4	2	3	8	8	9	8	8	8	5	2	72
CZ_M			3	0	1	4	8	9	7	5	8	6	4	0	55
CZ_F	Chester	2019	0	5	7	9	6	7	4	4	8	9	11	7	77
CZ_M			0	3	9	8	4	5	10	7	3	9	9	7	74
CZ_F	Chester	2020	11	7	5	3	14	12	5	1	3	3	0	0	64
CZ_M			10	3	5	3	14	6	8	4	5	0	0	0	58
F_F	Frankfurt	2022	8	12	12	14	11	10	9	14	14	10	11	5	130
F_M			8	12	3	5	9	11	11	16	12	9	10	9	115
F_F	Frankfurt	2023	7	0	0	0	0	0	0	0	0	0	0	0	7
F_M			5	0	0	0	0	0	0	0	0	0	0	0	5
M_F	Madrid	2022	0	0	0	4	9	7	9	9	9	4	0	0	51
M_M			0	0	1	5	8	8	9	8	8	4	0	0	51