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Diurnal variation of salivary cortisol in captive African elephants (*Loxodonta africana*) under routine management conditions and in relation to a translocation event

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Abstract

The present study assessed the diurnal variation in salivary cortisol in captive African elephants during routine management (baseline) and in relation to a potential stressor (translocation) to evaluate to what extent acute stress may affect diurnal cortisol patterns. Under baseline conditions, we collected morning and afternoon saliva samples of 10 animals (three zoos) on different days in two study periods ($n = 3$–10 per animal, daytime and period). Under stress conditions, we sampled the transported cow (newcomer) and the two cows of the destination zoo before and after the transport in the morning and afternoon ($n = 3$–9 per animal, daytime and transport phase), as well as after the first introduction of the newcomer to the bull ($n = 1$ per animal). Cortisol was measured in unextracted samples by enzyme immunoassay. Under baseline conditions, we observed the expected diurnal variation with higher cortisol levels in the morning than in the afternoon. Under stress conditions, neither a significant difference between pre- and posttransport, nor between morning and afternoon levels was found. The percentage difference between morning and afternoon cortisol after the transport, however, was remarkably lower than before the transport in the newcomer potentially indicating a stress response to familiarization. Saliva samples taken immediately after the introduction of the newcomer to the bull revealed a marked cortisol increase. Our findings indicate that stressors may disturb the diurnal cortisol rhythm. Furthermore, provided that samples can be collected promptly, salivary cortisol is a useful minimally invasive measure of physiological stress in the African elephant.

KEYWORDS
baseline, captive management, cortisol secretion, social introduction, stress

1 | INTRODUCTION

1.1 | Diurnal variation of cortisol secretion

In vertebrates, glucocorticoid (GC; cortisol/corticosterone) secretion from the hypothalamus–pituitary–adrenal axis (HPA-axis) follows an endogenous circadian rhythm. This rhythm is primarily entrained by the light–dark–cycle, but other periodic occurring environmental or behavioral stimuli may also influence the daily rhythm of HPA-axis secretory activity. The daily peak of GC secretion is synchronized with the onset of the major activity phase, which is associated with significant changes in environmental conditions and an increase in behavioral activity (for a review of the regulation of the circadian rhythm of the HPA-axis see Kalsbeek et al., 2012). In diurnal species, GC levels peak in the morning, decrease towards the afternoon, reach the trough around midnight and increase again in the second half of the night (Bohák et al., 2013; Coe & Levine, 1995; Ekkel et al., 1996; Kutsukake et al., 2009; Menargues, Urios, Limiñana, & Mauri, 2012, but see Giannetto et al., 2014 for dogs). This general pattern of diurnal GC dynamics has also been described for the African elephant, for which studies have shown that cortisol, the major glucocorticoid in this species, is secreted in high levels in the morning and low levels in the afternoon (Brown, Kersey, Freeman, & Wagener, 2010; Casares et al., 2016; Grand, Kuhar, Leighty, Bettinger, & Laudenslager, 2012; Kelling Swilley, 2008).

1.2 | Effect of stressors on the circadian GC rhythm

Stress-related and circadian HPA-axis activity mutually influence each other (reviewed in Nicolaides, Charmandari, Kino, & Chrousos, 2017). First, the stress responsiveness of the HPA-axis is greater during the trough than during the acrophase of the circadian rhythm (Charmandari et al., 2011). Consequently, short-term conditions that are perceived as stressful or physically demanding (here called acute stressors) are associated with a flattening of the circadian changes in GC secretion due to a greater increment of GC levels in the circadian trough than in the circadian peak (Bartlang et al., 2012; Ichikawa, Nishikai, Kawagoe, Yoshida, & Homma, 1972; Lassourd et al., 1996). Second, the effect on the circadian GC rhythm differs between acute and chronic stressors. Upon termination of an acute stressor, the circadian rhythm is reset within a few days (Balsalobre et al., 2000; Bartlang et al., 2014) and the flattening, therefore, is a transient phenomenon. Chronic stressors, however, disturb the circadian GC rhythm substantially, as the prolonged and/or deficient adaptive GC response to the stressor does not allow the quick resetting of the circadian rhythm (Nicolaides et al., 2017). This is reflected in the long-term flattening of the circadian rhythm, which, for example, was found in relation to poor housing conditions during rearing in pigs (De Jong et al., 2000) and different pathological conditions in humans and rodents (reviewed in Leliavski, Dumbell, Ott, & Oster, 2015). It has been suggested that, therefore, the circadian GC pattern could be used as another stress indicator beside the change in GC levels themselves (De Jong et al., 2000; Ekkel et al., 1996). In animals, transports are considered as stressors, as they are generally associated with GC level increases (Dembiec, Snider, & Zanella, 2004; Goymann, Möstl, Van't Hof, East, & Hofer, 1999; Laws et al., 2007; Schmidt et al., 2010a), which persist during the transport independent of daytime (Forhead, Smart, Smith, & Dobson, 1995; Schmidt et al., 2010b; Stull & Rodiek, 2000). To the best of our knowledge, the effect of transports on the diurnal cortisol rhythm was not examined yet. Changes in housing conditions, however, which are an inherent part of translocations, largely caused a transient flattening of the diurnal rhythm based on a greater increment of afternoon relative to morning levels (Barnett, Cronin, & Winfield, 1981; Becker et al., 1985; Irvine & Alexander, 1994; Pedersen, Rojkittikhun, Einarsson, & Edqvist, 1993). Thus, the change in housing in these studies produced rather acute than chronic stress. Transport is also associated with elevated GC secretion in African elephants as indicated by increases in levels of fecal GC metabolites in response to transportation (Millspaugh et al., 2007; Viljoen, Ganswindt, du Toit, & Langbauer, 2008). In addition, flattening of the diurnal GC profile in this species was attributed to an acute social stressor (Casares et al., 2016), personality (Grand et al., 2012) and potentially chronic stress conditions (Kelling Swilley, 2008). To what extent transportation stress also affects the diurnal GC dynamics in the African elephant has not yet been described.

1.3 | Measuring GC levels

GCs and their metabolites can be measured in different sampling matrices (reviewed in Heistermann, 2010 and Sheriff, Dantzer, Delehanty, Palme, & Boonstra, 2011). Blood allows real-time assessment of GC levels but requires invasive sampling, which limits its feasibility in wildlife. Sampling of urine and feces, in contrast, is noninvasive. However, fecal and urinary cortisol metabolites represent integrative measures of cortisol secretion and, concerning the African elephant, occur in the sample after a relatively long lag time of up to 38 hr in feces and 4.5 hr in urine (Ganswindt, Palme, Heistermann, Borragan, & Hodges, 2003). They are, therefore, not suitable to measure acute responses and are of limited use for understanding the diurnal variation of cortisol secretion. Saliva, in contrast, is ideally suited for these purposes. Salivary cortisol concentrations reliably reflect circulating cortisol in the blood (Grand et al., 2012) with a time lag of approximately 5–20 min in humans (Kirschbaum & Hellhammer, 2000) and 10 min in cattle (Hernandez et al., 2014). Furthermore, saliva can be collected minimally invasively and repeatedly from captive elephants provided that the animals are habituated to sampling through positive reinforcement training.

1.4 | Objectives

Before assessing the effect of a stressor on cortisol levels, the endogenous diurnal cortisol pattern in the absence of acute stressors has to be determined to be able to discriminate between acute responses and diurnal variation. The present study on captive African elephants had two objectives: (a) to assess the diurnal variation of salivary cortisol in ten animals housed in three different zoos under routine management

conditions and (b) to give first insights into the effect of a stressor, namely a transport and associated changes in housing conditions, on the diurnal variation of cortisol secretion in three animals involved in the translocation process. Concerning (a), we expected that the diurnal variation would be characterized by significantly higher salivary cortisol levels in the morning compared to the afternoon. Regarding (b), we expected a significant diurnal variation to occur before the transport and a disturbance (flattening or loss) of the diurnal variation in association with elevated afternoon levels to occur after the transport.

2 | METHODS

2.1 | Animals and management

Ten African elephants (*Loxodonta africana*) housed in three different German zoos (Opel-Zoo Kronberg, Grüner Zoo Wuppertal, Thüringer Zoopark Erfurt) were examined in two study periods separated by one year (2016, 2017). Animals of the same zoo were sampled in the same season in both years (Kronberg: spring, Wuppertal: summer, Erfurt: fall/winter). Table 1 summarizes information on the animals and the number of samples of each animal.

The elephant enclosures in all three zoos consisted of an outdoor enclosure and an indoor area with several boxes to separate individual animals. Enclosure furnishing included mud wallows, sandpits, and natural scratching surfaces. Hay was provided in hay nets or racks (Kronberg in 2017, Erfurt) respectively on the ground (Kronberg in 2016, Wuppertal). Additionally, browse, fruits, vegetables, and pellets were provided. Cows and their offspring were maintained in stable herds, whereas the bulls were separated during the night and temporarily during the day. Differences in management between 2016 and 2017 included change of feeding type (from ground to rack) and night housing (from outdoors to indoors) in AR and ZI (Kronberg), separation of the oldest young bull from the herd and socialization with TU (Wuppertal) and presence of a new cow (Erfurt, see Section 2.2.2).

2.2 | Saliva sampling

Before the beginning of the study, the animals were habituated to the sampling procedure through positive reinforcement training. To collect saliva, a swab (Salivette® Cortisol, Sarstedt, Nümbrecht, Germany) fixed in a clamp respectively a stainless-steel tube was used to wipe the inside of the mouth. The saliva samples were frozen at −18°C immediately following collection.

2.2.1 | Baseline diurnal cortisol variation

Baseline refers to cortisol levels during routine management conditions, that is the conditions the animals were regularly facing and habituated to. Therefore, we assumed that routine management did not present a stressful condition for the animals.

On a sampling day, a morning (08:00–08:30) and an afternoon (Kronberg: 18:00, Wuppertal: 14:00, Erfurt: 13:00) saliva sample was collected from one animal, which was separated from the group for this purpose. Cows, however, were not separated from their calves to prevent confounding social stress. This also applied to the animals SA and TI (mother and daughter), which were, therefore, sampled on the same day directly after one another. Between the morning and afternoon samples, the animal was handled according to the routine management of the respective zoo. The same animal was sampled in roughly weekly intervals. Three to ten samples per animal, daytime and study period were collected (Table 1) representing 3–10 assessments of diurnal variation per

TABLE 1 Information on animals (sorted by zoo) and number of samples of each animal in the morning (08:00) and in the afternoon (13:00, 14:00, 18:00) in the first (2016) and second (2017) study period

| Zoo | Animal-ID | Year of birth | Sex | Sample size 2016 | | Sample size 2017 | |
				08:00[a]	13:00[b] 14:00 18:00	08:00	13:00 14:00 18:00
Kronberg	AR	1979	Female	7	7	7	7
	ZI	1982	Female	6	6	10	7
	TA	2008	Male	5	6	7	7
Wuppertal	SA	1992	Female	6	6	6	5
	SW	1993	Female	3	6	6	5
	TI	2007	Female	6	6	5	5
	TU	1992	Male	5	6	6	6
Erfurt	SF	1971	Female	9	9	9	9
	CH	2003	Female	9	9	9	9
	KB	2005	Male	6	6	6	6

Note: Saliva samples were paired, which is on each sampling day a morning and an afternoon sample was collected from the same animal.
[a]Morning.
[b]Afternoon: 13:00, Erfurt; 14:00, Wuppertal; 18:00, Kronberg.

TABLE 2 Translocation sampling scheme

Animal-ID		PRE Day -18/-11 to -1[a]		POST1 Day 1–9		Introduction Day 20	POST2 Day 21–23	
		08:30/10:30[a]	14:30	10:30	14:30	11:45	10:30	14:30
CS	Newcomer	6	6	6	6	1	3	3
SF	Resident	6	6	9	7	1	3	3
CH	Resident	6	6	9	7	1	3	3

Note: Number and timing of saliva samples of the newcomer CS and the residents SF and CH in the four transport phases (PRE, POST1, Introduction, POST2), as well as timing of the transport phases in relation to Day 0 (arrival of CS). Not all animals were sampled on each day and not all daily samples were paired, which is on some sampling days either a morning or an afternoon sample was collected from an animal.

[a]CH, SF: 08:30 and Day -18 to -1; CS: 10:30 and Day -11 to -1.

animal. In 3.4% of samples saliva volume was too low (i.e., <50 µl) for the analysis, which reduced the sample size in some animals.

2.2.2 | Translocation (stressor)

We report the timeline of the transport in relation to the day of the arrival of the transported newcomer CS (Day 0). The adult cow CS was transported from Réserve Africaine du Sigean in France to Thüringer Zoopark Erfurt in Germany (distance traveled: 1,350 km). CS was loaded in the morning of Day -1 and arrived in the evening of Day 0 and therefore traveled about 36 hr including the compulsory stops to feed, drink and clean the elephant. Table 2 shows the timing of the four transport phases (PRE, POST1, Introduction, and POST2), the time of sampling in the morning and afternoon, as well as the number of samples of CS and the two resident cows CH and SF of the destination zoo in Erfurt. Due to arousal and refusal to respond to keepers, the first sample of CS after her transport could only be taken in the afternoon on Day 2. The first samples of CH and SF after CS's arrival were collected in the morning on Day 1. During the first phase after the transport (POST1: Day 1–9), CS and the resident elephants, including the bull, had visual and tactile contact through barriers. Moreover, CS was habituated gradually to the indoor and outdoor enclosures. From Day 12 onwards, CS, SF, and CH were housed together. On Day 20, CS was introduced to the bull KB for the first time. The introduction took place between 10:00 and 11:45 and the samples were collected immediately thereafter. CH and SF were separated in the outdoor bull enclosure, while CS and KB had access to the whole indoor area and the cow outdoor enclosure. Visual and tactile contact through barriers was possible between the two groups. On Day 21–23 (POST2), the cows were housed together and the bull was separated from the cows, allowing visual and tactile contact through barriers.

2.3 | Cortisol analysis

Salivary cortisol was determined by a microtiter plate enzyme immunoassay (EIA) using an antiserum against cortisol-3-CMO and biotinylated cortisol as a label. The EIA has been previously described and characterized (including cross-reactivity determinations) by Palme and Möstl (1997) and has been used successfully to monitor adrenocortical

activity from saliva samples in other species (Behringer et al., 2009, 2014). Before the study, we confirmed its biological validity for use in African elephants by demonstrating a rise in cortisol levels in response to washing, a potentially stressful event ($n = 3$ cases; cortisol level before: 1.28 ± 0.51 ng/ml; cortisol level after 2.28 ± 0.82 ng/ml; percentages of increase in response to each event: (a) 60.22%, (b) 50.00%, and (c) 133.96%).

For analysis, saliva samples were diluted 1:3 to 1:50 in assay buffer (phosphate-buffered saline with 1% bovine serum albumin, pH 7.2) to bring the cortisol concentrations into the working range of the assay and duplicate 50 µl aliquots of diluted samples and cortisol standard (50 µl, 0.6–40 pg/50 µl) were combined with biotinylated cortisol (50 µl) and cortisol antiserum (50 µl) and incubated overnight at 4°C. After incubation, the plates were washed four times, followed by the addition of 150 µl (667 ng) streptavidin–peroxidase (S5512; Sigma Aldrich Chemie GmbH, Deisenhofen, Germany) in assay buffer and a further incubation of the plate on a plate shaker for 1 hr at room temperature in the dark. Thereafter, the plate was washed again four times and TMB substrate solution (100 µl; 1-Step Ultra TMB, Thermo Fisher Scientific Inc., Rockford) was subsequently added and the plates incubated at room temperature in the dark for 45–60 min. The enzyme reaction was finally stopped by adding 50 µl of 2 M H_2SO_4 to each well and absorbance measured at 450 nm (reference 630 nm) in a spectrophotometer. Samples with a coefficient of variation (CV) >10% between duplicates were remeasured. The sensitivity of the assay at 90% binding was 12 pg/ml. Saliva samples of different animals gave displacement curves that run parallel to the cortisol standard curve. Inter-assay coefficients of variation (CV), assessed by replicate determinations of high- and low-value quality controls run in each assay, were 7.5% (high) and 10.4% (low), and intra-assay CVs were 5.0% (high) and 6.8% (low). Salivary cortisol concentrations are expressed as ng/ml.

2.4 | Data analysis

2.4.1 | Baseline diurnal cortisol variation

We applied a linear mixed model (LMM) using the function lmer of the R package lme4 (version 1.1-21; Bates, Mächler, Bolker, &

Walker, 2015) to examine if the daytime had an effect on the cortisol level. To meet LMM assumptions of homoscedasticity and normality of residuals, we log_{10}-transformed the cortisol data. Daytime (morning, afternoon) was included as a fixed effect. Furthermore, we controlled for the effect of the study period (2016, 2017) by fitting it as a covariate fixed effect. Each animal was measured twice per day on several sampling days. We accounted for this nested data structure by fitting sampling day nested in animal-ID as a random intercept. In this way, we controlled for effects on the cortisol levels generated by variation within and between animals, as well as within and between sampling days nested in animals. We used REML for model fitting and included the function BOBYQA (Bound Optimization BY Quadratic Approximation, Powell, 2009) as the optimizer. We applied t-tests (Satterthwaite's method) of the R package lmerTest (version 3.1-0; Kuznetsova, Brockhoff, & Christensen, 2017) to test for significance of fixed effects. For visualization of the average difference between morning and afternoon cortisol levels, we calculated the median morning and afternoon cortisol level of each animal separately for 2016 and 2017 using the original nontransformed data. Furthermore, we analyzed the diurnal variation on the individual level separately for 2016 and 2017. We used the Shapiro–Wilks test to check if data were normally distributed. Due to nonnormality of the original and log_{10}-transformed data and low sample sizes per animal we chose the nonparametric Wilcoxon signed-rank test for paired samples (Martin & Bateson, 2007; Siegel & Castellan, 1988) to test for differences in the central tendency of morning and afternoon cortisol levels.

In addition, we calculated the percentage difference between morning and afternoon cortisol levels by (morning–afternoon)/afternoon × 100 (formula 1) for each morning–afternoon pair to assess the average magnitude of the diurnal cortisol change of each animal. This was done to evaluate what magnitude of the difference between morning and afternoon cortisol levels corresponded to a nonsignificant, potentially disturbed, diurnal variation.

2.4.2 | Translocation (stressor)

To analyze the effect of the transport on the cortisol level, we used the Wilcoxon signed-rank test with Bonferroni-correction and compared the cortisol level in PRE to the cortisol level in POST1 separately for each animal and daytime (morning, afternoon). POST2 was not included in the statistical testing due to the low individual sample sizes in this transport phase. Regarding the introduction of CS to the bull, we calculated the percentage increase in relation to the individual median cortisol level in PRE for each animal and daytime with the formula: (Introduction-PRE)/PRE × 100. We considered the increase as significant if the cortisol level after the introduction was higher than the individual median morning respectively afternoon cortisol level in PRE plus 1.5 interquartile range. To analyze the effect of the transport on the diurnal variation, we used the same approach as

in the analysis of the baseline diurnal variation on the individual level (see Section 2.4.1). First, we applied the Wilcoxon signed-rank test with Bonferroni-correction to compare morning with afternoon levels separately for the transport phases PRE and POST1. Second, we calculated the average percentage diurnal variation with formula 1 and compared the values of the three transport phases. As not all morning and afternoon samples were paired, that is on some sampling days either a morning or an afternoon sample was collected from an animal, we determined the percentage diurnal variation based on morning and afternoon individual median cortisol levels.

Average salivary cortisol levels, respectively, percentage differences are reported as median (25% quartile, 75% quartile). The LMM and calculation of medians and exclusive quartiles were conducted in R Studio (version 1.2.1335). Shapiro–Wilks and Wilcoxon signed-rank tests were carried out in IBM SPSS (version 24). The significance level was $p < .05$. If $.05 \leq p \leq .09$, the tendency for significance is reported.

3 | RESULTS

3.1 | Baseline diurnal cortisol variation

The effect of daytime on salivary cortisol levels was highly significant when controlling for the effect of the study period and individual variation (Table 3). On average, salivary cortisol levels were lower in the afternoon than in the morning (Figure 1).

The same result was found on the individual level in most animals. In 2016, one animal (SW) was excluded from the statistical analysis due to low sample size ($n = 3$). From the remaining nine animals, seven animals had significantly higher morning than afternoon cortisol levels in 2016 (AR: $Z = -1.527$, $p = .128$, $n = 7$, ZI: $Z = -2.201$, $p = .028$, $n = 6$, TA: $Z = -2.023$, $p = .043$, $n = 5$, SA: $Z = -1.992$, $p = .046$, $n = 6$, TI: $Z = -1.997$, $p = .046$, $n = 6$, TU: $Z = -0.944$, $p = .345$, $n = 5$, SF: $Z = -2.073$, $p = .038$, $n = 9$,; CH: $Z = -2.547$, $p = .011$, $n = 9$; KB: $Z = -2.201$; $p = .028$; $n = 6$; Figure 2). In 2017, salivary cortisol levels significantly decreased from morning to afternoon in eight animals (AR: $Z = -2.117$, $p = .034$, $n = 7$, ZI: $Z = -2.366$, $p = .018$, $n = 7$, TA: $Z = -2.366$, $p = .018$, $n = 7$, TI: $Z = -2.023$, $p = .043$, $n = 5$, TU: $Z = -2.207$, $p = .027$, $n = 6$, SF: $Z = -2.310$, $p = .021$, $n = 9$, CH:

TABLE 3 Fixed effects resulting from the linear mixed model examining the effect of daytime (morning, afternoon) and study period (2016, 2017) on salivary cortisol concentrations ($n = 266$)

Fixed effect	Estimate	SE	df	t	p
Intercept	0.427	0.055	35.257	7.765	**<.001**
Daytime	−0.350	0.024	196.089	−14.465	**<.001**
Study period	−0.115	0.024	200.527	−4.750	**<.001**

Note: Animal-ID ($n = 10$) and sampling day nested in animal-ID ($n = 72$) were included as random effects. Bold values indicate statistical significance ($p < .05$) shown by t tests (Satterthwaite's method).
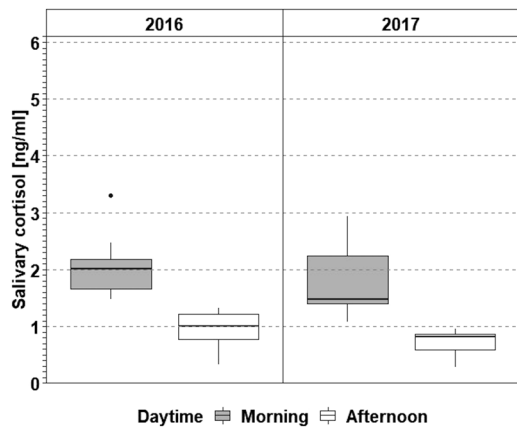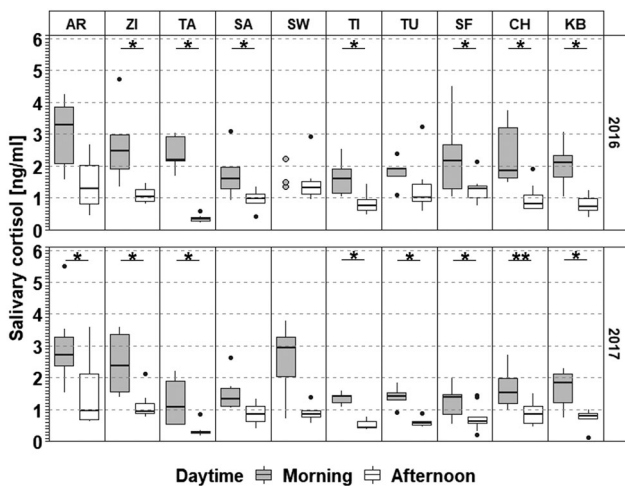
FIGURE 1 Difference between the average morning and afternoon salivary cortisol level in the first (2016, n = 10, left) and second study period (2017, n = 10, right). The boxes illustrate the 25% and 75% quartiles, bars indicate medians, the black dot indicates one outlier

$Z = -2.666$, $p = .008$, $n = 9$, KB: $Z = -2.201$, $p = .028$, $n = 6$; Figure 2) and showed a tendency for a significant decrease in the two remaining animals (SA: $Z = -1.753$, $p = .080$, $n = 5$, SW: $Z = -1.753$, $p = .080$, $n = 5$).

Table 4 shows the average percentage diurnal variation of each animal. The values respectively averages that corresponded to a nonsignificant, potentially absent, diurnal variation ranged from -7.05% and -54.42% [SW, 2016] to 245.24% (-2.14%, 445.88%) [SW, 2017].

FIGURE 2 Difference between the morning and afternoon salivary cortisol level of each animal in the first (2016, top) and second study period (2017, bottom). The boxes illustrate the 25% and 75% quartiles, bars indicate medians, black dots indicate outliers. Sample sizes are given in Table 1. In SW in 2016, single values instead of a boxplot are depicted and no statistical test was conducted due to low sample size. Significance level $p < .05$, stars indicate significance: *$p < .05$, **$p < .01$

TABLE 4 Average percentage diurnal variation (median [25% quartile, 75% quartile], n = sample size) of each animal in the first (2016) and the second (2017) study period

| Animal-ID | Study period | |
	2016	2017
AR	225.00% (−30.08%, 407.14%) n = 7	143.75% (57.14%, 338.71%) n = 7
ZI	144.51% (56.58%, 242.30%) n = 6	115.38% (39.22%, 276.19%) n = 7
TA	500.00% (404.52%, 837.97%) n = 5	200.00% (55.56%, 685.71%) n = 7
SA	74.86% (30.47%, 205.70%) n = 6	83.87% (15.59%, 242.33%) n = 5
SW	−7.50% (−54.42%, 113.46%)[a] n = 3	245.24% (−2.14%, 445.88%) n = 5
TI	135.33% (23.68%, 179.67%) n = 6	145.45% (117.08%, 224.16%) n = 5
TU	22.78% (−17.49%, 178.37%) n = 5	169.90% (97.23%, 193.33%) n = 6
SF	57.81% (12.92%, 195.66%) n = 9	75.00% (14.24%, 183.97%) n = 9
CH	171.01% (59.29%, 327.47%) n = 9	86.29% (39.12%, 141.68%) n = 9
KB	185.55% (81.48%, 278.15%) n = 6	161.67% (57.63%, 276.96%) n = 6

Note: Italic values indicate diurnal variation was not significant.
[a]Median (minimum, maximum).

3.2 | Cortisol in relation to translocation (stressor)

Figure 3 depicts the morning and afternoon cortisol level in PRE and POST1. The Bonferroni-corrected Wilcoxon test showed no significant difference between PRE and POST1 salivary cortisol levels at both daytimes in all cows but a tendency for significantly higher afternoon cortisol levels in POST1 than in PRE in CH (CS: morning: $Z = -0.314$, $p = 1.000$, $n = 6$; afternoon: $Z = -1.992$, $p = .093$, $n = 6$; SF: morning: $Z = -0.631$, $p = 1.000$, $n = 6$, afternoon: $Z = -1.153$, $p = .498$, $n = 6$; CH: morning: $Z = -0.943$, $p = .691$, $n = 6$; afternoon: $Z = -2.207$, $p = .055$, $n = 6$).

Figure 3 shows the cortisol concentrations of the three cows after the introduction to the bull. The introduction of CS to the bull
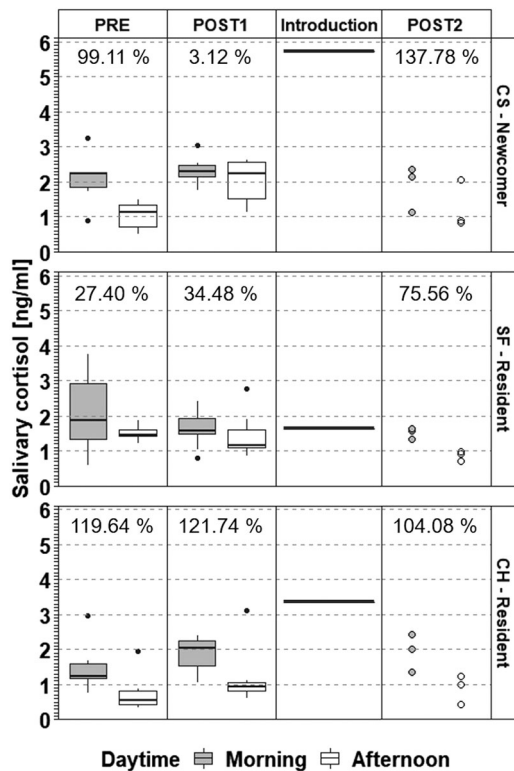
FIGURE 3 Morning and afternoon salivary cortisol level and median percentage difference between them in the newcomer cow (CS, top) and the two resident cows (SF, middle, CH, bottom) before the transport (PRE), after the transport between Day 1 and Day 9 (POST1) and between Day 21–23 (POST2), as well as the salivary cortisol concentrations of these animals after the first introduction of CS to the bull on Day 20 (Introduction). The boxes illustrate the 25% and 75% quartiles, bars indicate medians, black dots indicate outliers. Sample sizes are given in Table 2

resulted in a significant cortisol increase in CH and in CS compared to both morning and afternoon levels in PRE. In SF, cortisol was not significantly elevated after the introduction. Compared to afternoon PRE cortisol levels, CH and CS responded with a 496.43%, respectively, 410.71% increase to CS's introduction to the bull, whereas SF did hardly respond (12.33% increase). The response relative to the morning PRE cortisol level was lower than to the afternoon PRE cortisol level with a 171.54% increase in CH, a 156.50% increase in CS and no increase in SF.

Except for the strong tendency for a significant difference in CS before the transport (CS: PRE: $Z = -2.201$, $p = .055$, $n = 6$), morning and afternoon cortisol levels did not differ significantly in both PRE and POST1 in all cows (CH: PRE: $Z = -1.992$, $p = .093$, $n = 6$, POST1: $Z = -1.690$, $p = .182$, $n = 7$; CS: POST1: $Z = -0.943$, $p = .691$, $n = 6$; SF: PRE: $Z = -0.943$, $p = .691$, $n = 6$, POST1: $Z = -0.845$, $p = .796$, $n = 7$).

As shown in Figure 3, the percentage diurnal variation was slightly higher in POST1 than in PRE in the residents. CS, in contrast, showed a remarkably lower percentage diurnal variation in POST1 than in PRE. The percentage diurnal variation of POST2 was higher than in PRE and POST1 in CS and SF but lower than in the previous transport phases in CH.

4 | DISCUSSION

4.1 | Baseline diurnal cortisol variation

The present study found a significant diurnal variation of salivary cortisol levels when controlling for individual variation and the effect of the study period. The average cortisol level was higher in the morning than in the afternoon, which is in accordance with previous studies of the diurnal variation of cortisol in captive African elephants (Casares et al., 2016; Grand et al., 2012; Kelling Swilley, 2008) and numerous other mammal species (Aurich et al., 2015; Ekkel et al., 1996; Kutsukake et al., 2009; Suzuki et al., 2003). Generally, the high HPA-axis activity in the morning is considered as an anticipatory response to awakening (Born, Hansen, Marshall, Mölle, & Fehm, 1999; Moore-Ede, 1986). In the present study, the high morning cortisol level may presumably reflect the anticipation of management events occurring in the morning, which, therefore, may function as additional cues that entrain the diurnal cortisol rhythm (reviewed in Kalsbeek et al., 2012). These included feeding, shifting to outdoor enclosures, and changes in group composition.

The study period had a highly significant effect on the cortisol level. Assay drift may have accounted for a small proportion of this difference between study periods. Although the assay was stable across time, indicated by an average inter-assay CV value <10% across the two study periods, in 2016 quality control values were measured about 10% higher than in 2017/2018. Thus, sample concentrations may have been measured as well about 10% higher in 2016 than in 2017/2018. Furthermore, the sampling procedure might have produced a cortisol increase, which might have been lower in 2017 due to habituation to the procedure (reviewed in Grissom & Bhatnagar, 2009). Moreover, the management alterations in 2017 (see Section 2.1) may have been associated with an increase in environmental complexity (e.g., feeding on the ground to feeding from a rack), which was shown to lower cortisol levels (Carlstead, Brown, & Seidensticker, 1993). In addition, in Wuppertal, the keepers reported that the aggression in the group was higher in 2016 due to aggressive interactions between one cow and a young bull, which may have increased the average baseline cortisol level in this year (Alberts, Sapolsky, & Altman, 1992; Muller & Wrangham, 2004; Parrott & Misson, 1989). The temporary separation of the young bull reduced the aggression in the herd considerably, which may have contributed to overall lower cortisol levels in 2017.

On the individual level, most animals exhibited a significant diurnal variation in both study periods. The lack of a significant diurnal variation may have reflected a flat diurnal rhythm in the remaining animals. Flat diurnal rhythms, that is a low difference between morning and afternoon cortisol levels, may indicate compromised welfare due to chronic stress or disease (reviewed in Heim, Ehlert, & Hellhammer, 2000 and Oster et al., 2017). However, data must be interpreted with caution in this regard, as individuals differ in diurnal rhythms and flat rhythms do not necessarily reflect stress- or disease-related conditions (Cordero, Brorsen, & McFarlane, 2012; Smyth et al., 1997; Stone et al., 2001). In addition, assessing repeatability of the diurnal cortisol rhythm (Selmaoui

& Touitou, 2003) is important to distinguish between transient and stable flat rhythms and to ensure its validity as a potential welfare indicator (Stone et al., 2001). In the present study, we considered the flatness or loss of the expected diurnal variation in some animals as rather transient than permanent and assumed a statistical explanation for the non-significance of the diurnal variation. Despite nonsignificance, the average percentage diurnal variation was still positive in the respective animals. This means, individual average cortisol levels still followed the expected diurnal pattern with lower cortisol levels in the afternoon. The lack of significance was presumably based on one or two sampling days in the individual samples when the morning-afternoon difference was reversed with morning levels being lower than afternoon levels (i.e., a negative percentage diurnal variation). Therefore, it is highly likely that a larger sample size would have increased the power of the statistical test by balancing the variation between sampling days, that is the within-individual variation. Within-individual variation in salivary cortisol concentrations can arise from the cortisol responses to different acute stressors, which may have occurred 5–20 min (the time lag between peak cortisol levels in the blood and in saliva, Hernandez et al., 2014; Kirschbaum & Hellhammer, 2000) before sampling. In the present study, the animals were roaming in the enclosure at that point in time, which made it difficult to control for differences in the pre-sampling situation.

4.2 | Cortisol in relation to translocation

Although transports are evidently stressors producing cortisol elevations in different species including African elephants (Millspaugh et al., 2007; Viljoen et al., 2008), the salivary cortisol levels before (PRE) and directly after the transport (POST1) did not differ significantly in the present study. The tendency for a higher afternoon level in POST1 than in PRE was found in CH though. It is highly likely that the present study failed to find the predicted significant elevations after the transport due to the inability of collecting samples immediately after CS's arrival. In other studies, increased levels of fecal cortisol metabolites were found 6–48 hr after the transport depending on the digestion time of the species and the sampling interval (Goymann et al., 1999; Laws et al., 2007; Morrow, Kolver, Verkerk, & Matthews, 2002; Schmidt et al., 2010a, 2010b, 2010c). Salivary cortisol levels, in contrast, were reported to increase during the transport and to decrease within a few hours after the transport (Schmidt et al., 2010a, 2010b, 2010c). Unfortunately, the animals in the present study were not willing to cooperate immediately after CS's arrival, which made prompt saliva sampling impossible. Rather than reflecting the transport itself, sporadically elevated cortisol levels in POST1 could have been responses to acute stressors during the familiarization of the newcomer with the new enclosure and group (Alexander & Irvine, 1998; Carlstead et al., 1993; Dathe, Kuckelkorn, & Minnemann, 1992; Irvine & Alexander, 1994; Laws et al., 2007; Morrow et al., 2002).

The results regarding the introduction of CS to the bull supported the need for prompt sampling when measuring the salivary cortisol response. In this case, we were able to sample immediately after the stressor and found a marked increase in the cortisol levels of CS and CH. Similar results have been obtained in Asian elephants

(Dathe et al., 1992). SF already gained experience in stressors associated with transports and introductions, which may have explained her low cortisol response compared to the other resident CH (Dembiec et al., 2004; reviewed in Koolhaas et al., 2011).

The lack of a significant diurnal cortisol variation in PRE was unexpected considering that respective samples were collected 2 to 3 weeks before the translocation event, which makes an effect of management changes due to the upcoming transport unlikely. The lack of significance may be related to the statistical approach used. When uncorrected for multiple comparisons, the Wilcoxon test reached significance regarding the diurnal variation in PRE in CS and CH, but not in SF, whereas the diurnal variation in POST1 remained not significant in all animals. Furthermore, a larger sample size and a more balanced sampling design (i.e., consistently paired morning and afternoon samples) would have potentially lowered variability between samples clarifying the diurnal variation.

The minimal percentage diurnal variation between median morning and afternoon cortisol levels in POST1 indicated an effect of the transport on the diurnal variation in the newcomer CS. Based on comparisons between PRE, POST1 and POST2 in this animal and the residents, this flattening of the diurnal variation resulted from the elevation of the afternoon level, which was potentially caused by the changes in housing conditions (e.g., Irvine & Alexander, 1994). The increase of the percentage diurnal variation in POST2 may have indicated a normalization of the diurnal cortisol rhythm. Thus, the potential flattening of the diurnal rhythm in POST1 could be considered as a transient phenomenon reflecting acute stress. In both residents, the percentage diurnal variation in POST1 was slightly higher than in PRE making an effect of the transport rather unlikely. In POST2, the percentage diurnal variation of SF increased and approached the values in the baseline condition, which may have indicated a normalization of the diurnal variation like in CS. The percentage diurnal variation of CH decreased in POST2, but, like the values in PRE and POST1, it was still in the range of those during baseline management.

5 | CONCLUSION

The present study demonstrated that salivary cortisol levels of captive African elephants follow the expected diurnal variation characterized by high morning and low afternoon levels during baseline conditions (routine management). Salivary cortisol was sporadically but not significantly higher after the transport. Still, the translocation may have been a potential stressor for the transported animal, as the diurnal variation was absent based on elevated afternoon levels after the transport compared to before the transport. The first introduction of the transported animal to the bull, in contrast, produced significant cortisol increases. Furthermore, flattening of the diurnal variation was considered as transient both during baseline and in relation to the transport. Therefore, salivary cortisol is a useful minimally invasive measure of physiological stress provided that the animals are willing to cooperate and samples can be collected during or immediate (i.e., within a few hours) after the stressor.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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