

Comparative analysis of gestation in three rhinoceros species (*Diceros bicornis*; *Ceratotherium simum*; *Rhinoceros unicornis*)

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ABSTRACT

This comparative analysis evaluated endocrine profiles and gestation length data of captive pregnant black rhinoceros (*Diceros bicornis*), white rhinoceros (*Ceratotherium simum*), and greater one-horned (GOH) rhinoceros (*Rhinoceros unicornis*). Hormone profiles were collected over three decades as part of pregnancy diagnoses. After the third month of gestation, the luteo-placental shift in progesterone production in pregnant rhinoceroses causes a significant increase in the concentration of faecal progesterone metabolites. We defined a laboratory-specific value of 1000 ng/g faeces as a threshold for incipient feto-placental progesterone production. Using this value allowed a comparison between species and revealed significant individual differences within a species. The mean $\pm$ SEM gestation days for reaching the 1000 ng/g faeces threshold were 89.5 ± 2.9 (range 56–138 days; $n = 39$) in black, 96.0 ± 2.6 (58–138; $n = 39$) in white, and 117.8 ± 5.3 (74–173; $n = 19$) in GOH rhinoceroses. For the calculations of gestation length, we complemented our results from three decades of reproductive monitoring with data from the literature, resulting in about 70 values for each species. Gestation length in the black, the white and the GOH rhinoceros was 460.6 ± 1.5 (range: 436–486), 503.8 ± 1.3 (range: 480–525) and 480.5 ± 1.1 (range: 453–505) days, respectively. Daylight length significantly affected gestation length, while the sex of offspring had no effect. On average, pregnancies with parturitions in spring and summer were one week shorter than those in autumn and winter. Although rhinoceroses are non-seasonal breeders, most parturitions in captivity occur in autumn and winter. We also analysed preconception endocrine profiles in the white rhinoceros. Conceptions in this species occurred after oestrous cycles of approximately 35 days ($n = 18$), 70 days ($n = 3$), 15 days ($n = 1$), after periods of ovarian inactivity ($n = 5$), and during a foal heat within one month after stillbirth parturition ($n = 1$). In conclusion, this study provides a comprehensive overview of gestational parameters in three rhinoceros species.

1. Introduction

The family Rhinocerotidae includes five extant species native to Africa and Southern Asia. All rhinoceros species are threatened by poaching (Thapa et al., 2013; Knight, 2019) and, according to the IUCN red list (IUCN), are either critically endangered (the black rhinoceros *Diceros bicornis*; the Javan rhinoceros *Rhinoceros sondaicus*, and the Sumatran rhinoceros *Dicerorhinus sumatrensis*), vulnerable (greater one-horned rhinoceros *Rhinoceros unicornis*) or near threatened (Southern white rhinoceros *Ceratotherium simum simum*). Protecting in situ populations and captive breeding are essential measures for threatened species conservation. Furthermore, captive populations are crucial for studying their reproductive physiology and endocrinology (Roth, 2006;

Schwarzenberger and Brown, 2013). The implementation of physiological findings in internationally coordinated breeding programmes has contributed significantly to the increase in the number of reproducing rhinoceroses in zoos in recent decades (Foote and Wiese, 2006; Hutchins and Kreger, 2006; Reid et al., 2012; Houwald, 2018; Versteeg, 2018b).

The reproductive physiology of the oestrous cycle and pregnancy in different rhinoceros species has at first been analysed by non-invasive hormone analysis of urine samples (Kasman et al., 1986; Ramsay et al., 1987; Hodges and Green, 1989; Hindle et al., 1992; Stoops et al., 2014). Soon afterwards, faecal steroid analysis in female rhinoceros was established for all species managed in captivity, replacing urine hormone analysis, as it represented a more practicable way for longitudinal, routine sampling (Hindle and Hodges, 1990; Schwarzenberger et al.,

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1993, 1996, 1998, 2000; Berkeley et al., 1997; Radcliffe et al., 1997, 2001; Garnier et al., 1998, 2001, 2002; Heistermann et al., 1998; Patton et al., 1999; Brown et al., 2001; Graham et al., 2001; Lance et al., 2001; Roth et al., 2001, 2004; Hildebrandt et al., 2007, 2018; Hermes et al., 2009, 2012, 2016, 2021; Schwarz et al., 2014; Edwards et al., 2015, 2020; van der Goot et al., 2015; Santymire et al., 2016; Pennington et al., 2019a, 2019b; Kothmann et al., 2022). Today, urine hormone analysis is used to analyse protein hormones (FSH/LH) to determine the timing of ovulation (Stoops et al., 2004, 2014, 2016). In addition, analysis of reproductive hormones from saliva (Kuckelkorn and Dathe, 1990; Czekala and Callison, 1996; Gómez et al., 2004) and blood samples have been reported (Kock et al., 1991; Berkeley et al., 1997; Roth et al., 2001, 2004; Hildebrandt et al., 2007; Hermes et al., 2009, 2020). Regular blood sampling in rhinoceroses is increasing, but it is not yet a routine management practice, as is the case with elephants (Schwarzenberger and Brown, 2013).

Endocrine monitoring provides crucial information on oestrous cycle activity, the best timing for and success of mating, pregnancy, and the approximate period of parturition (Metrione and Eyres, 2014; Hermes et al., 2020; Versteeg, 2018a). However, it lacks information on the health of the reproductive organs. Reproductive ultrasound closed this knowledge gap by monitoring ovarian activity and diagnosing pregnancy, early embryonic loss, or reproductive pathology. Although rectal ultrasound examinations are not yet applied routinely, this technique allowed detailed insights into the ovarian dynamics of black, white, GOH and Sumatran rhinoceroses in recent years. (Berkeley et al., 1997; Radcliffe et al., 1997, 2001; Schaffer et al., 1998; Roth et al., 2001, 2004; Stoops et al., 2004, 2014, 2016; Hermes et al., 2005, 2006, 2009, 2012, 2016, 2020, 2021; Hildebrandt et al., 2007; Pennington and Durrant, 2019; Pennington et al., 2019a). As a result, the cumulated knowledge of the oestrous cycle and its related ovarian activity led to the development of ovulation induction (Hermes et al., 2012; Pennington et al., 2019a), artificial insemination in white and GOH rhinoceros (Hildebrandt et al., 2007; Hermes et al., 2009; Stoops et al., 2014, 2016; Pennington et al., 2019b), and the in vitro production of embryos and embryonic stem cells in an attempt to save the critically endangered Northern white rhinoceros (Hildebrandt et al., 2018).

The reproductive physiology is different among the four rhinoceros species studied, with marked differences in oestrous cycle and gestation length (Roth, 2006; Roth et al., 2018; Metrione and Eyres, 2014). Oestrous cycle length varies considerably between species and averages 21–25 days in the Sumatran (Heistermann et al., 1998; Roth et al., 2004), ~27 days in the black (Hindle et al., 1992; Schwarzenberger et al., 1993; Berkeley et al., 1997; Radcliffe et al., 1997; Brown et al., 2001; Graham et al., 2001; Garnier et al., 2002; Edwards et al., 2015), and approximately 40–48 days in the GOH rhinoceros (Kasman et al., 1986; Schwarzenberger et al., 2000; Gómez et al., 2004; Stoops et al., 2004). In the white rhinoceros, even two different cycle lengths of approximately 30–35 or 65–70 days have been described (Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001; van der Goot et al., 2015; Pennington et al., 2019b). In addition to the description of physiological oestrous cycle patterns, irregular oestrous cyclicity, persistent luteal activity, or extended periods of acyclicity often occur in black, white, GOH and Sumatran rhinoceroses (Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001; Roth et al., 2001; Garnier et al., 2002; Stoops et al., 2004; Hermes et al., 2006; Edwards et al., 2015, 2020). The effects of the different lengths of oestrous cycles on conception in white rhinoceros are not fully understood. Previous publications speculated that long cycles of approximately 70 days are infertile (Patton et al., 1999; Roth, 2006); yet, Pennington et al. (2019b) describe pregnancy as occurring after a 70-day cycle.

The length of gestation is about 15 months in the black (Jones, 1979; Ramsay et al., 1987; Schwarzenberger et al., 1993, 1996; Czekala and Callison, 1996; Berkeley et al., 1997; Garnier et al., 1998; Brown et al., 2001; Lance et al., 2001; Radcliffe et al., 2001; Roth, 2006; Schwarz et al., 2014; Santymire et al., 2016), 16 months in Sumatran (Roth et al.,

2001, 2004) and GOH rhinoceros (Jones, 1979; Kasman et al., 1986; Kuckelkorn and Dathe, 1990; Schwarzenberger et al., 2000; Roth, 2006; Stoops et al., 2014, 2016), and 16–17 months in white rhinoceros (Jones, 1979; Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001; Hildebrandt et al., 2007; Hermes et al., 2009, 2020; Pennington et al., 2019b). In all pregnant rhinoceros species studied to date, the feto-placental unit is a crucial source of progesterone production. The luteo-placental shift in progesterone production causes a marked increase in the faecal metabolite concentrations of this hormone and thus facilitates non-invasive endocrine pregnancy diagnosis. (Kasman et al., 1986; Hodges and Green, 1989; Kuckelkorn and Dathe, 1990; Kock et al., 1991; Schwarzenberger et al., 1993, 1996, 1998, 2000; Czekala and Callison, 1996; Berkeley et al., 1997; Garnier et al., 1998, 2001; Patton et al., 1999; Lance et al., 2001; Radcliffe et al., 2001; Roth et al., 2001, 2004; Hildebrandt et al., 2007; Hermes et al., 2009; Schwarz et al., 2014; Stoops et al., 2014; Pennington et al., 2019b; Kothmann et al., 2022). For pregnancy diagnosis, a combined assessment of individual hormone profiles together with a laboratory-specific threshold is applied. The timing when this threshold is exceeded is around 70, 90 and 120 days of gestation for white, black and GOH rhinoceros, respectively (Schwarzenberger et al., 1993, 1996, 1998, 2000; Hildebrandt et al., 2007; Hermes et al., 2009). However, this timing varies considerably between individuals, although this variation has not yet been comprehensively analysed.

The aims of this comparative analysis of gestation in black, white, and GOH rhinoceros were the evaluation of endocrine patterns during the first six months of gestation, the analysis of gestation length, and seasonal birth distribution. More specifically, we evaluated (a) intra- and interspecies differences in the increase in progestagen production during the first six months of gestation and calculated species-specific mean $\pm$ SEM hormone profiles. (b) Further, we analysed preconception endocrine profiles in white rhinoceros to evaluate which cycle length (approximately 30–35 or 70–75 days) promises the best chances of a conception; (c) determined intra- and interspecies variations in gestation length; (d) analysed the influence of season and offspring sex on gestation length.

2. Methods

2.1. Endocrine profiles during the first six months of gestation

The objectives of this study in three rhinoceros species were to evaluate intra- and interspecies differences in the increase of progestagen production during the first six months of gestation and to calculate species-specific mean $\pm$ SEM hormone profiles. For our evaluations, we used the results of endocrine profiles of pregnant rhinoceroses, which were analysed in the endocrine laboratory of VetMedUni Vienna during the last three decades. The sample series analysed in this study were collected for diagnostic purposes (pregnancy diagnosis) in the zoological facilities listed in the acknowledgements and [supplementary files](#). The sample series were each collected for about 3 to 4 months and sent in batches for analysis. Some sample series were collected with one sample per week, others with two samples per week. Once pregnancy was confirmed, no further samples were analysed. Therefore, sample series with different numbers of samples and sampling frequencies were available. As not every mating was successful, samples from the period preceding successful conception were also available from several animals. For other animals, sampling was started only after the second month of gestation or later.

Hormone metabolites in the black (Schwarzenberger et al., 1993, 1996) and white rhinoceroses (Schwarzenberger et al., 1998) were analysed using enzyme immunoassays for 20-oxo-pregnanes, whereas 20 α -OH-pregnanes were analysed in the greater one-horned (GOH) rhinoceros (Schwarzenberger et al., 2000). During the development phase of the analytical methods used, assays were tested for their suitability for reproductive monitoring in rhinoceroses. The assays most

suitable for diagnostic purposes are the 20-oxo-pregnane assay in African species and the 20 α -OH-pregnane assay in GOH rhinoceroses. The group-specific antibodies used in these assays, as well as the enzyme labels, were produced in-house. These assays have been used successfully in rhinoceros and numerous other species over the past decades (Schwarzenberger and Brown, 2013). The analytical methods for analysing the rhinoceros samples, including extraction, have remained the same since the methods were first introduced. The consistent approach to analysis over three decades allows us to compare individual results within a species and make comparisons between species.

Progestagen concentrations are reliable parameters for pregnancy diagnosis in the three rhinoceros species studied. The concentrations after mating indicate a luteal phase; a marked increase in concentrations to values above 1000 ng/g faeces occurs towards the end of the first trimester of pregnancy, reflecting the onset of feto-placental progesterone production. The selection criteria for including endocrine profiles in this study were that the results included the period when the 1000 ng/g faeces value was continuously exceeded and that at least 12 samples from the respective series were available. For the calculation of the 1000 ng/g faecal threshold, the longest interval between the two samples adjacent to the 1000 ng/g was eight days. Most of the hormone profiles included had 3–4 days between samples. The percentage of 6 to 8-day intervals for calculating the 1000 ng/g faeces threshold was 23.7, 25.6 and 47.4 % for black, white and GOH rhinoceroses, respectively. All other intervals were between one and four days.

We calculated the day when the threshold of 1000 ng/g faeces was reached and exceeded by smoothing the progestagen profiles of animals with known mating dates using a moving average of three values each. We determined the two days adjacent to the concentration of 1000 ng/g faeces in the resulting profiles. We then divided the interval between these days and added the resulting value to the lower of these days. Then we used the result as a reference value for the incipient feto-placental progestagen production. The number of profiles used for the calculations was $n = 39$, $n = 39$, and $n = 19$ for the black, white, and GOH rhinoceroses.

For the calculation of mean $\pm$ SEM progestagen concentrations used for the graphed pregnancy profiles, a time point for alignment of the individual profiles had to be defined. Possible options were to use the date of mating or the date of birth and, on the other hand, the hormonal increase above the 1000 ng/g threshold. Both gestation length and time of onset of feto-placental progestin production are subject to high individual variations. To reduce the effects of this variability on a best-fit pregnancy profile, we used the threshold of 1000 ng/g faeces as an anchor point for the alignment of the individual hormone profiles. This anchor point allowed us to include hormone profiles from $n = 14$ white rhinoceroses with unknown mating dates, thus further increasing the number of values available per calculation point. After we aligned the profiles, we used the original pregnane values (not the moving averages) to calculate the mean $\pm$ SEM values.

We calculated faecal progestagen mean $\pm$ SEM values during the first 190 days of gestation using $n = 39$, $n = 53$ (39 pregnancies with known and 14 with unknown gestation length), and $n = 19$ profiles of black, white, and GOH rhinoceroses, respectively. Before day 148, results of black and white rhinoceroses were combined at 3-day intervals and after that at 5-day intervals. In GOH rhinoceroses, fewer samples were available; therefore, we grouped results before day 148 at weekly and after that at two-week intervals. For the calculation of the mean $\pm$ SEM, we had 15 to 37 (median 23), 15 – 43 (median 29), and 9 – 35 (median 19) individual values for each time point of gestation for black, white, and GOH rhinoceroses, respectively.

2.2. Pre-conception endocrine profiles of white rhinoceroses

In white rhinoceroses, short and long oestrous cycles of approximately 35 or 70 days are commonly observed (Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001;

van der Goot et al., 2015; Pennington et al., 2019b). In addition to these oestrous cycle patterns, two other types of ovarian activity, missing luteal activity ('flatliners') or persistent luteal activity over periods more than three months, are monitored (Schwarzenberger et al., 1998). The effects of these different types of ovarian activity on a possible conception are unknown. However, it has been proven that infertility lasting for several years leads to ovarian and uterine pathologies (Hermes et al., 2006). In order to investigate the ovarian activity before conception, $n = 28$ hormone profiles of $n = 21$ individual white rhinoceroses were analysed, and results were assigned to ovarian function, such as varying lengths of oestrus cycles or ovarian inactivity.

2.3. Seasonal distribution of parturitions

Parturitions were not evenly distributed throughout the year. Births were grouped within two calendar month intervals and graphed.

2.4. Gestation length

Gestation length (mean $\pm$ SEM) was calculated using the days of observed mating (Day 0) and parturition. Data were collected during three decades of reproductive monitoring and validated through personal communication with the participating zoological institutions. This provided data of $n = 43$ black, 54 white, and 23 greater one-horned rhinoceroses. A detailed list of animals is included in the tables in the supplementary files. These results were complemented with gestation length data of $n = 11$ pregnancies from white rhinoceroses of the Southern hemisphere (Australia and New Zealand; C. Morrow – pers. comm.), with data gathered from the Rhino Resource Centre (RRC) database (Rookmaaker, 2022), and published literature (Jarvis, 1967; Lang et al., 1977; Kuckelkorn and Dathe, 1990; Nath et al., 1993; Berkeley et al., 1997; Patton et al., 1999; Radcliffe et al., 2001; Stoops et al., 2014, 2016; Santymire et al., 2016). The information on gestation length in the RRC database is usually a short press release from zoos that read like: "After 495 days of gestation, a white rhino calf was born at Zoo XY". Results were verified with the records in the International Studbooks of the respective rhinoceros species (Houwald, 2015; Kern, 2013; Versteeg, 2018b). All results combined, the total numbers for the gestation length calculations were $n = 68$, 73, and 75 in black, white, and GOH rhinoceroses, respectively. The range of variation in gestation length within a species was analysed using coefficients of variation of means and standard deviations.

We investigated the influence of the sex of offspring and daylight hours on the average gestation length. Results were calculated for male and female offspring and for pregnancies where the parturition occurred during short or long daylight. Seasonal transitions in spring (March 20/21) and fall (September 22/23) were used as discriminators. Gestation lengths of 11 white rhinoceroses kept in zoos in Australia and New Zealand were included; however, these results were time-shifted by half a year before analysis. The differences between groups (sex of offspring; daylight hours) were statistically analysed using a two-sample *t*-test.

The shortest gestations were observed on days with long daylight hours. We used a Pearson linear regression to analyse the correlation between gestation length and daylight. For this calculation, we transformed results into a linear correlation by positioning the gestation lengths data from January, February, and March to after December (April through December were set as months 1 to 9, and January to March as months 10–12).

2.5. Statistical analysis

Results were graphed and statistically analysed using SipmaPlot 14.5. Results are presented as means $\pm$ SEM with $p < 0.05$ considered significant. For ease of presentation, procedures for calculation of the 1000 ng/g faeces threshold, the preparation of pregnancy profiles, the influence of daylight hours and sex of offspring on gestation length, the

gestation length comparison by *t*-test, and the Pearson linear regression analysis are described in the previous paragraphs.

3. Results

3.1. Hormone profiles

We used a concentration of 1000 ng/g faeces as a reference value for the incipient feto-placental progesterone production. The day of gestation, when this threshold was exceeded, varied between species and considerably between individuals of one species (a list of individuals is provided in the [supplementary file](#)). The mean gestation days for the thresholds were 89.5 ± 2.9 days (range 56–138 days) in the black rhinoceros, 96.0 ± 2.6 days (58–138) in the white rhinoceros, and 117.8 ± 5.3 days (74–173) in the GOH rhinoceros ([Fig. 1](#)). The number of profiles used for the calculations was $n = 39$, $n = 39$ and $n = 19$ for black, white and GOH rhinoceroses, respectively. Results were obtained from $n = 23$, 31, and 12 female black, white and GOH rhinoceroses, respectively. Two or more births were obtained from $n = 6$, 6, and 2 black, white and GOH rhinoceroses, respectively. In the multiple-birth animals, there were significant differences between the pregnancies when the threshold value of 1000 ng/g was reached. The mean values of these differences were 19.8 and 19.7 days (median values 19 and 14.5 days) for black and white rhinoceroses, respectively. The mean value for the GOH rhinoceros was not calculated, as only two animals were represented more than once in the results. One GOH rhino was represented six times in our results. The days for this animal's 1000 ng/g faecal value were: mean 116; median 111.5; range 97–148.

For the 1000 ng/g faecal threshold calculation, we included hormone profiles of two white rhinoceroses that aborted male calves on days 136 and 395 after mating. The 1000 ng/g threshold for these animals was on days 90 and 112, respectively. We also included results from two black rhinoceroses and one GOH rhinoceros with premature births. These events in the black rhinoceroses occurred on gestation days 393 (male offspring) and 427 (female), respectively. The premature gestation of 425 days in a primiparous GOH rhinoceros resulted in a live birth; despite a small body size, this female calf developed into adulthood and became a proven breeder. In the gestation length analysis, we omitted the short gestations mentioned in this paragraph.

The marked individual variations in reaching the 1000 ng/g threshold would significantly impact the resulting mean $\pm$ SEM values of the species-specific pregnancy profiles. Therefore, individual results were aligned using the 1000 ng/g faeces threshold value. This approach allowed us to include hormone data from $n = 14$ white rhino pregnancies with unknown mating dates in the calculations of mean $\pm$ SEM.

The comparative results of the three rhinoceros species studied are presented in [Fig. 2](#); in this figure gestation days 70 to 125 are included as an inset on a larger scale. In early pregnancy, the average hormone concentrations in the three species studied range from (200) 300 to 600 ng/g feces. During the gradual transition from luteal to feto-placental hormone production, the concentrations increase to well over 1000 ng/g faeces within 2–3 (4) weeks. The increase in concentration in the African species is parallel, steeper, and more rapid compared to the GOH rhinoceros. After about 150 days of gestation, in both African rhinoceros species, mean faecal pregnane levels were about twice as high as in the GOH rhinoceros. However, 20-oxo pregnanes were analysed in the African species, whereas 20 α -OH pregnanes were analysed in the GOH rhinoceros.

Preconception ovarian activity ($n = 28$) in $n = 21$ white rhinoceroses indicated that all types of observed cycles were fertile. Females conceived after oestrous cycles of 35 days ($n = 18$) and 70 days ($n = 3$). Nevertheless, conceptions were also recorded after periods of ovarian inactivity ($n = 5$), a 15-day cycle ($n = 1$), and during a foal heat, within one month after stillbirth parturition ($n = 1$). Of the $n = 21$ animals, two individuals with results of 3 pregnancies each were included. One of these animals had two approximately 70-day cycles and one

approximately 35-day cycle prior to conception, and the second had two approximately 35-day cycles and one postpartum foal heat.

3.2. Seasonality of birth

Parturitions in captive rhinoceros species occurred throughout the year. Nevertheless, in terms of quantity, the highest number of births occurred between September and February, indicating that most conceptions took place in spring and summer ([Fig. 3](#)).

3.3. Gestation length

Gestation length data are summarized in [Table 1](#). The average values of gestation length vary by about three weeks between species and about seven weeks within species. The average values for black, white, and GOH rhinoceroses were 461, 504, and 480 days, respectively. The coefficients of variation of gestation length in the three species were 2.69 %, 2.12 %, and 2.07 %, respectively.

The gestation length results were obtained from $n = 37$, 45 and 38 black, white and GOH rhinoceroses, respectively. Two or more birth had $n = 17$, 19 and 18, and three or more births occurred in $n = 5$, 9 and 8 black, white and GOH rhinoceroses, respectively. There was a wide variation in gestation length in animals with three or more births. The mean values of gestation length variation of animals with three or more births were 18.0, 12.3 and 19.8 days (median values 20, 12 and 16 days) for black, white and white rhinoceroses, respectively.

Daylight length significantly affected gestation length; the shortest gestations were those in which parturitions occurred during the summer months. Categorizing results by births during short- or long-day periods revealed statistically significant differences in gestation length. Offspring sex had no statistically significant effect on gestation length in any of the three species ([Table 1](#)).

The listing of births by month illustrates the influence of daylight hours on gestation length ([Fig. 4](#)). Arranging data from January, February, and March to the position after December resulted in a positive linear correlation between the month of birth and gestation length. The results for the three species are as follows: black rhinoceros = $444.00 + (1.701 \cdot \text{month})$; $p < 0.001$, $R^2 = 0.152$; white rhinoceros = $498.69 + (0.525 \cdot \text{month})$; $p < 0.001$, $R^2 = 0.024$; GOH rhinoceroses = $471.94 + (0.859 \cdot \text{month})$; $p < 0.001$, $R^2 = 0.079$. The numbers in the calculations are Days of gestation.

4. Discussion

This study provides a comprehensive overview of gestational parameters in captive black, white, and GOH rhinoceros. The results on the endocrine profiles during pregnancy, gestation length, seasonal distribution of parturitions, and seasonal effects on gestation length represent the most extensive data analysis in rhinoceros species published to date. Although the differences in gestation length between species were known from previous studies, the pronounced within-species range of individual variations in gestation length and progesterone excretion during the luteo-placental transition was unexpected.

Our decade-long experience with hormone analysis in three rhinoceros species has shown that using a laboratory-specific value of 1000 ng/g faeces is a reliable indicator for an existing gestation. We used this 1000 ng/g faeces as a reference value for the incipient feto-placental progesterone production. This value is easily recognized when assessing hormone profiles, and in all pregnant individuals of the three rhinoceros species studied, it is exceeded during the onset of feto-placental progesterone production. A similar approach to diagnostic interpretation is used to evaluate plasma progesterone levels. For this hormone, in many species, a cut-off value of 1 ng/ml plasma is commonly used to determine the presence of a progesterone-producing corpus luteum.

The increase in progesterone concentration during the luteo-placental shift is well documented in the scientific literature on pregnant

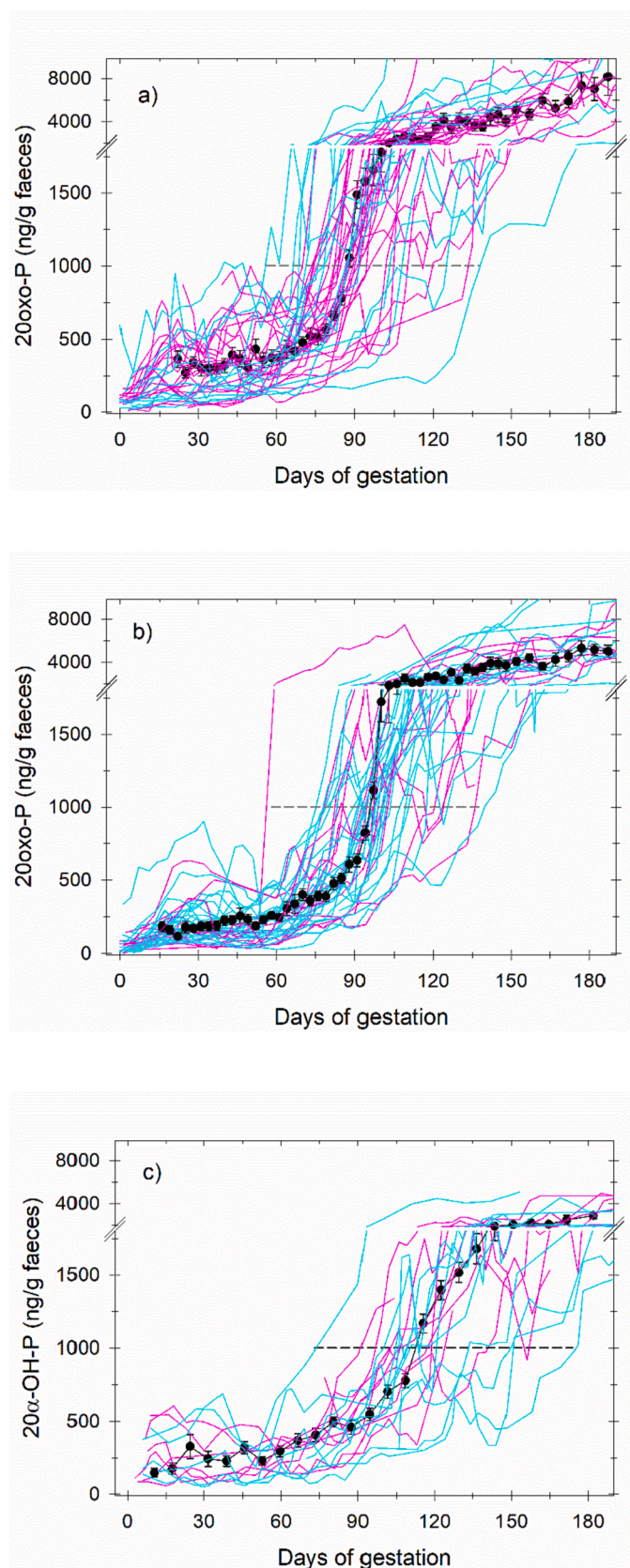


Fig. 1. Individual and mean $\pm$ SEM progestagen metabolite concentrations in pregnant (a) black, (b) white, and (c) GOH rhinoceroses with known gestation length. Each coloured line represents an individual gestation; colours indicate the sex of the offspring (red – female, blue – male offspring). The concentration of 1000 ng/g faeces was used as a reference for incipient fetoplacental progesterone production. For the calculation of mean $\pm$ SEM values (bold black line), the individual profiles were aligned with the 1000 ng/g faeces threshold. The horizontal dashed line indicates the range of variation in individual results when this threshold was reached and exceeded. The means of these threshold values were 89.5, 96.0, and 117.8 days in the black, the white and the GOH rhinoceros, respectively.

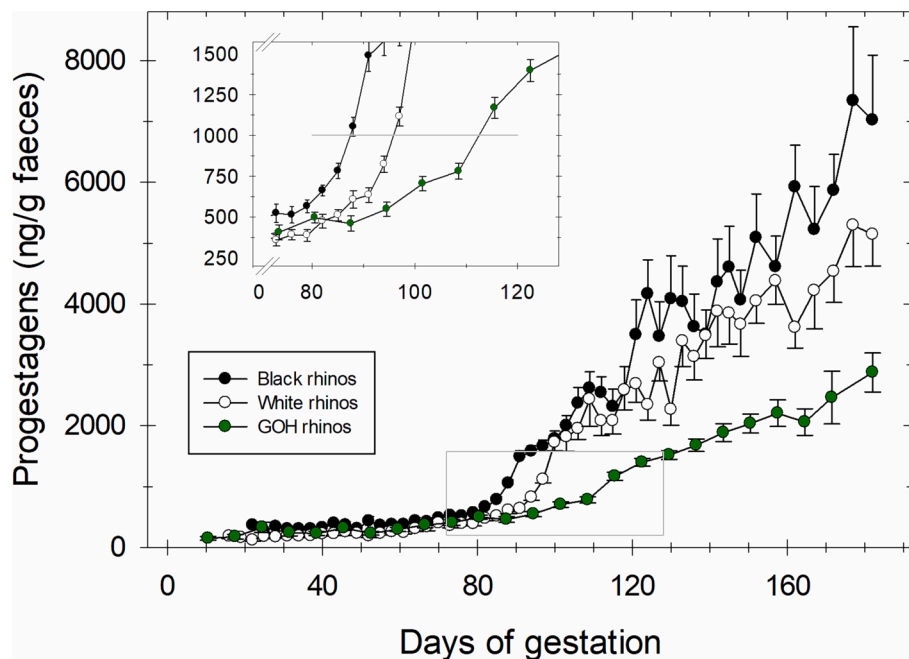


Fig. 2. Mean $\pm$ SEM progestagen metabolite concentrations in pregnant (a) black, (b) white, and (c) GOH rhinoceroses. Individual profiles were aligned with the 1000 ng/g faeces threshold. Gestation days 75 to 125 are shown on a larger scale in the inserted graph. In the overall graph, the results depicted in the insert are marked as a grey rectangle.

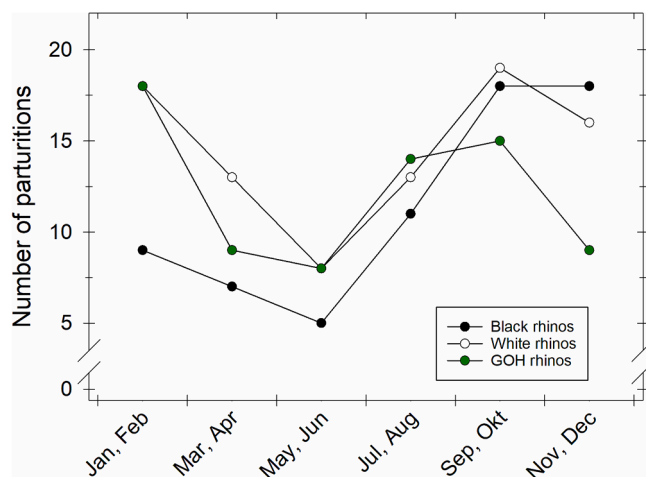


Fig. 3. Seasonal distribution of parturitions in black, white, and GOH rhinoceroses; the number of parturitions was grouped in bimonthly intervals. Results include pregnancies used for gestation length calculations and those of white rhinoceroses ($n = 14$) with unknown gestation lengths that were included in the calculation of the mean $\pm$ SEM pregnancy profiles.

rhinoceroses and is compatible with our results. However, published studies' results refer to a single or only a few animals. Comparing the published absolute faecal progestagen concentrations is also problematic because of the use of different antibodies in the immunoassays. The partial use of freeze-dried samples and a partially logarithmic presentation of the results are additional factors that make it difficult to compare the published results (Kasman et al., 1986; Czekala and Callison, 1996; Berkeley et al., 1997; Garnier et al., 1998, 2001; Patton et al., 1999; Lance et al., 2001; Radcliffe et al., 2001; Roth et al., 2001, 2004; Stoops et al., 2014; Pennington et al., 2019b). The differences in the onset of feto-placental hormone production in the published literature are, therefore, less detailed in time than in this study, with almost 100 hormone profiles and a threshold of 1000 ng/g faeces. It should be

emphasised again that our hormone analyses were always carried out using identical methods, so the individual results are readily comparable. Although different assays were used for the analysis of the African species (20-oxo-pregnane) and the GOH rhinoceros (20 α -OH-pregnane), a statistically significant correlation was found between the two assays when the analytical method for the GOH rhinoceros was established (Schwarzenberger et al., 2000).

The faecal steroid hormone metabolites used to create the hormone profiles in this study are 5 α -reduced pregnanes (Schwarzenberger et al., 1993, 1996, 1998; Lance et al., 2001). It is worth mentioning that these faecal pregnanes (i.e. dihydropregesterone and its hydroxylated 5 α -pregnane metabolites) are similar to pregnanes in the plasma of pregnant mares (Schwarzenberger et al., 1991; Scholtz et al., 2014; Legacki et al., 2016, 2020; Conley and Ball, 2019) and toothed whale species (Legacki et al., 2020). The confirmed source of these pregnanes in mares is the feto-placental unit (Scholtz et al., 2014; Legacki et al., 2016, 2020; Conley and Ball, 2019). In pregnant rhinoceroses, the wide range of variation in the timing of the luteo-placental shift in hormone production has not yet been described. To our knowledge, comparable results have also not been investigated in other families of the order Perissodactyla, including Equidae, Rhinocerotidae, and Tapiridae.

The transition from luteal to feto-placental hormone production is a gradual process, and the time from which the corpus luteum no longer produces progesterone cannot be narrowed down to one single day. In addition to the gradual transition, our enzyme-immunoassays used broad-spectrum antibodies that simultaneously detect multiple pregnane metabolites. The threshold of 1000 ng/g faeces comprised an early time point of feto-placental hormone production. It remains to be seen whether and how analytically specific methods such as GC-MS/MS will be used in future studies to analyse specific pregnane metabolites in blood and faecal samples. With such studies, the predicted individual differences in the onset of placental progestagen production can be confirmed or refuted. It also remains to be seen whether a specific analytical method will detect the somewhat longer-lasting and flatter increase in progestagen metabolites in the GOH rhinoceros. In any case, over the past three decades, it would have been unlikely to collect blood samples from nearly 40 pregnancies in each African species and from

Table 1

Gestation length (mean $\pm$ SEM) in days in black, white and GOH rhinoceros; 'n=' refers to the number of gestations. The numbers in the parentheses are the minimum and maximum values.

Species	Mean	Daylight length*		Offspring gender			
Black rhinoceros (n = 68)	460.6 $\pm$ 1.5 (436 – 486)	Short	464.0 $\pm$ 1.7 (440–486) n = 39	t(66) = 2.69 p = 0.009	Male	458.5 $\pm$ 1.9 (438–482) n = 31	t(66) = 1.33 p = 0.187
		Long	456.2 $\pm$ 2.4 (436–480) n = 29		Female	462.5 $\pm$ 2.2 (436–486) n = 37	
White rhinoceros (n = 73)	503.8 $\pm$ 1.3 (480 – 525)	Short	506.0 $\pm$ 1.5 (484–525) n = 41	t(71) = 2.05 p = 0.044	Male	505.6 $\pm$ 1.6 (481–525) n = 41	t(71) = 1.59 p = 0.120
		Long	500.9 $\pm$ 2.0 (480–525) n = 32		Female	501.6 $\pm$ 1.9 (480–525) n = 32	
GOH rhinoceros (n = 75)	480.5 $\pm$ 1.1 (453 – 505)	Short	482.7 $\pm$ 1.5 (453–497) n = 39	t(73) = 2.07 p = 0.042	Male	481.1 $\pm$ 1.7 (453–505) n = 40	t(73) = 0.53 p = 0.595
		Long	478.1 $\pm$ 1.7 (461–505) n = 36		Female	479.9 $\pm$ 1.4 (464–497) n = 35	

*Short vs long days refer to the season – autumn/winter vs spring/summer.

nearly 20 pregnancies of the GOH rhinoceros to generate the profiles used for analysis in this study.

The pronounced individual differences in gestation length and the onset of hormone production by the feto-placental unit presented confounding factors for calculating the mean $\pm$ SEM values. Therefore, the individual profiles were aligned at the 1000 ng/g faeces threshold. In previous studies, the date of conception or birth was used for the alignment (Schwarzenberger et al., 1996; Garnier et al., 1998). The 1000 ng/g faecal threshold of progesterone excretion is correlated with the luteo-placental shift in hormone production. However, since in some individuals, the threshold was already exceeded as early as the approximate 50th day of pregnancy, it seems plausible that in rhinoceroses, hormone-producing accessory corpora lutea can be formed at an early stage of gestation. The timing of reaching the threshold of 1000 ng/g faeces did not correlate with the duration of gestation. Furthermore, in some individuals in successive pregnancies, we found different dates for exceeding the threshold of 1000 ng/g.

Overall, the relatively large individual variations in reaching the threshold of 1000 ng/g and the duration of gestation are biologically interesting and of diagnostic importance, primarily if mating was not observed. This is rare in individually kept black and GOH rhinos, as a pairing between the sexes only occurs when the female is in oestrus. In contrast, mating often goes unnoticed in the gregarious white rhinoceroses, as this species is kept as a group of several females with offspring and a breeding bull (Metrione et al., 2007; Metrione and Eyres, 2014; Versteeg, 2018a). In such cases, pregnancy can be diagnosed using the 1000 ng/g threshold. However, because of the large variability in gestation length (Jones, 1979; Metrione and Eyres, 2014; Pilgrim and Biddle, 2014; Houwald, 2018; Versteeg, 2018a; Hermes et al., 2020) and in reaching the 1000 ng/g threshold, the timing of parturition can only be tentatively predicted. In such cases, it is advantageous to examine the decline in daily plasma progesterone concentrations during the week before parturition (Hermes et al., 2020).

Our results in white rhinoceroses demonstrate that pregnancies occur independently of prior ovarian activity. Conceptions occurred after cycles of approximately 35- or 70-day, periods of ovarian inactivity, an approximately 15-day cycle, and a foal heat following a still-birth. Our results thus contradict previous considerations that oestrus cycles of approximately 70 days would be infertile (Patton et al., 1999; Roth, 2006) and corroborate published findings by Pennington et al. (2019b).

Gestation length in species appears to be a basic and easy to obtain physiologic parameter. To date, detailed information on gestation length in rhinoceroses is available in editions of the International Zoo Yearbook

(Jarvis, 1967; Lang et al., 1977), in husbandry guidelines (Metrione and Eyres, 2014; Pilgrim and Biddle, 2014; Versteeg, 2018a), in the Rhino Resource Centre database (Rookmaaker, 2022), and reviews on rhinoceros reproduction (Roth, 2006; Pilgrim and Biddle, 2014; Roth et al., 2018; Pennington and Durrant, 2019). However, these data are dispersed and not compiled in a way comparable to this study. The mean gestation length of black, white and GOH rhinoceros reported here has a narrow standard error of fewer than two days. Nonetheless, the range of gestation length of about 50 days in each of the three species demonstrates the dimension of individual margins left despite a narrow mean value. These differences are comparable to gestation length variations reported, i.e. in different horse breeds (Satué et al., 2011; Heck et al., 2017; Ewert et al., 2018), donkeys (Galisteo and Perez-Marin, 2010), dromedaries (Nagy and Juhász, 2019), or in captive dolphins (O'Brien and Robeck, 2012) and killer whales (Robeck et al., 2016). One of the best-studied species in terms of gestation length is the domesticated horse, with very accurate studbook records. Mean gestation length in more than 20 horse breeds ranged between 332 and 344. Individual values for gestation length were 294 to 419 days, although most foalings occur between 325 and 368 days (Satué et al., 2011; Heck et al., 2017; Ewert et al., 2018). Like in rhinoceroses, these variations of several weeks appear to be relatively high; however, the coefficient of variation for gestation length variations in most species is in the range of 2–3 %, and thus comparable to our results in rhinoceroses. Tabulated data on gestation length in various mammalian species in the orders of Insectivore, Primates, Lagomorpha, Rodentia, Carnivore, Proboscidea, Perissodactyla, and Artiodactyla give coefficients of variation of 0.4, and up to 6.3 % (Kiltie, 1982).

The season and length of daylight significantly influenced the duration of gestation in all three rhinoceros species studied; the shortest pregnancies occurred in the months with the most extended daylight. The influence of fetal sex on gestation length was statistically not significant. Our results suggest that gestation in captive rhinoceros is, on average, one week shorter when the birth occurs in summer as opposed to winter. The influence of daylight on gestation length in black, white and GOH rhinoceroses has not yet been described. In a herd of six white rhinoceros with 12 pregnancies managed in Uganda, near the equator, gestation length was 493.3 $\pm$ 2.8 days (mean $\pm$ SEM), hence ten days shorter than in this study (Felix Patton, RFU - Rhino Fund Uganda, pers. communication). Missing seasonal fluctuations near the equator indicate that daylight length variations in temperate zones may influence gestation length in rhinoceros species. In domestic horses, the influence of daylight on gestation length has been well documented, i.e. in Thoroughbred mares, with a data set of >10,000 gestations (Ewert et al.,

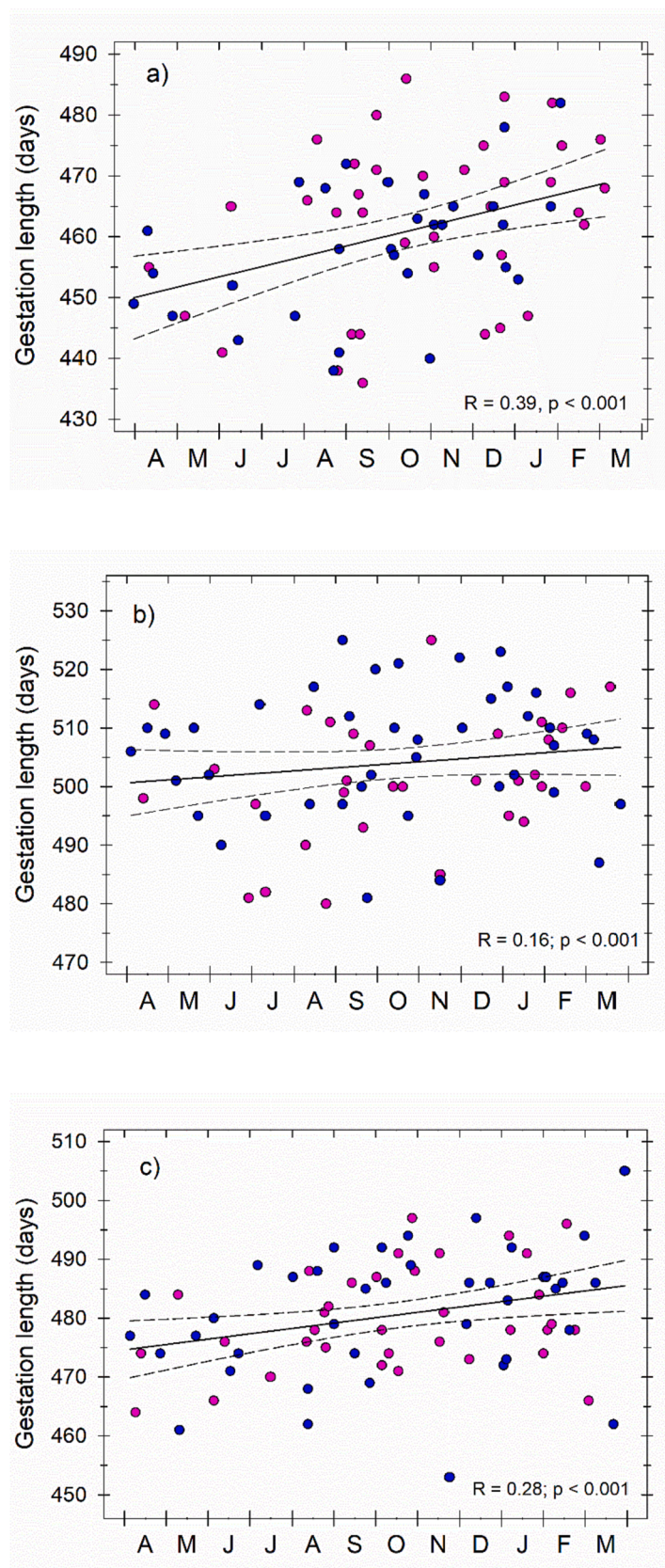


Fig. 4. Gestation length vs month of birth in (a) black, (b) white, and (c) GOH rhinoceroses. Gestations of male and female calves are depicted in blue and purple. Regression lines and 95% confidence intervals were calculated using all birth of one species.

2018). Gestation length of mares shortens as daylight hours during the spring foaling season increase. In addition to daylight length, environmental (mating period within the breeding season), foetal (sex of the offspring), and maternal factors (horse breed) influence gestation length in mares (Satué et al., 2011; Heck et al., 2017).

Rhinoceroses are considered non-seasonal breeders, although there is a particular seasonal distribution of parturitions (Metrione and Eyres, 2014; Pilgrim and Biddle, 2014; Houwald, 2015; Versteeg, 2018b). We observed a seasonal accumulation of parturitions in autumn and winter in the three captive species studied here. This seasonal accumulation of parturitions in rhinoceroses in zoological facilities in the temperate climate zones is likely influenced by favourable breeding opportunities during the spring and summer. The seasonal distribution of parturitions in the wild is attributed to variations in food availability at the end of the rainy season in spring (Owen-Smith, 1992; Berkeley and Linklater, 2010; Metrione and Eyres, 2014). Consequently, births of black and white rhinoceros in the wild occur predominantly during the dry season in autumn and winter (Owen-Smith, 1992; Garnier et al., 2002; Hrabar and Du Toit, 2005; Berkeley and Linklater, 2010). However, in another study on black rhinoceros in South Africa, the influence of precipitation on the conception rate was indeterminate (Freeman et al., 2014). On the contrary, an analysis of >500 calvings in white rhinoceros on a game farm in South Africa determined an apparent seasonal accumulation of birth in the summer and autumn months (Ververs et al., 2017). Compared to the wild, this shifted distribution of parturitions was attributed to supplementary feeding during the dry season. In the GOH rhinoceros, the distribution of births in the Chitwan National Park was consistent with the seasonal distribution of births in zoos in this study (Dinerstein and Price, 1991; Subedi et al., 2017). In contrast, birth dates listed in the regional studbook for the GOH rhinoceros on the Indian subcontinent (Wildlife Institute of India, 2016) yielded a peak in May and June, and thus during a period when the number of births in zoos in temperate zones was lowest. We, therefore, conclude that there is currently no definitive answer to whether the seasonal distribution of births in rhinoceroses is influenced more by daylight length or food supply.

5. Conclusions

This study provides a comprehensive overview of gestational parameters in captive black, white and GOH rhinoceroses. We describe species-specific differences in the onset of feto-placental progesterone production and gestation length, which are also subject to pronounced differences within each species. While rhinoceroses are considered non-seasonal breeders, there is a particular seasonal distribution of parturitions, most of which occur in autumn and winter. The season and, thus, the daylight hours at the time of birth significantly influenced the duration of gestation. Births occurring in spring and summer had about a one-week shorter gestation length than those occurring in autumn and winter.

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Data availability

The data supporting this study's findings are listed in a [supplementary file](#) and are available from the corresponding author upon reasonable request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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