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# Evidence for seasonal shift in the reproduction of Aldabra giant tortoises (*Aldabrachelys gigantea*) in managed care in the Northern hemisphere compared to the natural habitat in the Southern hemisphere

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## Abstract

*Ex situ* breeding constitutes an important tool for species conservation; however, many reptile species are not managed sustainably under human care due to poor fecundity in *ex situ* settings. In this study, we tested whether the translocation of a seasonally reproducing species to a different environment results in decoupling of extrinsic signals and intrinsic conditions. The endocrinological patterns of plasma steroid sex hormones, follicular development, and mating behaviour of two female and two male sexually mature Aldabra tortoises (*Aldabrachelys gigantea*) in a zoological institution in the Northern hemisphere was aligned with enclosure climate data (mean monthly daylight duration, temperature, and precipitation) and compared with respective hormone patterns of wild individuals and climate conditions in the native habitat on the Aldabra Atoll in the Southern hemisphere. Whereas occurrence of mating behaviour was not considered a limiting factor, lack of ovulation and subsequent follicular atresia was the main reason for the lack of reproductive output. While it was impossible to elucidate the triggering factors of ovulation and the multifactorial complexity of reproduction was not fully addressed, this study indicates suboptimal temperature conditions and relative temporal shifts of interacting external triggers (temperature and photoperiod) in the zoo setting.

## KEYWORDS

follicular atresia, photothermal cueing, steroid hormone

## 1 | INTRODUCTION

Giant tortoises were once present in thriving populations throughout the western Indian Ocean Islands, composed of 8–12 species spread across the islands of Madagascar to the Mascarene Islands, and many

of the Seychelles Islands. However, the spread of human colonisation resulted in a drastic decline and extinction of most species, be it due to overhunting, translocation or predation of hatchlings by newly introduced predators such as cats, dogs, and rats. Today, only one species of giant tortoise remains in the region, the Aldabra giant

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tortoise (*Aldabrachelys gigantea*), present in a large population on the Seychelles Islands with a population of well over 100'000 individuals, mainly attributable to the population on the Aldabra Atoll (Bourn et al., 1999; Gerlach et al., 2013; Hansen et al., 2010; Turnbull et al., 2015). Though currently listed as Vulnerable by the IUCN Red List of Threatened Species (IUCN, 2022), a further 40%–65% population decline is estimated over the next 100 years due to negative impacts of sea-level rise, which would render the species Endangered (Gerlach et al., 2013).

Conservation efforts by the Seychelles government and the Royal Society of London on the Aldabra Atoll have successfully been ongoing since 1977 (Stoddart et al., 1982). *Ex situ* conservation efforts on the other hand have been considerably less successful. Although a popular exhibit species held by 183 accredited zoological institutions worldwide and currently managed by accredited European Zoos as an EEP (*ex situ* breeding programme), only a few zoos (e.g., Tulsa, Taipei, and recently Beauval) have succeeded in breeding this species, and even fewer (Tulsa) have succeeded in multiyear breeding and distribution to other zoos over the past fifteen years (Species360, 2023). Occasional breeding success has been reported in small numbers by private owners (Merz, 2022).

This poor fecundity under *ex situ* settings in zoological institutions stands in sharp contrast to the *in situ* breeding output. A previous study monitoring the follicular cycle of both Galapagos and Aldabra giant tortoises at Zurich Zoo documented the presence of vitellogenic and atretic follicles all year round, without clear indication for seasonality (Casares et al., 1997). Follicular atresia is the process of resorption of mature follicles that have failed to ovulate and persist on the ovary in late vitellogenic state. Therefore, failure of ovulation rather than ovarian inactivity is suspected as the cause for the lack of reproductive output. Several studies hypothesise that follicular atresia is likely a physiological process, allowing for adaptation to suboptimal environmental conditions and inadequate resources (Kummrow et al., 2010; Swingland & Coe, 1978; Vitousek et al., 2010), but the pathophysiological background of ovulatory failure itself has not yet been elucidated.

In a previous study, plasma levels of gonadal steroid hormone, estradiol, testosterone, and progesterone, were recorded in wild Aldabra giant tortoises across seasons (Kummrow et al., 2020). In reptiles, estradiol and testosterone are associated with gonadal growth and gamete development, and progesterone with follicular ovulation (Branson et al., 2016; Kummrow et al., 2020). A clear seasonal pattern was demonstrated in both sexes, coinciding with previously reported morphological and physiological changes (e.g. change in testicular and ovarian weight, breeding activity) associated with reproduction (Bourn, 1977; Kummrow et al., 2020; Swingland & Coe, 1978). In the wild, reproductive activity is strictly seasonal, with mating occurring during the hot and rainy season (February–May), nesting during the cool and dry season (June–September), and offspring hatching during the following hot and rainy season (Bourn, 1977; Swingland & Coe, 1978). Reproductive events in *ex situ* managed populations in the Northern hemisphere are recorded with a seasonal delay: mating also generally occurs in the hot summer

months (June–September) and nesting in cool winter months (November–February). The events are, however, less clearly separated with temporal overlaps (e.g., mating observed all year round) and the few successful events of hatching were observed during the cold winter season (Collins, 1984; Ebersbach, 2001; Merz, 2022; Stearns, 1988). For the only reported successful *ex situ* breeding in a Southern hemisphere managed population, reproductive seasonality corresponded to the wild pattern (Peters & Finnie, 1979). The same deviation of breeding patterns in *ex situ* populations in the Northern hemisphere compared to the wild habitat applies to the second extant giant tortoise species, the Galapagos tortoise (*Geochelone nigra*) (Bacon, 1980; Rostal et al., 1998). In these species, poor fecundity in captivity was hypothesised to originate from inadequate husbandry, likely coupled with a disconnect between reproductive seasonality and managed environmental conditions (Casares et al., 1995; Ebersbach, 2001).

While reproduction is a complex multifactorial interplay influenced by a large number extrinsic and intrinsic factors acting directly or indirectly on the physiology of reptiles, environmental factors are particularly intriguing in seasonally reproducing species. In mammals, there are, broadly speaking, two ways that a seasonal breeding pattern in a natural habitat can be reflected in zoo animals (Claus et al., 2021). If the reproductive seasonality is triggered by innate responses to a phototrigger, it should—to some degree—still occur in zoos, albeit shifted according to the photoperiods of the respective hemispheres. If the reproductive seasonality is a reaction to fluctuating resource availability (body condition-threshold controlled reproduction), one would expect more year-round reproductive patterns in zoos where resources are generally not limited. This concept applies to reptiles alike, with the addition of temperature as a naturally crucial factor in ectotherm physiology and reproduction (Falcón et al., 2018a; Licht, 1972). As ambient temperature and photoperiod are inevitably closely connected, they act on reptilian reproduction as interconnected “photothermal cueing”, and it remains unclear, which of the two represents the main external trigger in seasonal reptile reproduction (Licht, 1967a, 1967b).

This study served to investigate whether there is a difference in the interplay of extrinsic (daylight duration, mean daily temperature, precipitation) and intrinsic (hormonal) conditions between the natural habitat on Aldabra and the *ex situ* habitat at Zurich Zoo, and how these relate to reproductive behaviour. We did this by aligning seasonal climate data, and endocrinological patterns of plasma steroid sex hormones as well as mating activity and follicular development of two female and two male sexually mature Aldabra tortoises in the tropical Masoala Rainforest Ecosystem exhibit at Zurich Zoo. The follicular development of the females and the mating behaviour of the group throughout the year was also recorded. These observations were compared to available corresponding information from the natural habitat. Our prediction was that a translocation of a seasonally reproducing species to a different hemisphere should, in theory, simply lead to a shift of reproductive events in the annual cycle, with consistent reactions of sex hormones, follicular development, and sexual behaviour to shifts in the external cues.

## 2 | MATERIALS AND METHODS

For this study, the following data were evaluated over the duration of an entire year: plasma gonadal steroid hormones (estradiol and progesterone in females, testosterone in males; monthly), ultrasound imaging of follicular development in females (monthly), daily environmental data (daylight duration, temperature, humidity and artificial precipitation), and daily behavioural observations. The presented data on hormone measurements and diagnostic imaging were compiled from the routine veterinary diagnostic reproductive monitoring programme, in alignment with institutional welfare and research regulations.

### 2.1 | Animals and husbandry

Four sexually mature adult Aldabra giant tortoises of wild origin, two males (M1, 41 yrs, 187 kg; M2, 41 years, 140 kg) and two females (F1, 51 years, 94 kg; F2, 71 years, 108 kg), were monitored over a 1-year period. The animals were kept year-round in the Masoala Rainforest Ecosystem exhibit at Zurich Zoo, Switzerland (Bauert et al., 2007). The enclosure design included multiple basking sites with heat and UV lamps, access to a water pond, mud wallow, and sand area for oviposition. The substrate consists of a mix of earth and gravel. The Masoala Rainforest Ecosystem exhibit is covered with a UV-permeable foil, allowing outside daylight conditions to be reflected inside the exhibit.

Throughout the year, approximately 0.5 kg of hay-based pellets (Pre Alpin Testudo Fibre, Agrobs GmbH) and freshly chopped vegetables (Swiss chard, bell peppers, cucumbers, carrots) were provided per animal daily. Calcium and mineral supplementation consisted of ground sepia or Herpetal Complete-T/D3 (Keweloh Animal Health GmbH) along with rehydrated algal fronds twice weekly. Either hay or freshly cut grass and foliage (*Ficus benjamini*, *Terminalia cappa*) trimmings from the rainforest exhibit were provided for ad libitum consumption in the winter months and summer months, respectively.

### 2.2 | Behavioural observations

Four cameras (PlotWatcher Pro, Day 6 Outdoors, Diamond, USA) were placed to survey the entire enclosure and record mating frequency throughout the monitoring period. The cameras recorded pictures every 5 s in dependence of the natural daylight cycle, which coincided with the activity phase of the animals. A research assistant of the Zurich Zoo, familiar with the morphological appearance of the individual animals, evaluated the video material and tallied mating attempts for all videos using the software BORIS (Friard & Gamba, 2016). Mating attempts were defined as in (Bourn, 1977): male approaches female and mounts (typically from the rear) with an outstretched neck and hindlimbs on the ground in an attempt to copulate.

### 2.3 | Diagnostic imaging

A portable ultrasound (GE Logiq E Veterinary, GE Medical Systems Co.) with a convex 2.0–5.0 MHz probe applied bilaterally to the prefemoral fossa was used to monitor the follicular development of the two female Aldabra giant tortoises once a month, in parallel to plasma sampling, without sedation.

Follicles were categorised into three main groups (early vitellogenic, late vitellogenic and atretic) based on ultrasonographic appearance as described in previous publications (Casares et al., 1997; Cigler et al., 2023; Rostal et al., 1996). Briefly, early vitellogenic follicles were small, round and homogenous, visible from about 0.4 cm in diameter onwards. Late vitellogenic follicles were identified based on their larger size and hypoechoic centre. Atretic follicles were determined by their round-to-uneven shapes, heterogeneous content and/or presence of anechoic cavities. Previtellogenic follicles could not be identified.

### 2.4 | Hormone analyses

Monthly blood sampling was performed from the *Vena brachialis* with 3 mL syringes and hypodermic 23G needles. For blood sampling, male animals presented voluntarily for feeding rewards, while phlebotomy in females was performed during physical immobilisation for ultrasound examination by placing the animals on a stand that lifted their feet off the ground to allow for bilateral access to the prefemoral fossa (Hatt, 2008). Blood was transferred immediately into lithium-heparin containing tubes (Vacuette Tubes, Greiner Bio-One, Kremsmünster, Austria) and plasma was centrifuged within 2 h of collection at 2000 g for 10 min and stored at  $-20^{\circ}\text{C}$  until further analysis. Blood sampling was performed between 9 and 11 AM to minimise the effect of circadian variations of blood hormone levels.

In males, plasma testosterone (T) was measured, and in females oestradiol (E) and progesterone (P). Progesterone and testosterone concentrations were quantified using EIA methods. Antisera (C. Munro, University of California) were diluted as follows: T (polyclonal R156/7), 1:10,500 and P (monoclonal CL425, Quidel Corp., with final purification by C. Munro), 1:8,900. Horseradish peroxidase conjugates (C. Munro) were diluted as follows: T-HRP, 1:20,000 and P-HRP, 1:40,000. Standards used were T (A6950; 48–12,500 pg/mL; Steraloids Inc.) and P (Q2600-000; 15.6–4,000 pg/mL; Steraloids Inc.). Oestradiol was measured using a plasma  $17\beta$ -estradiol EIA (KB30-H1, Arbor Assays). In contrast to the previously described EIA method analysing neat plasma samples in assay buffer (Kummrow et al., 2020), plasma samples of this study were double extracted using diethyl ether in 7 mL glass vials. Briefly, plasma (T: 10–75  $\mu\text{L}$ ; P: 75  $\mu\text{L}$ ; E: 20–60  $\mu\text{L}$ ) was added to 200  $\mu\text{L}$  of  $\text{dH}_2\text{O}$  followed by 1 mL of diethyl ether, vortexed and left to phase separate for 20 min. The aqueous ether phase was removed from plasma using a dry ice/methanol bath and collected into a clean glass vial. The plasma was allowed to thaw, and the process was repeated. Ether fractions from both steps were combined, evaporated, and reconstituted in assay buffer (T: 150–200  $\mu\text{L}$  EIA buffer for a final dilution of 1–1:20; P: 150  $\mu\text{L}$  of EIA buffer, final dilution

1:2; E: 240  $\mu$ L kit assay buffer, final dilution 1:4–1:12) immediately before analysis. Standards, samples, and controls were run in duplicate with <12% CV between duplicates. Intra- and inter-assay CVs were <10% for all assays. Due to the differences in hormone extraction methods between the studies (Aldabra: Kummrow et al., 2020; Zurich Zoo: current study), we did not compare absolute values; however, temporal hormone patterns can nevertheless be reliably assessed.

## 2.5 | Environmental data

Daily sunshine duration of the Zurich area during the entire monitoring period was commissioned from the Swiss Meteo database (MeteoSchweiz, 2022). Indoor temperature and humidity were monitored using four loggers (IPMH20R logger, Swittrace SA) placed in representative areas of the tortoise enclosure. Readings were recorded every half an hour over the entire monitoring period. Indoor rainfall records were retrieved from the artificial daily rain dashboard (ControlMaestro SCADA, Elutions) of the Masoala Rainforest Ecosystem Technology Centre. All environmental data were calculated and graphed as monthly means.

## 2.6 | Data evaluation and statistics

The collected data were contrasted against published endocrinological, behavioural and anatomical observations in the native habitat, Aldabra Atoll, from other study groups (Bourn, 1977; Kummrow et al., 2020; Swingland & Coe, 1978). For each site (Zurich, Aldabra Atoll), and additionally for each individual Zurich animal, monthly averages of daylength, climate and hormonal measures were assessed for nonparametric correlations using Spearman's correlation coefficient using R (Team, 2020), to assess whether the same correlations were evident at both sites. The difference in peak daylength, climatic and hormonal measures between the sites was only evaluated graphically. For a statistically validated quantitative difference in these peaks that goes beyond the qualitative differences provided in our study, a larger data set would have to be generated.

## 3 | RESULTS

### 3.1 | Environmental parameters in association with mating and nesting activity

The Aldabra Atoll is located closely to and south of the equator (9.4237° S, 46.3433° E) and therefore shows little variation in daylength duration throughout the year (12.5 - 13.5 h/day), whereas Zurich, Switzerland (46.8182° N, 8.2275° E) shows variations in daylight from 10 h in December to 17 h in June. The hot and wet summer season (December–May) and the cool and dry winter season (June–November) on the Aldabra Atoll are, in general, mimicked in the Masoala Rainforest Ecosystem exhibit in Zurich with a 3-month

delay, corresponding to the respective long-day and short-day seasons. However, peak temperature and precipitation coincide in Zurich with the summer solstice, and are highly positively correlated with daylength (temperature  $p < .001$ ; precipitation  $p = .001$ ). By contrast, on Aldabra, daylength and climate variables are dissociated (temperature  $p = .125$ ; precipitation  $p = .152$ ), with the temperature and precipitation maximum occurring approximately 2 months after the longest daylength has passed (Figure 1a, Figure 2a).

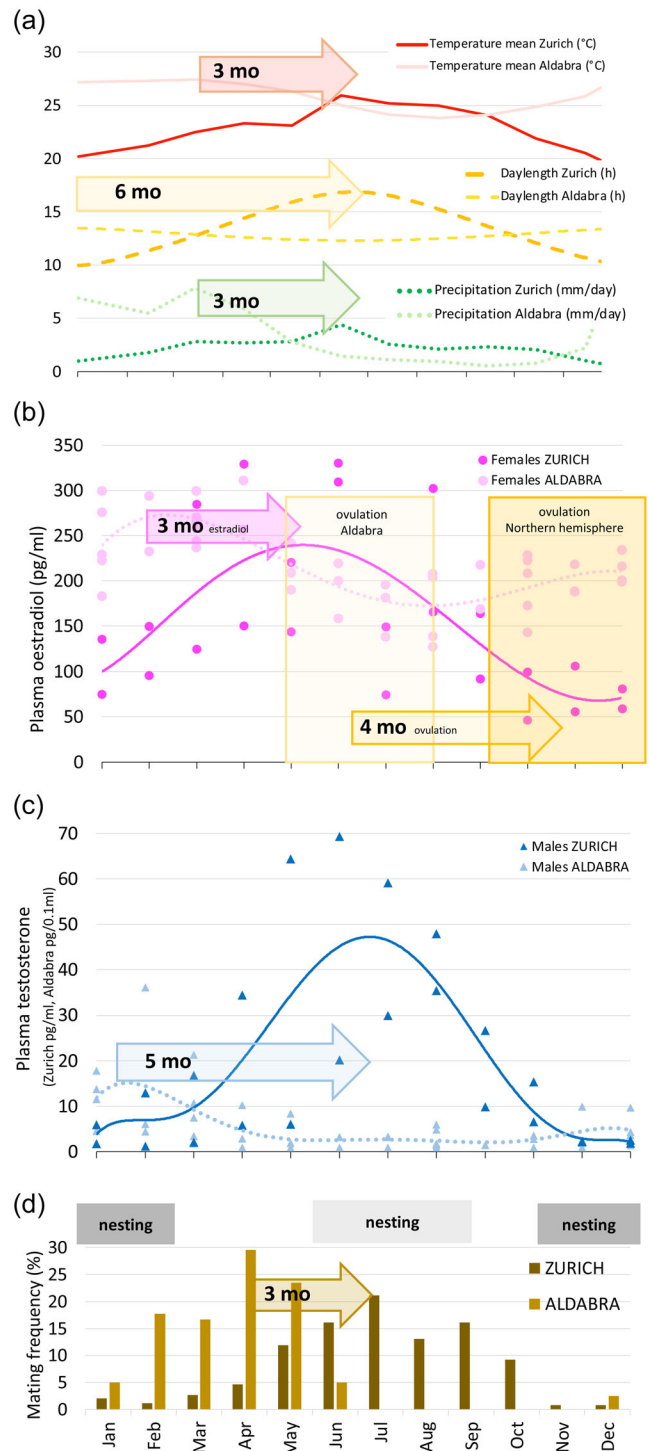
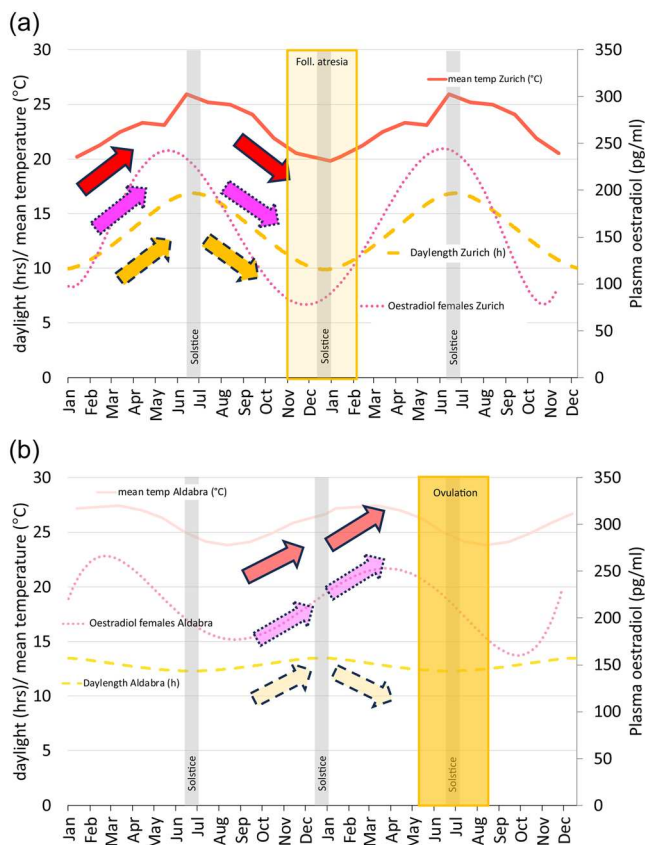


FIGURE 1 (See caption on next page).



**FIGURE 2** Photothermal cueing and follicular development represented by oestradiol plasma levels in female Aldabra tortoises (*Aldabrachelys gigantea*). Data duplicated over two years for better visibility of annual patterns. In Zurich (a) and on Aldabra (b), increasing daylight durations (dashed line) and temperatures (solid lines) are leading up to summer solstice. In Zurich (a), temperature is dropping after summer solstice along with declining oestradiol values (dotted lines) followed by follicular atresia around winter solstice, whereas on Aldabra (b), increasing plasma oestradiol values are going along with further increasing temperatures after summer solstice, followed by the ovulation period around winter solstice. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

**FIGURE 1** Environmental, hormonal and behavioural data during an entire year in zoo-managed (dark lines and boxes) and wild (light lines and boxes) Aldabra giant tortoises (*Aldabrachelys gigantea*; AGT). (a) Mean monthly temperature, daylight duration, and precipitation on the Aldabra Atoll (grey lines) and in the Masoala Exhibit Zurich (black lines). (b) Plasma oestradiol of zoo-managed female AGT ( $n = 2$ ; black circles with solid polynomic trendline) and wild female AGT ( $n = 4$ ; grey circles with dotted polynomic trendline; from Kummrow, 2020), rectangular boxes indicating anticipated ovulation time (light grey = Aldabra; dark grey = Zurich). (c) plasma testosterone of zoo-managed male AGT ( $n = 2$ ; black triangles with solid polynomic trendlines) and wild male AGT ( $n = 4$ ; grey triangles with dotted polynomic trendline; from Kummrow, 2020). (d) mating frequencies (from Bourn, 1977; grey columns) and nesting periods (from Bourn, 1977, light grey boxes) of the wild population on Aldabra and mating frequencies observed in the four Zurich zoo AGT (black columns) and nesting as reported from other zoo populations in the Northern hemisphere (dark grey boxes). [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

As previously reported (Bourn, 1977; Kuchling & Griffiths, 2012; Swingland & Coe, 1978), mating season in the natural habitat on the Aldabra Atoll occurs at the end of the warm wet summer months (February–May) and nesting ensues during the cool and dry winter months (June–September) (Figure 1d). In the Zurich population, heightened mating activity was also observed during the summer months with high temperature and precipitation (June–September), and although no nesting or oviposition was observed in Zurich during the study period, the nesting season (November–February) reported in other Northern hemisphere zoos (Collins, 1984; Ebersbach, 2001; Merz, 2022; Stearns, 1988; personal communication private breeders and zoological institutions in US, France, Switzerland, and Germany) coincided with the cool winter season with little precipitation (Figure 1d).

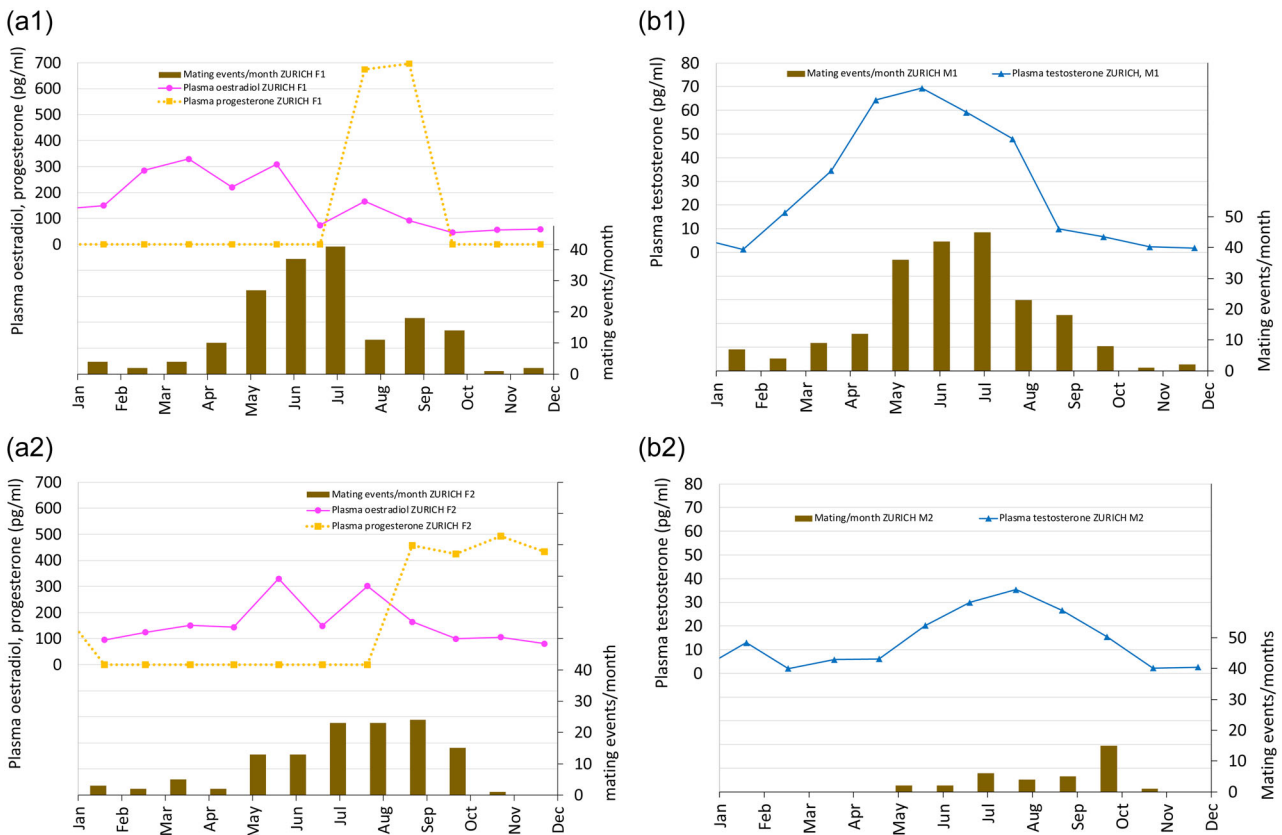
### 3.2 | Hormone patterns and follicular development

Zoo female plasma oestradiol levels are depicted in Figure 1b and showed a seasonal pattern with highest values from April to June, a decline in August and September, followed by the nadir in November and December, and increasing values in February. Compared to data from the wild population (Kummrow et al., 2020), there is a delay in seasonality of 3 months in the oestradiol plasma peak level.

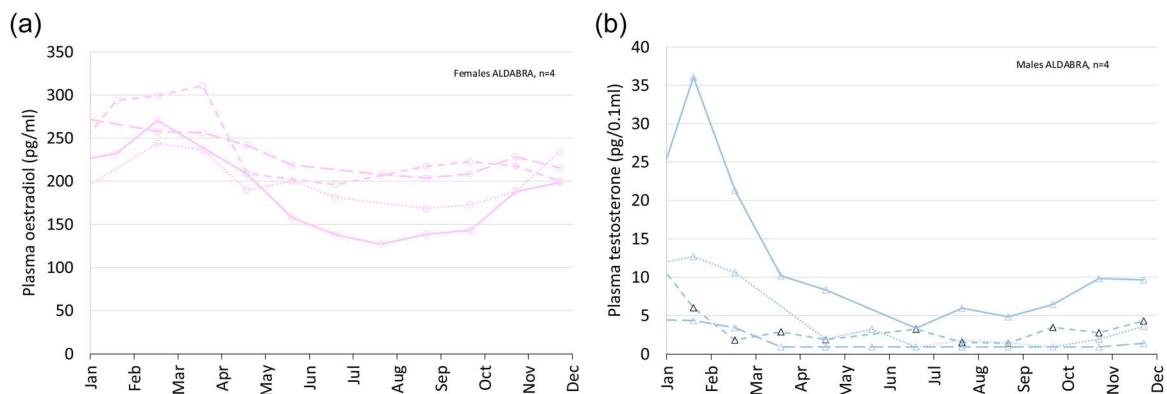
Plasma testosterone levels in the two zoo-managed male Aldabra giant tortoises showed a seasonal variation with highest values in May through July and lowest values in December through January (Figure 1c). Compared to data from the wild population (Kummrow et al., 2020), there is a delay of 5 months in testosterone plasma peak level.

On Aldabra, both female and male hormone patterns showed stronger positive correlation with temperature ( $p = .003$  for oestradiol;  $p < .001$  for testosterone) than with daylight ( $p = .039$  for oestradiol;  $p = .019$  for testosterone), while in the Zurich animals, due to the parallel course of temperature and daylight, hormones correlated equally, positively with temperature ( $p < .001$  for estradiol;  $p = .005$  for testosterone) and daylight ( $p < .001$  for estradiol and testosterone) equally (Figure 2).

By plotting the individual hormone curves of the study animals, a difference of 2–3 months in hormones peaks between the two females and the two males is visible (Figure 3). Although the females and males on the Aldabra Atoll appeared to be in synchrony, there were also temporal differences in hormone peaks of up to 3 months between individual females and 1 month between males (Figure 4). Both in wild and zoo animals, absolute hormone levels were of a similar magnitude among the females, whereas differences were visible between individual males (Figure 3, Figure 4). Plasma progesterone levels in zoo females were measurable in late summer/fall (August–September) for F1 and fall/winter months (September–January) for F2 and coincided with decreasing estradiol values (Figure 3a).



**FIGURE 3** Plasma hormone levels in 2 female (a1, a2) and 2 male (b1, b2) zoo-managed Aldabra tortoises (*Aldabrachelys gigantea*) in the Masoala Rainforest Ecosystem exhibit in Zurich, in alignment with individual mating attempts/month. Solid circles = estradiol, open squares = progesterone, solid triangles = testosterone, bars = mating attempts/month. [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/zoo.21851)]



**FIGURE 4** Individual plotting of plasma hormones in wild female (a) and male (b) Aldabra tortoises (*Aldabrachelys gigantea*) on the Aldabra Atoll (from Kummrow et al. 2020). [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/zoo.21851)]

### 3.3 | Mating activity

Peak of mating activity in the zoo-managed animals showed a delay of 3 months (Figure 1d). On Aldabra, mating activity showed weak, although stronger positive correlation with temperature ( $p = .013$ ) than with daylength ( $p = .577$ ); in Zurich, mating activity was highly positively correlated with both factors ( $p < .001$ ). Mating activity showed positive correlation with female and male hormone patterns

( $p = .017$  for estradiol,  $p < .001$  for testosterone) in Zurich. In Aldabra, assessment of correlation of mating activity and hormone patterns was limited to descriptive graphical display due to the origin of datasets from different studies precluding statistical analysis (Bourn, 1977; Kummrow et al., 2020; Swingland & Coe, 1978).

Records of individual mating events between the four animal in Zurich revealed considerably more mating activity by F1 and M1 (Table 1).

### 3.4 | Follicular development

Monthly ultrasonographic examinations of the ovaries of zoo-managed females confirmed ovarian activity throughout the year, yet no evidence for egg formation was noted. Both early and late vitellogenic follicles were identified in both females and changing ratios of early and late follicular stages were subjectively noticed; however, the presence of numerous batches of atretic follicles impeded detailed and objective assessment of concerted follicular progression and therefore, no distinct correlation could be drawn between periods of follicular growth and the hormonal or environmental factors described. Atretic follicles of varying sizes and structures, including cystic atretic follicles, were present throughout the year and represented the predominant form of follicles observed. Previtellogenic follicles could not be identified (Figure 5).

## 4 | DISCUSSION

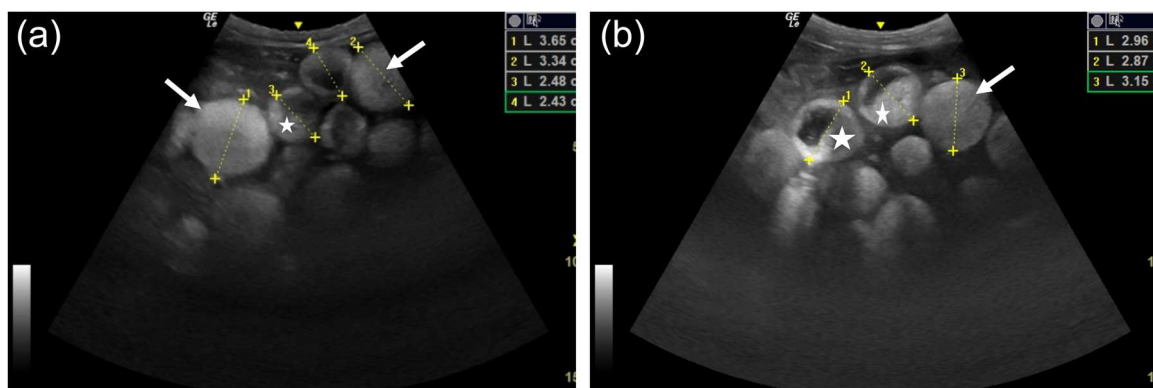
This study provides additional evidence that reproduction of giant tortoises follows a seasonal pattern, in zoos as well as in their natural habitat. This is evident from both physiological and behavioural measurements. This study can only generate hypotheses about the lack of reproduction in zoos with similar conditions as described here, yet in doing so opens avenues for further management modifications that should be tested as next steps in addressing the scarcity of reproduction in the species in managed populations.

**TABLE 1** Mating attempts/year between in two female and two male Aldabra giant tortoises (*Aldabrachelys gigantea*) in the Masoala Rainforest Ecosystem exhibit in Zurich during a 12 months period.

	F1	F2
M1	142	56
M2	12	23

Reproductive physiology is governed by a large number of extrinsic and intrinsic factors. When investigating the impact of factors, is important to scrutinise whether they act directly (metabolic or endocrinological pathways) or indirectly (e.g. precipitation changes nutritional resources and substrate consistency for oviposition), and which reproductive processes are targeted (e.g. in females preovulatory vs. post-ovulatory processes). Barometric pressure, which has been reported to impact activity levels in reptiles (Ariano-Sánchez et al., 2022), was not addressed in the present study.

This study focused on investigating seasonality-associated factors, but several limitations need to be acknowledged. Only four zoo individuals were assessed. Evidently, results from such a low number of animals must be considered preliminary. However, given that this number of animals may well reflect the size of a single zoo group, the results nevertheless offer some guidance to understanding of the species' reproductive physiology and for their *ex situ* management. The association of gonadal steroid hormone values with the respective follicular stages is based on extrapolations from the other extant giant tortoise species, the Galapagos tortoise (Branson et al., 2016; Rostal et al., 1998; Schramm et al., 1999), and reptiles in general (Custodia-Lora & Callard, 2002; Ho et al., 1982), but has not been examined for Aldabra tortoises directly. Quantitative variations in hormone values are strongly dependent on the assay methodology used and should be scrutinised carefully when comparing values between different studies. Testosterone values in the zoo-managed individuals of this study significantly differed from the testosterone levels measured in the wild population (Kummrow et al., 2020) due to different extraction methods: the plasma samples collected from wild animals were assayed neat and without prior extraction, compared to the zoo-collected samples presented here in which the hormones were ether-extracted from the plasma to concentrate steroid levels and improve detection on the EIA assays. The different extraction methods were the result of continuous development of reptile plasma hormone analysis with the goal of improving hormone extraction efficiency. For this reason, only temporal patterns of hormone peaks and baselines may be compared between the two studies but not magnitude of



**FIGURE 5** Representative ultrasonographic examinations of ovaries of two zoo-managed Aldabra tortoises (*Aldabrachelys gigantea*). Large, late vitellogenic (arrow) and cystic atretic follicles (star) dominating the ovaries throughout the year as shown here in (a) dioestrus phase in November and (b) late proestrus phase in June. [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/zoo.21851)]



hormone levels. Differences in magnitude and temporal patterns of hormone levels between individuals of the same study are influenced by both intrinsic factors including genetic and maternal effect or the age of the individual, and extrinsic factors including the time of day of blood sampling or the social environment of the individual (Kempnaers et al., 2008), the latter two of which were excluded in the present study due to the consistent morning sampling and the stable group composition.

In this study, seasonal reproductive steroid hormone patterns of zoo-managed individuals coincided temporally between males and females and, to a certain degree, with the expected seasonal climatic conditions when compared to the wild counterparts. Mating activity was associated with testosterone and oestrogen patterns in males and females, respectively, coinciding with the expected late vitellogenic follicular phase in the females and testicular growth phase in the males (Kummrow et al., 2020). Yet, no reproductive output has been observed in this population for the last three decades. On the Aldabra Atoll, distinct seasonal variations in ovarian weight and follicular development stages, including atretic follicles, were recorded (Bourn, 1977; Swingland & Coe, 1978). Monthly ultrasonographic examinations of the zoo-managed females demonstrated ongoing ovarian activity by the presence and changing ratio of early and late vitellogenic follicles. However, due to the predominating mass of atretic follicles obscuring the identification of seasonal recruitment and maturation of new follicles, no distinct ovarian seasonality could be identified within this study period. In particular, there was no evidence for egg formation, despite the distinct seasonal pattern of ovarian steroid hormones. In reptiles, estradiol is responsible for the recruitment and maturation of follicles, whereas progesterone is involved in the ovulation of follicles (Custodia-Lora & Callard, 2002; Ho et al., 1982). Based on the seasonal cycling pattern of estradiol, a concerted development of an annual batch of follicles was still to be expected in the study subjects. Distinct progesterone peaks were measured in the zoo-managed females coinciding with declining oestrogen values, as expected during ovulation (Rostal et al., 1998; Schramm et al., 1999), yet progesterone peaks have been measured despite an absence of ovulation in other reptile species as well, indicating that relative magnitude and temporal association of progesterone is more relevant than the mere presence or absence of the hormone (Fox & Guillette, 1987; Kummrow et al., 2010). In the study of the wild Aldabra tortoise females, plasma progesterone levels were only measurable above detection level in April in one of the four monitored animals, but the field study was limited by monthly encounters with individual animals without continuous monitoring, possibly missing short-time hormone peaks or ovipositions (Kummrow et al., 2020). Examinations of anatomic and physiologic factors in females and males, such as investigation of reproductive pathologies or sperm quality assessment, were not performed, but the observations made in this study coincide with previously published data, in which the lack of ovulation and resulting follicular atresia was found to be the main diagnostic correlate with reproductive failure in managed Aldabra tortoise populations in the Northern hemisphere (Casares et al., 1995; Casares et al., 1997; Ebersbach, 2001).

The costly energy investment into follicular development and maturation without ovulation is a well-documented, yet poorly understood phenomenon observed in numerous reptile species, both in the wild as well as in managed care (Cigler et al., 2023; Swingland & Coe, 1978). Follicular atresia is not considered pathological per se, but rather a consequence of the suppression of ovulation in response to environmental or social conditions (Kummrow et al., 2010; Vitousek et al., 2010). Many studies on reptile reproductive seasonality focused on investigating the triggers of gonadal recrudescence and mating (Licht, 1966, 1967a, 1969); however, these processes appear less critical for *ex situ* breeding programmes of giant tortoises than the triggers for ovulation in females. Environmental triggers for inhibition of ovulation are not known, yet they must necessarily be endocrinologically mediated. And although not only gonadal steroid hormones but a complex system of various endocrinological axes and neurotransmitters are likely involved (Dayger et al., 2018; Desan et al., 1992), the association of the measured steroid sex hormones with potential triggers might allow some insight into the regulation. Frequency and timing of ovulations were never directly observed (in zoos due to their rare occurrence, and in the wild due to the lack of external cues) but they can be inferred from observations of ovarian development (Bourn, 1977; Casares et al., 1997; Swingland & Coe, 1978), timing of nesting (Ebersbach, 2001), and hormone patterns (Kummrow et al., 2020). Ovulation periods of Aldabra tortoises are therefore expected during May–July in the native habitat and from August–December in the Northern hemisphere populations (Figure 1b). Environmental or social cues before or during the critical time of ovulation are therefore of particular interest with regard to the extrinsic regulation of reproduction (Figure 2).

Lack of mating behaviour could not explain the absence of reproductive output in the examined zoo-managed population. On the contrary, the mating attempts of M1, perceived as the more dominant of the two males, are considerable in number. Hierarchical social structures have been described in giant tortoises and observations about social status impacting reproductive behaviour were reported in both zoo-managed Galapagos tortoises (*Geochelone elephantopus*) (Bacon, 1980; Hairston & Burchfield, 1989; Hatt, 2008) and Aldabra tortoises (Chida, 1998; Gerlach, 2003). On the other hand, F1 was described by keepers as more timid and solitary than F2. The higher mating activity of F1 compared to F2 may therefore be interpreted as the incapability of the weaker female to fend off mating attempts by the dominant male, rather than a dominance in reproductive behaviour. To our knowledge, there is no comparable data on individual mating events from the natural habitat. Structural adjustments to the enclosure upon recording this data revealed female evasion of the frequent male mating attempts, suggesting social stress with a possible negative impact on ovulation in the mating season. Individual temporal variability of hormone patterns and reproductive behaviour of 2–3 months, as observed in both the wild and zoo-managed individuals, might result in the lack of temporal conformance of available mating partners in the rather small breeding population common to

zoo-managed situations, as opposed to the large congregations of animals in the wild (Swingland & Coe, 1978).

In the current study, the pattern of reproductive events associated with precipitation and temperature *ex situ* were concurrent with the patterns of the wild habitat: in the wild, mating occurs during the rainy and hot season (February–May), as was seen in the Zurich Masoala Rainforest Ecosystem exhibit (March–September). Though no ovulation or oviposition was observed during the study period, data from other zoological and private institutions in the Northern hemisphere indicated that oviposition occurs in the dry and cool season both in *ex situ* (October–February), as well as in the wild (June–September) (Collins, 1984; Ebersbach, 2001; Merz, 2022; Stearns, 1988). These observations could point towards a condition-threshold controlled reproduction, both for the onset of mating and for ovulation and oviposition. On the Aldabra Atoll, nutritional resources, in particular “tortoise turf”, a sward providing the main forage for tortoises, are highly seasonal dependent on precipitation (Gibson & Hamilton, 1983; Gibson & Phillipson, 1983). In a small-scale experiment with controlled feeding of two groups of five females Aldabra tortoises each on the Aldabra Atoll in April, i.e. shortly before the expected ovulation time, a 7–12 day limitation of food already increased rates of follicular atresia in the individuals, supporting the condition-threshold hypothesis (Swingland & Coe, 1978). These results suggest that seasonally variable body condition is a plausible scenario for the natural habitat, and also indicate that body reserves may not be sufficient to buffer even short periods of time. Feeding protocols in the Masoala Rainforest Ecosystem exhibit at Zurich Zoo differ seasonally by replacing fresh greens (seasonally and locally available grass and browse) in the summer with low quality hay in the winter. It must be noted that whereas precipitation has a clear effect on the available nutritional resources on the Aldabra atoll (Gibson & Phillipson, 1983), the precipitation in the Masoala Rainforest Ecosystem is decoupled from the food offered to the tortoises, where amounts offered allow for *ad libitum* consumption. Yet, seasonality in body condition in managed care is likely less directly linked to the food offered, but to environmental temperatures and their effect on intake and assimilation efficiency; in reptiles, both usually decline at declining temperatures (Seebacher & Franklin, 2005). Given the suspected decrease in both intake and digestibility with a drop in temperature at Zurich Zoo, investigating the effect of offering both, additional support in September–December in terms of heat sources and a diet that maintains the energetic intake assumed for the preceding summer months, should be further investigated.

As previously reported in other *ex situ* Aldabra tortoise populations in the Northern hemisphere (Collins, 1984; Ebersbach, 2001; Merz, 2022; Stearns, 1988), the current study suggests a seasonal delay of reproductive events (endocrine patterns and mating frequency). Observed seasonal patterns of reproduction raise the question for the underlying trigger. In mammals, the main trigger is considered to be photoperiod, with various versions of phototriggers having been described, revolving around changes in daylength and association with solstice (Clauss et al., 2021). For reptiles, the

contribution of photoperiod is less clear, and various combinations of photo and temperature signals have been described, coining the term “photothermal cueing” (Licht, 1967a, 1967b, 1969). On the Aldabra Atoll, there is a 2-month delay between daylength and temperature patterns, whereas in Zurich, there is a general parallelism between the two. Differentiating between the likely interacting photo- and temperature signal is therefore impossible in a study like ours, which, in addition, follows only a few animals over only a single seasonal cycle with relatively low resolution. Our discussion must therefore be considered speculative, and the conclusions drawn in this study are based on the measured data without elucidating the underlying regulatory processes.

In theory, a hemisphere change should lead to a 6-month shift of reproduction. Therefore, the 3 to 5-month shift of hormonal patterns and mating behaviour between Aldabra and Zurich could be construed as an indication of the importance of the temperature signal. This is also supported by the stronger correlation of endocrine patterns and mating frequency with temperature than with day length in the native habitat, and agrees with previous studies in lizards and chelonian species, where combinations of various photo and a temperature signals revealed differential temperature sensitivity of different reproductive processes, temperature-dependent sensitivity to phototriggers, and both photo- and thermorefractory phases (Ganzhorn & Licht, 1983; Licht, 1967a, 1967b, 1969; Mendonça, 1987; Sarkar et al., 1996). To what degree the effect of temperature is direct, or an indirect trigger via its effect on both food intake and digestive efficiency, remains debatable.

An increase in oestrogen was observed in both Aldabra and Zurich females after the winter solstice, reflecting follicular growth possibly triggered and supported by the increasing photoperiod, temperatures and/or the associated increase in energy uptake. After the summer solstice, the photoperiod in both locations is naturally declining. However, oestrogen levels on Aldabra continued to rise, while they decreased in Zurich, in parallel with the enclosure temperature. A hypothetically stimulating function of temperature (possibly, via energy uptake) allows for full follicular development and pre-ovulatory follicular maturation in the Aldabra situation, whereas follicular development may be cut short by the decline of temperature in Zurich (Figure 2).

Independent of temporal association with photoperiodism, mean ambient temperature peak values during the hot season in Zurich merely reached temperatures of the cool season on Aldabra, and during the cool season in Zurich, mean temperatures (<21°C) were clearly below the temperature range of Aldabra tortoises to maximise their activity (25.8–31.7°C) (Falcón et al., 2018a). A previous study in the Masoala Rainforest Ecosystem revealed that these animals effectively made use of additionally offered focal heating areas (Falcón et al., 2018b). While these artificial thermal sources proved effective for behavioural thermoregulation, availability is impacted by confounding factors, such as social stress. Additionally, in comparison to ambient temperatures, behavioural thermoregulation on focal heat sources results in increased body temperature fluctuations, in particular in smaller animals with lower thermal inertia. During

mating season, females were often hiding for many days in the pond of the exhibit, away from the males occupying the artificial heat areas. Although core body temperature was not recorded in this study, suboptimal thermoregulation may have impeded the endocrinological signalling for ovulation directly, or indirectly via effects on energy uptake. In contrast to the photo signal, a temperature signal is not unambiguous in its effect in reptiles. Relative temperature changes might act as a trigger influencing the endocrinological cascade (Licht, 1972). But the absolute magnitude of temperature influences body condition via food intake and assimilation efficiency (McConnachie & Alexander, 2004; Plasman et al., 2019) and sets the pace of the overall metabolism, possibly preventing appropriate hormone production at too low-temperatures (Licht et al., 1989).

## 5 | CONCLUSION

Regulation of ovulation and reproduction is a complex multifactorial process and certain aspects have not been included in this study (e.g. nutritional analyses, daylight intensity, individual social interactions and hierarchies, impact of quantitative differences in triggers, and additional endocrinological factors). Nevertheless, this study provides evidence for temporal discrepancies of seasonal extrinsic and intrinsic factors in a reptile species managed in an *ex situ* zoological institution in the opposite hemisphere to the natural habitat. The systematic investigation of possible reproduction-associated factors and their interplay constitutes an evidence-based approach, which replaces the commonly applied, rather random and uncoordinated changes of single management measures. In this study, the following conclusions can be drawn:

- Parallelism of temperature and photoperiod in the zoo-managed population as opposed to the temporal delay of these triggers in the native habitat might lead to inhibiting combinations of environmental trigger and endocrinologically refractory periods.
- Without deliberate experimentation, triggering factors such as temperature, photoperiod, and resources cannot be disentangled.

Recommendation for the zoo-managed population at hand would therefore constitute of (1) increase of ambient environmental temperature to the optimal activity phase of Aldabra tortoises, (2) decoupling of temperature and photoperiod in approximation to the situation in the wild habitat, (3) ensuring the maintenance of energy uptake during follicular maturation, and (4) larger breeding groups and structural niches for females to escape social stress by excessive male mating attempts.

### AUTHOR CONTRIBUTIONS

Pia Cigler analysed hormonal, meteorological and ultrasound data and compiled all data to a complete study. Leyla R. Davis and Sarah-Lisa Gmür provided the behavioural and meteorological data. Marcus Clauss performed statistical analyses and contributed to study design, compilation of the data, and writing of the manuscript. Jean-Michel

Hatt and Stephanie Ohlerth contributed to data collection (blood and ultrasound examination). Gabriela Mastromonaco consulted on and performed the endocrinological analyses. Maya Kummrow provided the funding, conceived, and designed the study. All authors contributed critically to the development and writing of the final manuscript and approved the final manuscript.

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### CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

### DATA AVAILABILITY STATEMENT

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

### ETHICS STATEMENT

The presented data on hormone measurements and diagnostic imaging were compiled from the routine veterinary diagnostic reproductive monitoring programme, in alignment with institutional welfare and research regulations.

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