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Pregnancy length and health in giant pandas: What can metabolic and urinary endocrine markers unveil?

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ABSTRACT

Mature female giant pandas usually ovulate once a year. This is followed by an obligatory luteal phase, consisting of a long-lasting corpus luteum dormancy phase (CLD; primary increase in progestogens) and a much shorter active luteal phase (AL; secondary increase in progestogens). Varying duration of both the dormant (embryonic diapause) and AL (post-embryo reactivation) phases has hampered unambiguous pregnancy length determination in giant pandas until today. Additionally, progestogen profiles have been considered not to differ between pregnant and pseudopregnant cycles. Only ceruloplasmin, 13,14-dihydro-15-keto-PGF$_{2\alpha}$ (PGFM) and – more recently – estrogens have been assigned diagnostic power so far. Our study investigated the competence of

Abbreviations: CLD, corpus luteum dormancy; AL, active luteal; PGFM, 13,14-dihydro-15-keto-PGF$_{2\alpha}$ (PGFM); USpG, Urinary Specific Gravity; GCM, glucocorticoids; AI, artificial insemination; LOD, limit of detection; LOQ, limit of quantification; SD, standard deviation; CV, coefficient of variation; CL, corpus luteum.

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metabolic (fecal output) and Urinary Specific Gravity (USpG)-normalized urinary endocrine (progestogens, PGFM, glucocorticoids (GCM) and ceruloplasmin) markers for pregnancy monitoring including defining the duration of the AL phase length. Research on 24 (6 pregnant, 8 pseudopregnant and 10 non-birth) cycles of 6 giant pandas revealed a fixed AL phase length of 42 days in giant pandas, e.g. representing 6 weeks of post-diapause development in case of pregnancy. Progestogen concentrations were significantly higher in pregnant cycles throughout the majority of the AL phase, with significant higher values during the AL phase in healthy twin compared to singleton pregnancies. GCM concentrations were also markedly higher in giant pandas expecting offspring, with a clear increase towards birth in the final 2 weeks of pregnancy. This increase in GCM was running in parallel with elevating estrogen and PGFM concentrations, and decreasing progestogens. In addition, during the AL phase, a more pronounced decrease in fecal output was obvious for pregnant females. The combined profiles of non-invasive metabolic and endocrine markers, the latter normalized based on USpG, showed a true pregnancy signature during the AL phase. The findings of this study are applicable to retrospective evaluations of non-birth cycles facilitating categorizing those into pseudopregnant or lost pregnancies, with USpG-normalization of the urinary endocrine markers as a prerequisite.

1. Introduction

Giant pandas (*Ailuropoda melanoleuca*) are charismatic bears and flagship representatives of the biodiversity of the People's Republic of China. Due to their conservation status, e.g. formerly 'endangered' and more recently downgraded to 'vulnerable' on the IUCN red list of threatened species [1], national and international research groups have been collaborating successfully to disclose essential information on their reproductive biology with the aim to assist in giant panda breeding, both in situ and ex situ [2,3].

Non-invasive endocrine methods in urine and feces were established decades ago to monitor ovarian function and pregnancy in this species. Radio and enzyme immunoassays (RIAs and EIAs) focusing on estrogen- and progestogen- metabolites have been methods of choice to monitor both the follicular and luteal phases of the estrous cycle [4–17].

In female giant pandas, this follicular phase is described to last on average 7–14 days and is characterized by a change in behavior (e.g. waterplay, increased activity, scent marking and specific vocalizations) [14], physiology (e.g. reddening and swelling of vulva) [18] and hormone levels (predominantly increase in estrogens) [4,6,8,15] (Fig. 1 A and Supplementary figure 1).

The start of the luteal phase is announced by a modest increase in progestogen levels, which remains relatively stable during 2–3 months. This period was previously defined as the primary progestogen rise or the corpus luteum dormancy phase (CLD) and corresponds to the period of diapause in pregnant cycles [12]. A subsequent abrupt acceleration in progestogen concentrations is the typical signature for the onset of the secondary progestogen rise or active luteal (AL) phase. This is the phase in which the embryo and membranes resume development in pregnant individuals [12]. All mature female giant pandas, whether bred or not, show this biphasic progestogen profile after ovulation. Giant pandas are thus obligate pseudopregnant in the absence of conception [19] (Fig. 1 A and Supplementary figure 1).

The occurrence of embryonic diapause and pseudopregnancy are compromising pregnancy diagnosis in giant pandas. Indeed, until recently, progestogen profiles have been reported to be non-discriminative between pregnant and non-pregnant cycles [12,20–22]. However, three other markers have been suggested as potential pregnancy markers. Kersey et al. [10,23,24] investigated fecal glucocorticoid metabolites (fGCM) during different reproductive phases and compared pregnant and non-pregnant cycles. Averaged higher fGCM values were reported during the AL phase of pregnancy. Willis et al. (2011) [25] described the use of the acute phase reactant ceruloplasmin as an early (CLD) and late (AL) pregnancy marker in giant panda urine, with elevated values from 1 week after ovulation until 20–24 days prior to parturition. Roberts et al. (2018) [26] elaborated on the urinary prostaglandin $F_{2\alpha}$-metabolite 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) as a late (AL) pregnancy marker and – for the first time – offered a tool to predict the day of birth in pregnant cycles, e.g. 23–25 days after a first luteolytic peak in PGFM. Although a late gestational rise in estrogens was mentioned by Hodges et al. in 1984 [8], potentially reflecting placental production and/or fetal/maternal biosynthesis, this was not confirmed in more recent studies investigating the diagnostic potential of estrogens during pregnancy [27]. A major game-changer however, was the introduction of urinary specific gravity (USpG) for urinary concentration normalization instead of creatinine, introduced for giant panda reproductive monitoring in 2018 [28]. Urinary creatinine (Cr) has been universally accepted as 'gold standard' because of its constant daily excretion rate [29,30]. Consequently, Cr was used for urinary analyte correction in all species where urinary hormones have been analyzed so far, including giant pandas [4–6,8,10,13,14,25–27,31,32]. However, Cr is a by-product of muscle metabolism cleared via the kidneys through urinary excretion [33]. Despite previously being described with a constant excretion, there are contradictory studies suggesting Cr varies in relation to sex, age, weight, diet, exercise, race, injury and stress [29,33–40]. Changes in sex steroid hormones can also be a factor in regulating potential drivers of Cr modulation, including appetite, activity levels and metabolic status, thus potentially altering urinary Cr excretion [37,38,41–49]. In addition, pregnancy hormones such as progesterone and relaxin alter reabsorption, filtration and osmotic regularity functions of the kidney [46,50,51]. Moreover, maternal adaptations to pregnancy include changes to renal anatomy and function. This suggests a number of limitations to using Cr for urinary analyte correction for reproductive monitoring. In a former study validating USpG-normalization as an alternative to creatinine, we clearly demonstrated significant deviation between urinary longitudinal profiles corrected by USpG versus creatinine corrected data during estrus and in the two to three weeks prior to onset of the AL phase (as defined by Wauters et al., 2018 [28]). These metabolic shifts in the creatinine-corrected profile have been hampering an unambiguous definition of the onset and duration of the AL, e.g. the post-embryo reactivation period, in giant pandas. In addition, recently, USpG-corrected estrogen profiles – but not creatinine-corrected - during late AL phase were found discriminative for pregnancy, confirming the earlier observations by Hodges et al. (1984) [8,52].

In response to the highlighted pitfalls in giant panda monitoring, we investigated the applicability of an easy-to-determine metabolic marker (daily fecal output) as a tool to align the onset and end of the active luteal phase, with the aim to facilitate determining the length of the post-diapause development phase and pregnancy monitoring/diagnosis in giant pandas. Using this novel alignment strategy, we investigated 4 endocrine urinary markers normalized by USpG, with a focus on their potential for pregnancy diagnosis and monitoring during the AL phase. Progestogens, PGFM, glucocorticoids and ceruloplasmin were analyzed for 24 cycles (6 pregnancy, 8 pseudopregnancy and 10 non-births) from 6 female giant pandas housed across Europe. We compared our findings to previous reports based on conventional creatinine correction of urinary concentrations.

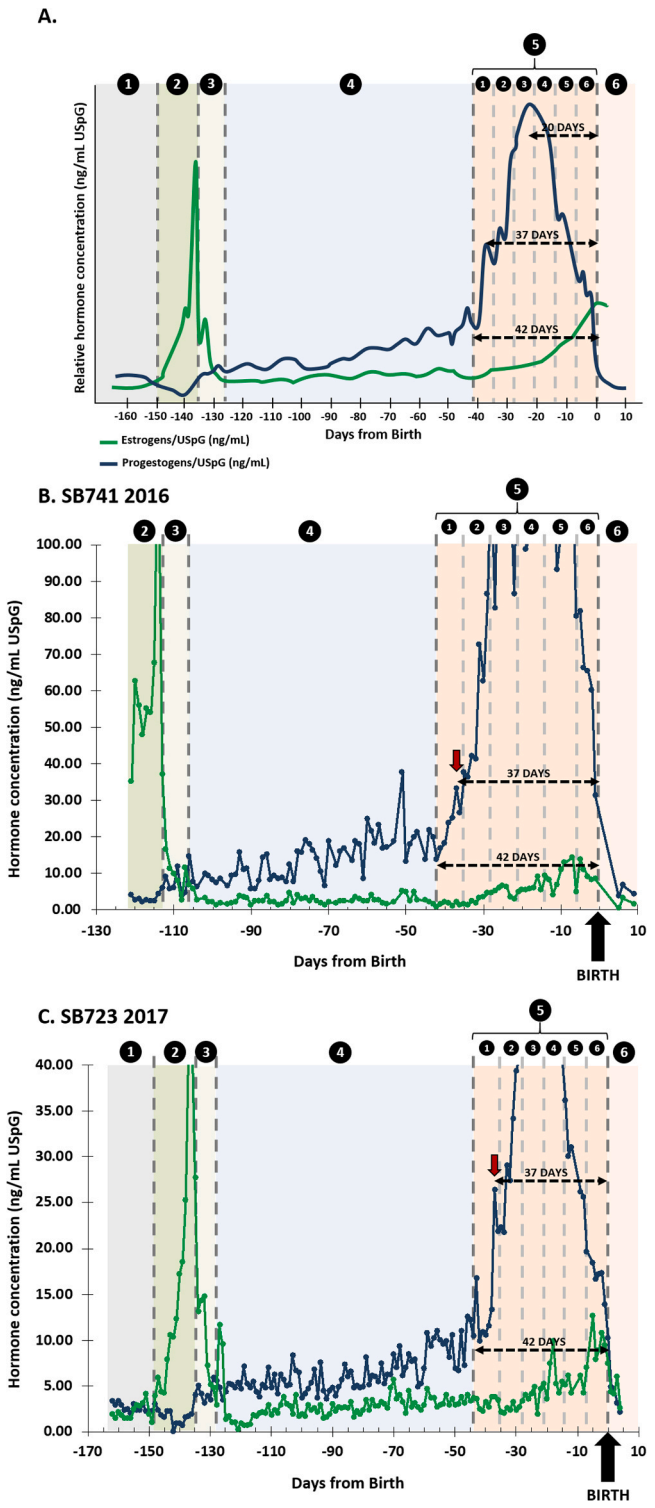

Fig. 1. (A) Schematic overview of the different periods of interest in the female giant panda reproductive cycle. (B) SB741 2016 pregnancy cycle divided by the different periods of interest. (C) SB723 2017 pregnancy cycle divided by the different periods of interest. (1 = anestrus; 2 = pro-estrus; 3 = postestrus; 4 = corpus luteum dormancy phase (CLD); 5 = active luteal phase (AL), further divided into 6 weekly periods; 6 = post-end-of-cycle). The black arrow indicates the day of birth, the red arrow corresponds to D-37/D-36.

2. Material and methods

2.1. Animals

Six zoological institutions provided metabolic data (fecal output, kg) and urine samples for the reproductive monitoring of their resident female giant pandas. 24 cycles (2013–2021) of 6 pandas were included in this study (Table 1). 2618 luteal phase urine samples (n = 109 ± 5.8 per cycle) were analyzed, including 867 samples (n = 36 ± 2.3 per cycle) from the AL phase. Cycles included six pregnancies, eight pseudopregnancies (non-bred cycles) and ten non-birth cycles (AI without a cub), summarized in Supplementary Table 1.

Urine samples (n = 34) from a male giant panda, SB564, housed at RZSS Edinburgh Zoo were also included as part of the glucocorticoid EIA validation.

Each female giant panda was housed near 1 male giant panda, in most cases allowing auditory, olfactory and visual contact. Physical contact was possible in most cases during the time of estrus. The giant pandas were accommodated based on best practice guidelines for animal husbandry as well as recommendations given by the respective supporting Chinese giant panda experts. Giant pandas had ad libitum access to water and were fed a diet consisting of mainly bamboo supplemented with protein rich cake or biscuits, apples and carrots.

The pandas had free access to an indoor (ambient humidity; average temperature 18 °C) and outdoor enclosure. More details on the giant pandas' husbandry is summarized in Supplementary Table 2.

All animal-related work has been conducted according to relevant national and international guidelines and no specific ethical approval was mandated for this study.

2.2. Non-invasively obtained records of metabolic factors

For all 6 zoological institutions, daily fecal output was recorded by weighing (kg) the amount of feces produced, and values were disclosed at least for the duration of the reproductive cycle, e.g. from pro-estrus until parturition or the end of the AL phase.

2.3. Urine samples

2.3.1. Non-invasive urine sampling

Urine was aspirated from the ground with a clean syringe and transferred to a collection vial [28]. Sample collection was pursued daily at least from pro-estrus until parturition or end of cycle.

Frequently, samples were lacking during critical periods, such as the transition from CLD phase into AL phase, as well as during weeks 2–4 of the AL phase (Supplementary Table 3). During the pro-estrus and the presumed prepartum period, numerous samples were collected throughout the day and immediately analyzed (including USpG normalization) at the zoos' facilities. In such cases, daily averages were used to generate single representative outcomes. All other samples were first stored at − 20 °C after sampling until analysis at the laboratories (see below).

2.3.2. Urinary Specific Gravity

Urinary hormone metabolite concentrations were determined after normalization by Urinary Specific Gravity (USpG) [28].

USpG was measured as previously described using a handheld digital refractometer (ATAGO, Japan), designed either for human (PAL-10S, range 1.000–1.060) or cat urine (PAL-USG (Cat), range 1.000–1.080). The use of either refractometer for the measurement of giant panda USpG has been validated previously. The same refractometer was used for all samples of the same panda. Briefly, deionized water (300 μL) calibrated the instrument prior to the measurement of USpG of the sample (300 μL). Hormones were corrected for USpG as described for giant pandas, making use of the formula described by Miller et al. (2004) [53].

Table 1
Population details on the female giant pandas included in this study (D.O.B. = date of birth). (n.a. = not applicable).

Zoological Institution	Latitude	Studbook nr	Animal ID	D.O.B.	Pregnant	Pseudo pregnant	Non-birth
Edinburgh Zoo – RZSS	55.95°N	SB569	Tian Tian	24/08/2003	n.a.	2018, 2020	2013–2017, 2019, 2021
Zooparc de Beauval	47.59°N	SB723	Huan Huan	10/08/2008	2017, 2021	n.a.	2016, 2020
Pairi Daiza	50.60°N	SB741	Hao Hao	7/07/2009	2016, 2019	n.a.	2018
Ouwehand	51.96°N	SB884	Wu Wen	5/08/2013	2020	2017–2019	n.a.
Berlin Zoo	52.52°N	SB868	Meng Meng	10/07/2013	2019	n.a.	n.a.
Ähtäri Zoo	62.55°N	SB941	Jin Baobao	21/09/2014	n.a.	2019–2021	n.a.

2.4. Non-invasive urinary endocrine monitoring

The analysis of progestogens, PGFM and ceruloplasmin were shared between 3 laboratories. Urine samples of SB569 and reproductive years 2017 and 2018 of SB884 were analyzed at the MRC Centre for Reproductive Health, University of Edinburgh, Edinburgh, United Kingdom. Samples of SB741, SB723, SB884 (2019, 2020) and SB941 were analyzed at the Laboratory of Integrative Metabolomics, Ghent University, Belgium, whereas samples of SB868 were partially analyzed by the latter lab and largely by the Department of Reproduction Biology, Leibniz Institute for Zoo and Wildlife Research, Germany, rigorously adhering to the methodologies and guidelines.

All glucocorticoid metabolite (GCM) analysis was performed by the MRC Centre for Reproductive Health, University of Edinburgh, Edinburgh, United Kingdom.

2.4.1. Progestogens

Urinary progestogens were assessed using the Arbor Assays Progesterone DetectX® Enzyme Immunoassay (EIA) kits (K025-H5; Arbor Assays™, Ann Arbor, Michigan, USA) as previously described [28,52]. Samples were measured at x10 dilution in assay buffer during the CLD, dilutions of at least x100 to x500 were required during the AL. Inter- and intra-assay coefficients of variation (CV; averaged between labs; all individual lab CVs were below 15% and 10%, respectively) were calculated as 13.8% and 2.0%, respectively.

2.4.2. PGFM

Urinary PGFM was assessed using the Arbor Assays PGFM DetectX® Enzyme Immunoassay (EIA) kits (K022-H5; Arbor Assays™) as previously described [28,52]. Samples were measured at x10 dilution in assay buffer during the CLD and at the beginning of the AL, dilutions of at least x100 to x500 were required across the progression of the AL. Inter- and intra-assay coefficients of variation (CV; averaged between labs; all individual lab CVs were below 15%) were calculated as 11.1% and 11.3%, respectively.

2.4.3. Glucocorticoids

2.4.3.1. Glucocorticoid EIA validations. Two glucocorticoid EIAs, an assay designed for detection of cortisol and another for the detection of corticosterone, were selected for biological validation. The objective was to identify the assay best qualified for urinary GCM monitoring in giant pandas. The biological validation was performed with carefully selected urine samples (before and after a stressful event) from the male giant panda SB564. In July 2019, this panda was moved to a new enclosure across the site at RZSS Edinburgh Zoo and during the 'settling in' period accidentally brushed against the electric fence (an electric fence was not present in the previous enclosure due to a different outdoor design). Keepers reported that after the incident the male had a loud vocal reaction and showed an obvious 'flight' response by running inside. The male remained nervous and exhibited some alarm calling for at least 2 days following the incident and was still sensitive to noise for a further 2 days. This incident was thus identified as a potential stressor activating the HPA axis. Samples were analyzed with both assays, and the best performing assay (e.g. the cortisol assay) was selected for further GCM determinations in female samples (See Supplementary results, Supplementary Table 4 and Supplementary figure 11).

Subsequently, the 'cortisol' EIA was analytically validated through several validation experiments (See supplementary results, Supplementary table 2 and Supplementary figure 7). The limit of detection (LOD) was determined by running 10 wells of the blank and calculated as two standard deviations (SD) above the mean value. The limit of quantification (LOQ) was calculated by running a 96-well plate only of the standard curve (total 12 times), including two additional lower standards than typically used (0.78 and 0.36 ng/mL), plotting the percent coefficient of variation (%CV) of each standard, and determining the concentration point when the %CV was 20% or lower. At a % CV of 20%, this was regarded as the LOQ [54]. Thirty-three steroids (estrogens, progestogens, androgens and glucocorticoids) were tested on the assays to determine potential cross-reactivities (Supplementary Table 4). Three giant panda urine samples were serially diluted (neat, x2, x4, x8, x16, x32, x64) in assay buffer to determine parallelism. Six freeze-thaw cycles of both neat urine and pre-diluted urine at x5 (three urine samples) were analyzed to determine the potential effect on the readings.

2.4.3.2. Cortisol EIA. Urinary GCM was assessed using an 'in-house' developed EIA for cortisol at the University of Edinburgh. Briefly, EIA plates (96-well; Greiner Bio-One, GmbH, Germany) were coated overnight with 5.0 μg/mL goat anti-mouse IgG (A008; Arbor Assays™) in 100 mM sodium bicarbonate buffer (100 μL, 4 °C). Plates were washed twice with wash buffer (300 μL; tris buffered saline (TBS) with 0.05% Tween), then blocked with blocking buffer (220 μL; 0.5% bovine serum albumin (BSA) phosphate buffered saline (PBS)) for 1 h at room temperature. Standards (between 1.56 and 100 ng/mL prepared from powdered cortisol (Sigma-Aldrich) in blocking buffer), quality controls (also prepared from powdered cortisol (Sigma-Aldrich) in blocking buffer) and samples, diluted x5 in 0.1% BSA PBS (assay buffer), were added in duplicate to the plate (20 μL). Cortisol-horse radish peroxidase (C-HRP, 80 μL of 1:4000; #12–01, Astra Biotech, Germany) diluted in assay buffer was added and mixed briefly. Cortisol antibody (C-Ab, 50 μL; #10–10, Astra Biotech) at 0.176 μg/mL diluted in assay buffer was added, and plates were incubated (2 h, 28 °C, with shaking). After washing 4 times, 3,3',5,5'-tetramethylbenzidine (TMB; 120 μL; Millipore, UK) was added to each well for 10 min of incubation (dark, room temperature, with shaking). The reaction was stopped with a stop solution (80 μL; 1 N sulphuric acid). The absorbance was quantified at 450 nm on a LT-4500 Microplate Absorbance Reader (LabTech, Version 7 2010, Tecan Group Ltd., Switzerland). EIA readings were analyzed using a 4-Parameter Logistic (4PLC) nonlinear regression model on SoftMaxPro Software (Version 7.1, Molecular Devices, California, USA). Inter- and intra-assay CVs were 10.5% and 3.6%, respectively.

2.4.3.3. Corticosterone EIA. Urinary GCM was measured in the male giant panda urine samples by an Arbor Assays Corticosterone DetectX® EIA (K014-H1; Arbor Assays™) following the manufacturers guidelines as part of the confirmation of which GCM EIA to use for giant panda urinary GCM monitoring. Assay parallelism was tested with two serially diluted giant panda urine samples (neat, x2, x4, x8, x16), and a dilution

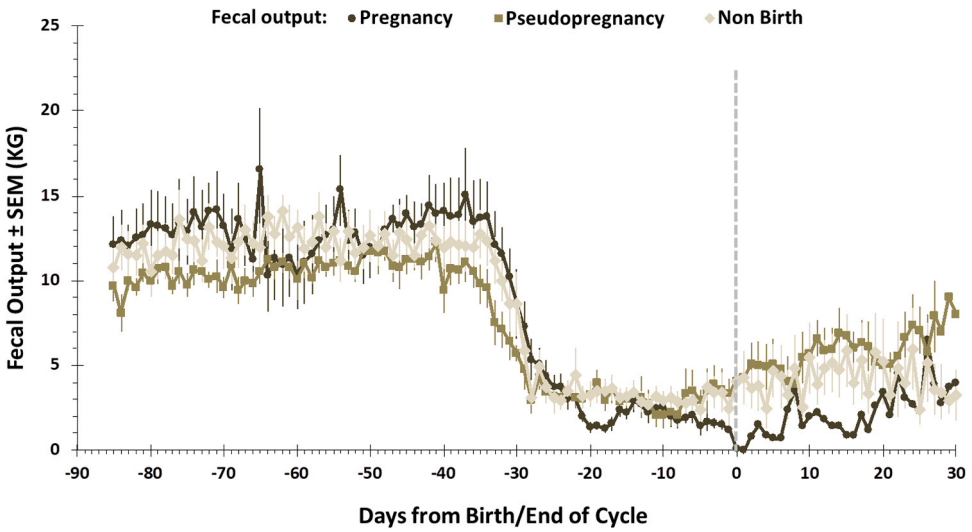

Fig. 2. : Alignment of fecal output profiles for pregnant (n = 6), pseudopregnant (n = 8) and non-birth (n = 10) cycles. The same figure including the respective progestogen profiles is available in Supplementary figure 2. Error bars = standard error of the mean (SEM).

of x5 was chosen for analysis of all samples (Supplementary figure 11). The inter-assay CV was calculated as 10.9%.

2.4.4. Ceruloplasmin

Urinary ceruloplasmin was assessed using either the Arbor Assays DetectX® Ceruloplasmin Colorimetric Activity kit (K035-H5; Arbor Assays™) following the manufacturers guidelines for analyses undertaken at Ghent University and Leibniz Institute for Zoo and Wildlife Research, or by an 'in house' method as optimized by Sunderman and Nomoto (1970) [55] and adapted for use on a Cobas Fara centrifugal analyzer for analyses undertaken at the University of Edinburgh, as previously described [28,52]. More details on the methods, including method comparison, can be consulted in Supplementary figure 12 and Supplementary methods.

2.5. Data processing

2.5.1. Definition of subperiods based on the reproductive cycle

Each giant panda cycle is typically divided into at least 6 subperiods: anestrus (start of observations until cross over between progestogens and estrogens; [28]), pro-estrus (cross-over until day of peak estrogen), postestrus (7 days following day of peak estrogen), corpus luteum dormancy phase (CLD; from 7 days after day of peak estrogen until start of AL phase), AL phase (AL; from early onset of consistent increase in progestogens until end-of-cycle) and post-end-of-cycle (from end of AL phase until max. 30 days later) (Supplementary figure 1).

The onset of the AL, which is the period of interest in this study, was defined with the help of changes in progestogen levels [12] and 'fecal output' as a metabolic marker (see below; 3.1). For non-invasive marker comparison, the AL was subsequently subdivided into 6 weekly intervals (Fig. 1 A).

2.5.2. Data analysis

In terms of data analysis, it is critical to acknowledge the unequal distribution of specific giant panda cycles into certain groups of interest, e.g. the pregnant (2 of 6 cycles each from SB723 and SB741), pseudopregnant (3/8 cycles each from SB884 and SB941) and particularly, non-birth cycles (7 of 10 cycles are from SB569) (Supplementary Tables 1 and 3). These outcomes are important, since magnitude and behavior of a specific metabolic or endocrine marker during the cycle may be influenced by giant panda maturity (adolescent versus adult), individual metabolic differences, acclimatization (newly arrived pandas versus established), climatological influences (within a cycle, but also between years) and other factors. Additionally, interactions may occur between several of those factors. Therefore, urinary biomarkers and fecal output data are presented as a mean fold change from the average CLD concentration or value of each cycle (figures based on averaged nominal concentration values can be consulted in the Supplementary figures 4–10). This was to take into consideration the natural variation in excretion levels observed across pandas and cycles, and allowed for clear patterns in biomarkers to be observed. For this, the average of each biomarker across the CLD was calculated for each cycle. The concentration/amount for each sample across the AL (and the few shown for CLD) was then divided by the average CLD concentration/amount per biomarker. Finally, this mean fold change value was used for plotting the data, statistics, and data interpretation.

For each biomarker, cycles were categorized by outcome (pregnancy, pseudopregnancy or non-birth) and the total average per outcome per week of the AL was taken. These data were deemed normally distributed (Shapiro-Wilk normality test). Differences between outcomes within weeks of the AL were then compared by two-way ANOVAs with Bonferroni's multiple comparisons. Within the pregnancy data, singleton births (n = 2) and twin births (where both cubs were surviving, n = 3) were further compared. These data were also deemed normally distributed (Shapiro-Wilk normality test), and the two groups were compared by multiple unpaired t-tests with Welch correction.

3. Results and discussion

3.1. Defining the duration of the AL phase (secondary progestogen rise)

3.1.1. Alignment of the AL phase in pseudopregnant and non-birth cycles

For this study, it was important to define the transition into the AL phase, e.g. the switch into the secondary progestogen rise, accurately. Observations in the 6 pregnant cycles permitted to define post-diapause pregnancy length in giant pandas. For these cycles, D0 was defined by the day of parturition. Based on descriptive data assessment, the early start of the transition into the AL phase was set at D-42/D-41, identified as the lowest point of progestogen concentration prior to onset of accelerated increase towards peak (D-20/D-19) in all pregnant cycles (Fig. 1 A & 3 A). A second point of confirmation was D-37/D-36, typically matching with the previously reported chosen formula by Kersey et al. (2010) with arbitrarily calculated values of onset of secondary progestogen rise (AL phase) [12].

In pseudopregnant and non-birth cycles, the definition of D0 is however disputable. Additionally, frequently lacking samples

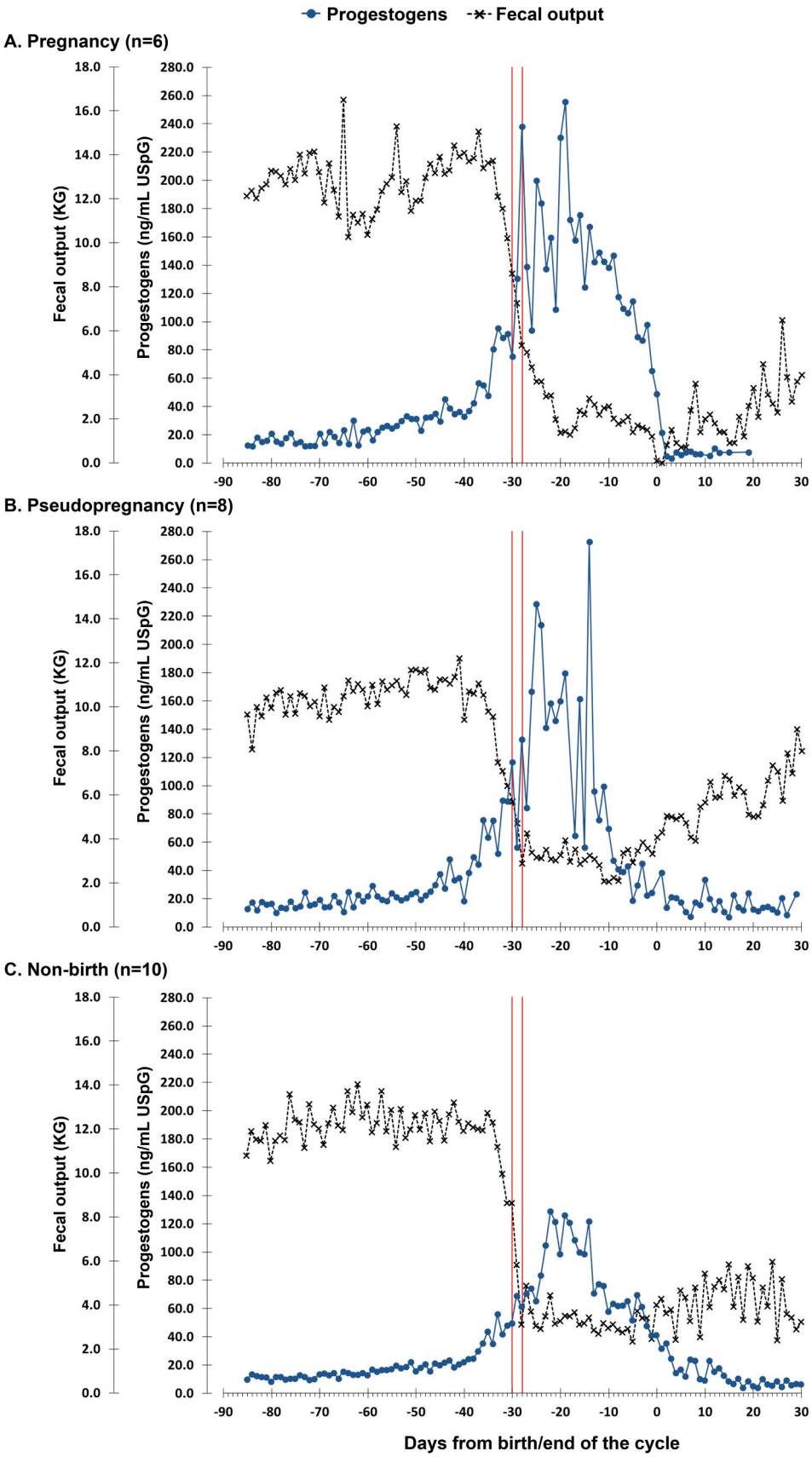

Fig. 3. : Average urinary progestogen profiles and average fecal output profiles for pregnant (n = 6), pseudopregnant (n = 8) and non-birth (n = 10) cycles in female giant pandas. Red lines at D-30 and D-28 respectively indicating timing of reduction of fecal output with approx. 50% and timing corresponding with stabilizing low levels before further decline (pregnancy) or balancing low levels (pseudopregnancy and non-birth). The same figure including error bars (standard error of the mean; SEM) is available in Supplementary figure 3.

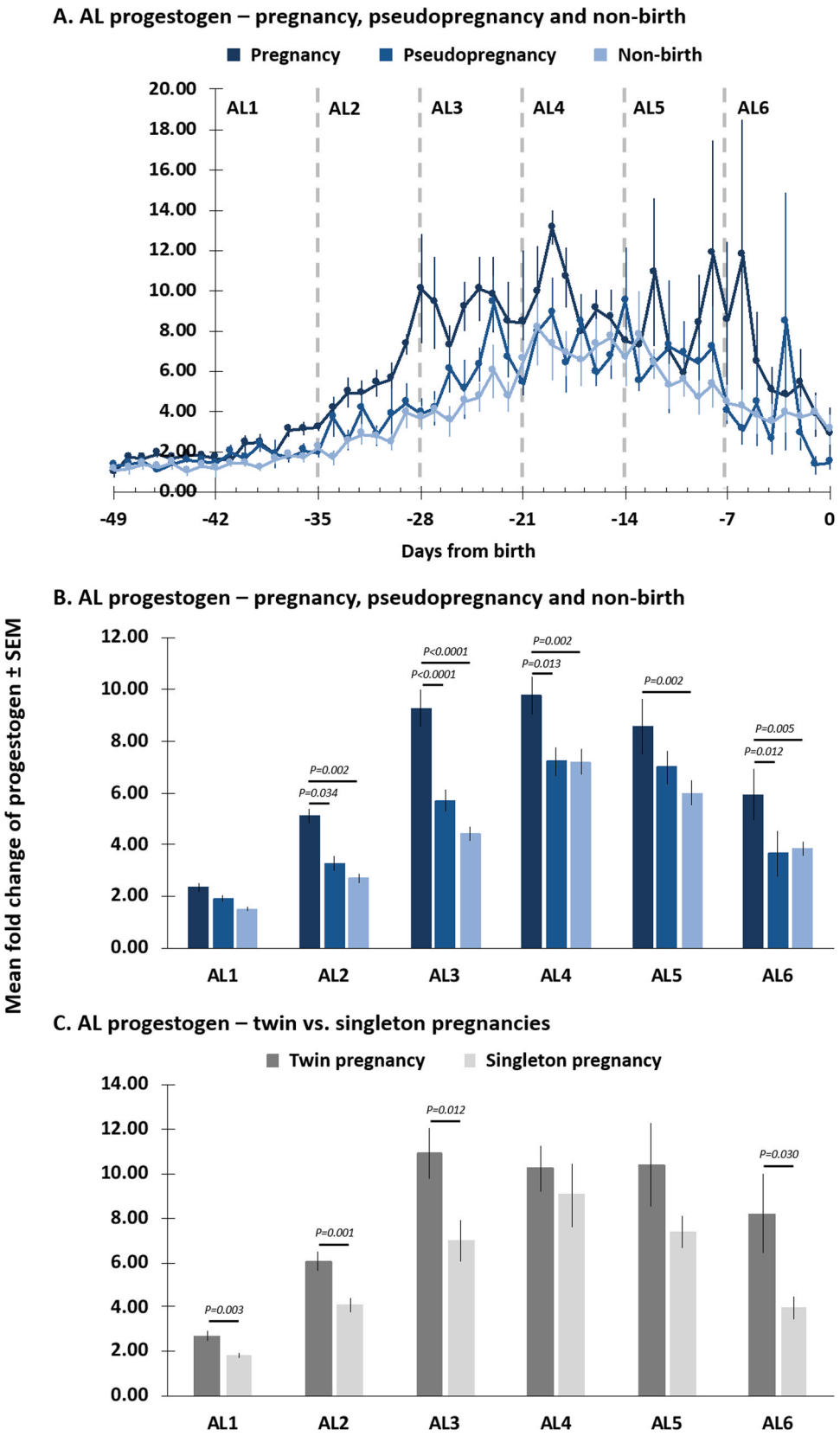

Fig. 4. : Overview of urinary mean fold change progestogen values divided over the 6 weeks of the AL phase. (A) is showing the full averaged profile of pregnant, pseudopregnant and non-birth cycles, (B) is showing the comparison between AL weeks for pregnant, pseudopregnant and non-birth cycles, (C) makes the comparison between singleton and twin pregnancies. (error bars = SEM).

Table 2
Descriptives (mean, SEM, n = number of cycles, N = number of samples) for mean fold change for pregnant, pseudopregnant and non-birth cycles for the respective endocrine and metabolic markers per week of the AL phase (note: AL 1–5 include 7 days each; AL6 has 8 days included).

		Pregnancy (n = 6)			Pseudopregnancy (n = 8)			Non-birth (n = 10)		
		Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
Progestogens	***AL1***	2.35	0.15	41	1.91	0.14	38	1.51	0.09	55
	AL2	5.12	0.29	40	3.28	0.30	44	2.70	0.20	51
	AL3	9.27	0.71	37	5.72	0.45	35	4.44	0.29	56
	AL4	9.75	0.74	32	7.21	0.53	25	7.20	0.50	55
	AL5	8.55	1.11	31	6.99	0.66	24	6.00	0.50	57
	AL6	5.93	1.00	42	3.67	0.89	31	3.86	0.30	65
PGFM	***AL1***	0.96	0.07	41	1.08	0.20	36	0.82	0.08	41
	AL2	1.21	0.10	40	1.10	0.14	44	1.21	0.14	44
	AL3	5.00	0.72	36	4.25	1.17	35	4.08	0.67	56
	AL4	13.23	1.25	30	12.45	1.62	26	12.34	1.01	55
	AL5	11.59	1.33	32	6.37	1.37	25	5.04	0.50	58
	AL6	57.54	13.81	43	2.99	0.41	31	4.36	0.38	67
Glucocorticoids	***AL1***	0.97	0.14	34	0.96	0.09	34	0.93	0.14	51
	AL2	1.28	0.22	33	0.91	0.10	38	0.71	0.10	48
	AL3	1.91	0.84	28	1.28	0.16	30	0.87	0.14	49
	AL4	1.85	0.53	22	0.60	0.10	23	1.06	0.12	48
	AL5	1.87	0.32	25	0.52	0.07	24	0.73	0.05	53
	AL6	3.37	0.58	27	0.64	0.10	29	0.87	0.12	58
Ceruloplasmin	***AL1***	1.14	0.09	41	1.57	0.22	31	0.96	0.09	48
	AL2	0.98	0.09	40	1.86	0.30	35	0.77	0.08	52
	AL3	0.50	0.05	37	1.12	0.32	27	0.47	0.06	55
	AL4	0.37	0.05	30	0.18	0.04	21	0.37	0.06	55
	AL5	0.39	0.05	30	0.32	0.10	23	0.33	0.06	57
	AL6	0.36	0.06	34	0.19	0.05	23	0.42	0.07	56
Fecal output	***AL1***	1.10	0.04	42	1.07	0.02	54	1.11	0.03	67
	AL2	0.87	0.05	42	0.93	0.04	55	0.95	0.04	66
	AL3	0.32	0.02	42	0.60	0.04	56	0.44	0.04	66
	AL4	0.15	0.01	42	0.32	0.02	46	0.29	0.02	66
	AL5	0.19	0.02	37	0.31	0.01	53	0.27	0.02	68
	AL6	0.12	0.01	42	0.25	0.02	54	0.26	0.02	73

sometimes made it challenging to identify the D-42/D-41 and the D-37/D-36 confirmation points correctly, particularly in pseudopregnant cycles. For the latter cycles, urine sample sets were often incomplete while fecal output numbers provided the most complete dataset.

Longitudinal monitoring of several giant pandas during consecutive cycles demonstrated that the early stage of AL phase revealed typical distinct changes in fecal output. During this same period, decreased activity and low bamboo intake was also observed. The decreasing fecal output was inversely related to a congruent increase in progestogen (Supplementary figure 2). This was surprising, since progesterone is associated with appetite enhancing properties in other species [56–58]. However, factors such as decreased water intake and overall hydration status, may further have compromised gut motility, consequently negatively influencing gut transition time and fecal output numbers [59,60]. A closer investigation of the daily fecal output numbers in pregnant cycles allowed defining an alignment procedure for all non-pregnant cycles (Fig. 2). More specifically, 2 reproducible points of recognition were identified. In pregnant cycles, daily fecal output was typically at half-maximum 30.10 ± 1.43 days prior to birth (= one week after the onset of accelerated increase in progestogen; 12 days after the onset of the AL). It then remained around the same level for 1–3 days, prior to a further lower plateauing point (1–3 days) at 27.60 ± 1.50 days prior to birth (Fig. 3A & Fig. 2). In pregnant cycles, fecal output numbers then typically continued to decline (Fig. 3A), whereas stabilization of fecal output numbers was observed in non-pregnant cycles (Fig. 3 A&B). For this reason, in non-pregnant cycles, D-28 was matched with the 'lowest' point of fecal output (Fig. 3 B&C). Based on this alignment, half-maximum numbers were observed at 30.50 ± 0.50 and 30.44 ± 0.63 in non-birth and pseudopregnant profiles, respectively, which corresponded to the pregnant cycles (Fig. 3A). In only one non-birth cycle (SB723 2020), the D-28 criterion was hard to match, most likely because of the seasonal impact on this atypical cycle (estrus occurred in December, at a time of seasonally increased bamboo intake, and cycle length was significantly shorter compared to previous cycles and ending in March 2020).

3.1.2. *Reactivation of CLs 42 days prior to birth: post-diapause development takes 42 days in giant pandas*

As described above, a quantitative metabolic marker (fecal output) was identified to align all cycles. Other bear studies have similarly used alternative non-invasively obtained, non-endocrine markers (for example temperature and activity data) to estimate implantation and birth date during hibernation [61,62].

Herein, a decrease in fecal output occurred almost in accordance with a significant progestogen increase, known as the start of the secondary progesterone increase (AL) (Supplementary figure 3). We confirmed distinct changes, exclusively monitored in the USpG-corrected progestogen profile, from D-42 onwards, with D-42 representing a nadir in progestogen prior to accelerated increase, becoming statistically significant from the averaged primary rise values, typically at D-37 (Fig. 1 B&C and Fig. 3A). Based on this information, we suggest post-diapause development in giant pandas to be of fixed duration, taking not more than 42/43 (D-42) days from early reactivation of the corpus luteum (CL). This CL reactivation is leading to significant luteal progestogen production, promoting embryonic growth from 37 to 38 (D-37) days before the birth of an altricial cub.

A post-diapause development of maximum 42 days is significantly shorter than reported for any of the other 7 bear species. Exact information on other bear species' CL dynamics and pregnancy durations is extremely limited and restricted to estimations of potential timing of CL reactivation (approximately 2–4 weeks prior to implantation) and embryonic implantation (60 days prior to birth) [61,63–67]. In the brown bear, the mean date of implantation was documented as the 1st of December (SD = 12 days), with subsequent parturition around 26th of January (SD = 12 days) and thus an average duration of post-implantation gestation of 56 days (SD = 2 days) [61]. Based on the scarce information available in other bear species, the post-embryo reactivation period – including pre-implantation events such as

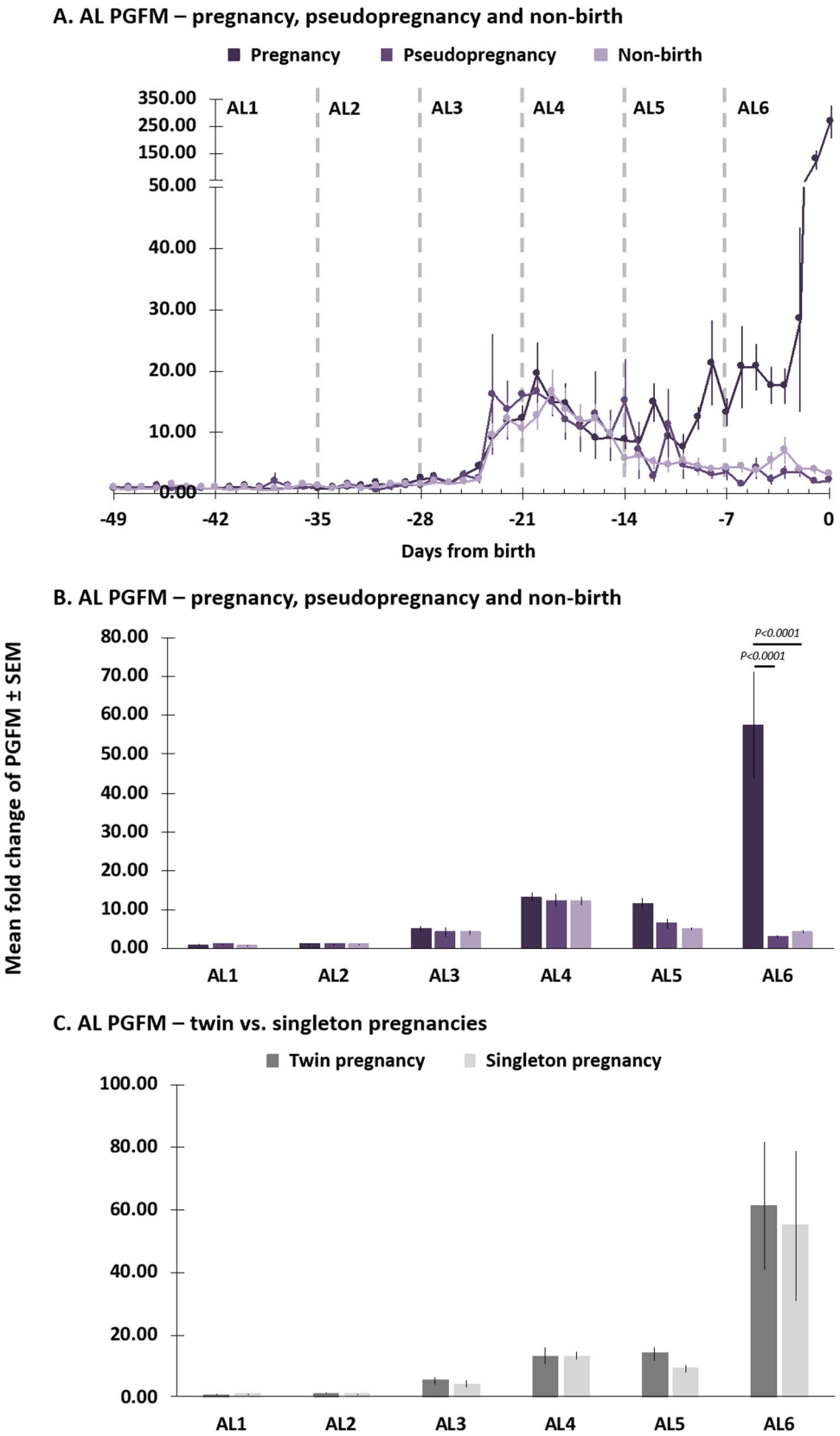

Fig. 5. : Overview of urinary mean fold change PGFM values divided over the 6 weeks of the AL phase. (A) is showing the full averaged profile of pregnant, pseudopregnant and non-birth cycles, (B) is showing the comparison between AL weeks for pregnant, pseudopregnant and non-birth cycles, (C) makes the comparison between singleton and twin pregnancies. (error bars = SEM).

development of the embryonic folds – lasts approximately 70–88 days and is thus at least 4 weeks longer compared to the post-diapause length defined in this study for giant pandas [61,63–65,67–69]. The shortening of this post-diapause development phase by almost one month may explain the findings of Griffiths et al. (2015) [70]. The latter study reported that transition from colostrum to main phase lactation only took place approximately 30 days *post-partum* in giant pandas [70], which corresponds to the period that cubs from other bear species still develop *in utero*. The validity of this short *in utero* development model, particularly the exact duration of 42 days, is additionally supported by the study results of Li and Smith (2020) [71]. These authors investigated ossification and skeletal anatomy in neonatal ursid cubs (brown bear and polar bear) and concluded that these other bear species' cubs are not exceptionally altricial relative to other caniforms, despite the impressive difference in body size between newborns and adults. In contrast, for the two newborn giant panda cubs investigated in this study, skeletal maturity levels matched up to beagle fetuses of only 42–45 days old *in utero*, corresponding to 70% of the total gestation period in dogs. In addition, in canine species (*Canis familiaris)*, skin pigmentation may start to develop from 37 days onwards *in utero*, but is only noticeable over the whole body from day 46 onwards [72]. This is in accordance with the development of the black and white patches in giant pandas during the first week *post-partum*. Body hair presence is evident between 40 and 46 days of *in utero* development in dogs, aligning with the physical appearances of giant panda cubs at birth [72]. A post-diapause development of 42 days is thus scientifically plausible for giant pandas and most likely results from the evolutionary adaptation towards a herbivorous diet, additionally explaining the absence of hibernation in this bear species [73].

3.2. Pregnancy diagnosis and evaluation based on non-invasive monitoring

3.2.1. Progestogens

Urinary (normalized with creatinine) and fecal progestogen profiles previously have been reported to be indistinguishable between pregnant and pseudopregnant giant pandas [12,22].

In this study, however, overall higher average mean fold change urinary progestogen concentrations (normalized with USpG) were observed in the pregnant cycles, with significant differences between pregnant versus pseudopregnant and non-birth cycles during the majority of the luteal phase (Fig. 4 A&B; Table 2). Non-birth cycles did not differ significantly in progestogen metabolite concentrations compared to pseudopregnant cycles. These results likely indicate that luteal progesterone production in the early and mid AL phase is supported by luteotropic fetal factors, counteracting the presumed luteolytic activity of the first PGFM spike, while more active feto-placental contributions are likely responsible for higher and slower decreasing progestogen levels closer towards the end of cycle.

Compared with the absolute concentration profile (Supplementary figure 4; Supplementary Table 5), the results are clearer from the dataset based on mean fold change concentrations (e.g. ratio between the measured level per sample and the average baseline levels from the CLD, for each individual cycle). This latter approach may allow defining thresholds for pregnancy diagnosis.

Remarkably, for twin pregnancies higher mean fold change progestogen concentrations were observed compared to singleton pregnancies, particularly during the early stages of post-diapause development of the embryo (AL1-AL3, e.g. before increasing PGFM concentrations). This period corresponds to the time window prior to attachment and placental development (Fig. 4C). Wimsatt (1974) [67] studied placental development in black bears and indeed confirmed a considerable development of both embryo and embryonic folds prior to proper implantation, fueled by histiotrophic nutrition (uterine milk). He found that in the early stage of attachment a well vascularized choriovitelline placenta is formed, still with a predominant histiotrophic function. High progestogen concentrations during this phase most likely support the production of uterine milk by the endometrium. At a later stage – only a week later – proper chorionic penetration of the endometrium occurs with the conversion into a chorioallantoic placenta. In our profiles, progestogen concentrations indeed start to decline from AL4-AL5 onwards, indicating that hemotrophic nutrition established.

In other species, progestogen levels have been found to correlate with litter size most likely because of an increased number of CL and/or placentas participating in the progestogen production, which easily explains our study results for twin pregnancies compared to singletons [74,75].

Similar to the differences observed between pregnant and non-pregnant cycles, AL6 is also characterized by higher progestogen metabolite levels in twins compared to singletons, again pointing towards feto-placental involvement. Progestogen profiles remaining at high progestogen levels at the end of a presumed pregnancy, and pending at higher levels during AL5 and AL6, with the decrease slowing down until the pre-birth PGFM spike, are indicative for a healthy pregnancy. Any sudden decrease of progestogens during this, or any earlier phase, should raise concerns about the viability or survival chances of (a) potential cub(s).

3.2.2. PGFM

The USpG-normalized PGFM profile, corrected by individual baseline values (e.g. mean fold change), shows to be very similar between pregnant, pseudopregnant and non-birth cycles, at least for the first 4 weeks of the 6-week AL phase. The profile is characterized by a first luteolytic PGFM peak, occurring approximately 3 weeks prior to the end of cycle, with values remaining elevated during mid-AL3 to mid-AL4 before declining. This is a different observation compared to the results obtained with creatinine normalization. Roberts et al. (2018) [26] reported a different timing of the first PGFM peak in pregnant versus pseudopregnant cycles and disclosed higher averaged PGFM values throughout the remaining weeks of the AL phase for pregnancy cycles.

In our study, the pregnant profile only starts to diverge from the pseudopregnant and non-birth profile during AL5, showing an average increase towards birth, becoming significant in the last week prior to birth (Fig. 5 A&B; Table 2). The latter increase is running in parallel with estrogens starting to increase around the same time [52] (Supplementary figure 5 and 6). It is well described in literature that elevated estrogens, and in particular increasing estrogen/progesterone ratios, activate prostaglandin synthases in the placenta, as such initiating $PGF_{2\alpha}$ to increase [76,77]. An impressive peak in PGFM at the end of this phase is announcing birth to occur in the next hours or day, as previously reported by Roberts et al. (2018) [26].

In the final 3 weeks of the AL, the pseudopregnant and non-birth profile show a rather decreasing trend, with low PGFM values maintained during the final 2 weeks of the cycle. Nevertheless, both non birth and pseudopregnant cycles often show a temporary small increase a few hours to a day prior to the predicted end-of-cycle, most likely from ovarian or uterine (endometrium) origin [77–79] (Supplementary figure 7). This increase risks to be misinterpret as the start of a pre-birth PGFM increase. Currently adapted dataset (by mean fold change) however clearly demonstrates that drafting the corrected profile may help to prevent misinterpretations based on absolute PGFM concentration levels and profiles (Fig. 5A versus Supplementary figure 7).

There is a trend for slightly higher PGFM concentrations in the final 2 weeks of the AL in twin pregnancies compared to singletons, but no significant difference could be demonstrated in this study.

3.2.3. Glucocorticoids

Not surprisingly, significantly higher mean fold USpG-normalized GCM concentrations were observed in pregnant cycles from 2 weeks prior to birth (AL5 and AL6). A trend of these higher values in pregnant animals was already monitored from AL2 onwards. This matches observations of Kersey et al. (2011) [23] in fecal samples. They reported

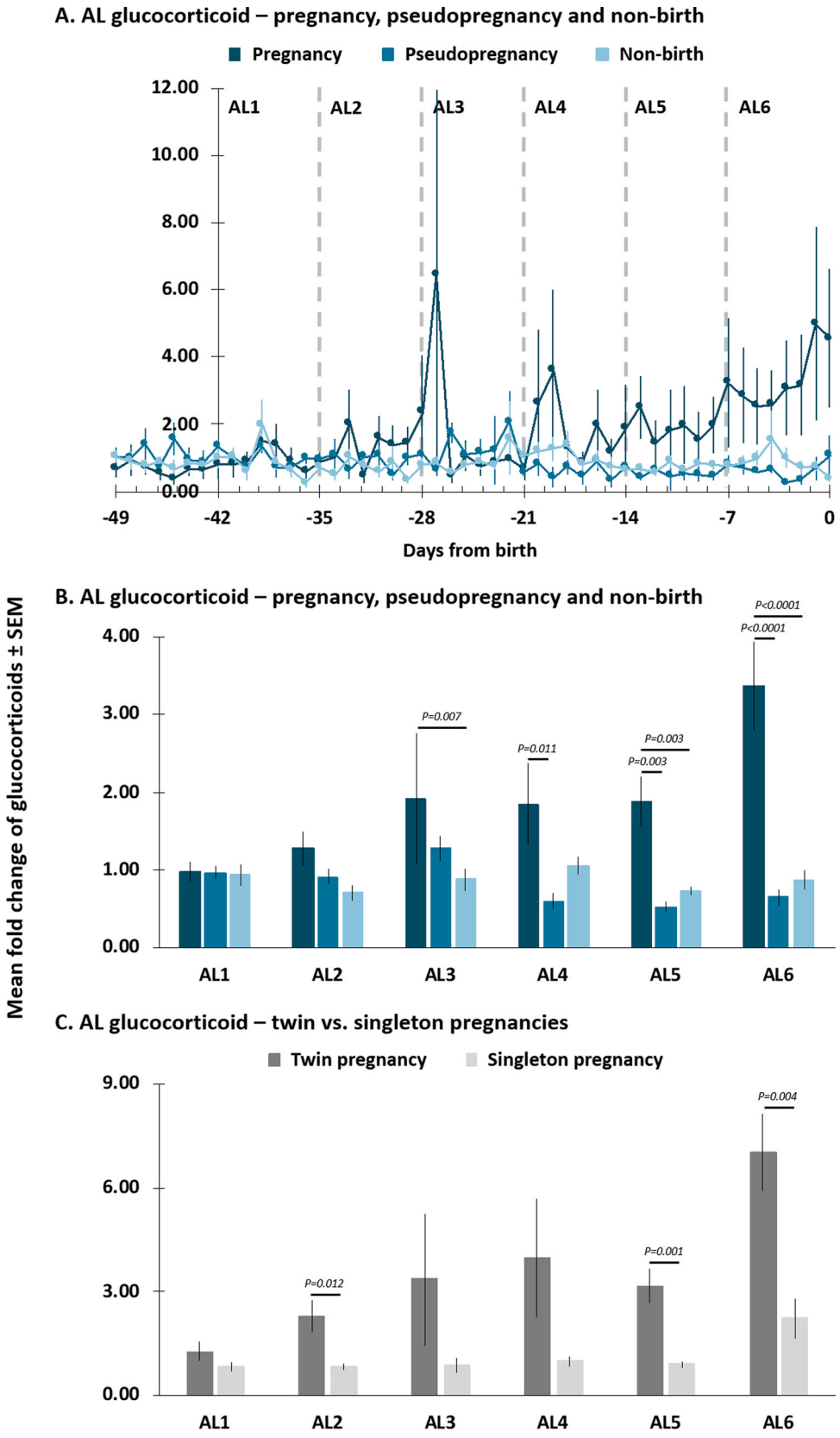

Fig. 6. : Overview of urinary mean fold change GCM values divided over the 6 weeks of the AL phase. (A) is showing the full averaged profile of pregnant, pseudopregnant and non-birth cycles, (B) is showing the comparison between AL weeks for pregnant, pseudopregnant and non-birth cycles, (C) makes the comparison between singleton and twin pregnancies. (error bars = SEM).

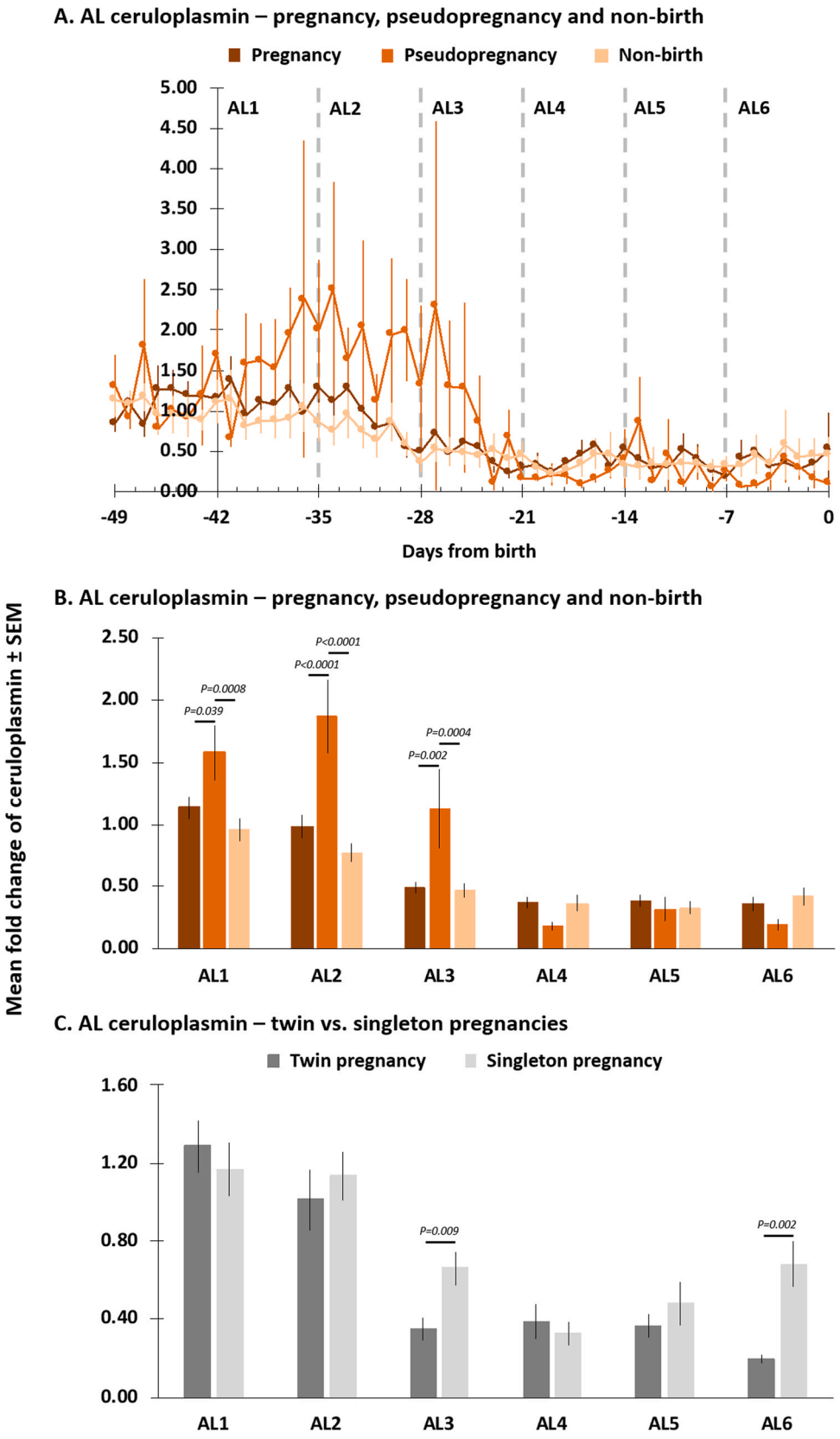

Fig. 7. : Overview of urinary mean fold change ceruloplasmin values divided over the 6 weeks of the AL phase. (A) is showing the full averaged profile of pregnant, pseudopregnant and non-birth cycles, (B) is showing the comparison between AL weeks for pregnant, pseudopregnant and non-birth cycles, (C) makes the comparison between singleton and twin pregnancies. (error bars = SEM).

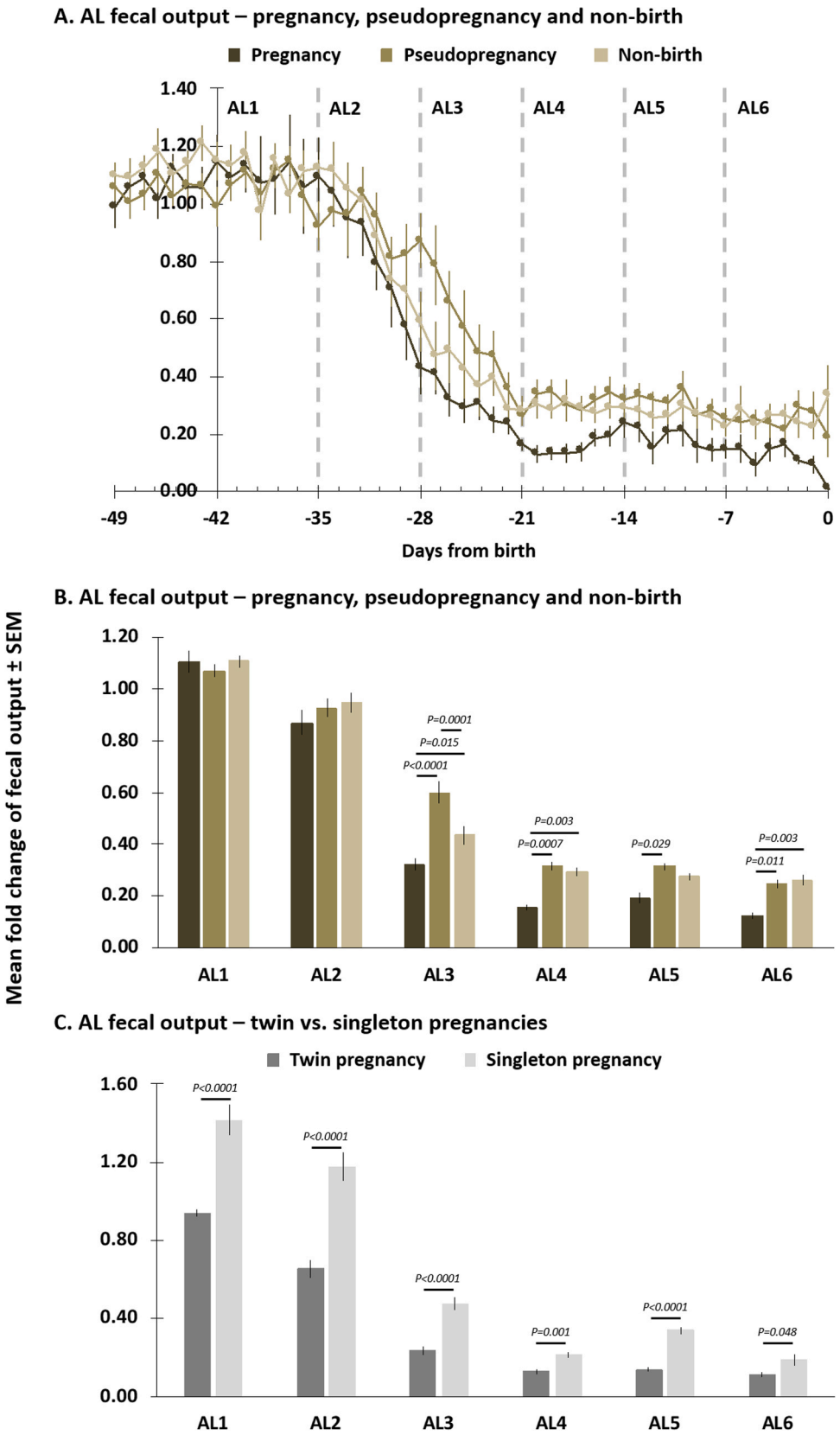

Fig. 8. : Overview of mean fold change fecal output values divided over the 6 weeks of the AL phase. (A) is showing the full averaged profile of pregnant, pseudopregnant and non-birth cycles, (B) is showing the comparison between AL weeks for pregnant, pseudopregnant and non-birth cycles, (C) makes the comparison between singleton and twin pregnancies. (error bars = SEM).

higher concentrations of GCM in parturient compared to non-parturient females during the later stages of the luteal phase. In contrast, a similar study in female polar bears showed no different fGCM levels between pregnant and pseudopregnant cycles [80].

It should be considered that the higher GCM values during the first weeks post attachment (AL3/AL4) may be predominantly maternal. During this phase of pregnancy, a giant panda fetus is growing exponentially, therefore most likely causing a shift in metabolism. Cortisol is involved in biochemical pathways that result in increased serum glucose availability. Increased cortisol concentrations, likely from maternal origin, may therefore represent altered metabolism in favor of pregnancy [81]. It can also explain why higher values were recorded for twin-pregnancies, as the demand for nutrients is twice as high.

While values seem to remain relatively constant for pregnant cycles during AL3-AL5, a steep further increase with peaking values, not observed in pseudopregnant and non-birth cycles, is seen in the final week prior to birth (Fig. 6 A&B; Table 2). Jenkin and Young (2004) [76] explain that in all studied species a late pregnancy increase in fetal glucocorticoids has been encountered so far. For example, in fetal sheep plasma, glucocorticoid concentrations are increasing exponentially over the last month of gestation. During this time window, glucocorticoids are essential for the accelerated maturation of organ systems required for life outside of the uterus (e.g. lungs and liver) [82]. In addition, the increase in glucocorticoids induces endocrine metabolic pathways that are mandatory for the initiation of parturition. We thus hypothesize that the additional increase in GCM during AL5-AL6 is mainly from fetal origin. In other species, glucocorticoids stimulate the expression of placental steroidogenic enzymes, which redirect the steroidogenic pathways in favor of secretion of estrogens, in turn activating prostaglandin synthetases in the placenta/uterine endometrium [77–79]. It is therefore not surprising that both estrogens and PGFM (Fig. 5 A&B) show increases during the same window compared to GCM (AL5-AL6). The actions of both $PGF_{2\alpha}$ and estrogens eventually lead to a regression of the CL, removal of the P4 block, activation of the myometrium, eventually initiating parturition.

Significant higher GCM values were observed for a twin pregnancy compared to a single pregnancy, particularly in the final 2 weeks prior to birth (Fig. 6C). This offers another indirect proof for the feto-placental origin during late pregnancy.

3.2.4. Ceruloplasmin

Willis et al. (2011) [25] described a pregnancy-specific profile for ceruloplasmin (normalized by creatinine) with values decreasing in the early weeks of the AL phase. Our study however demonstrates clearly higher values in pseudopregnant cycles during the early AL phase, with a decreasing mean fold change ceruloplasmin profile present for all 3 reproductive groups (Fig. 7 A&B; Table 2).

At approximately 23 days prior to the end of cycle (during AL3), ceruloplasmin remains low until the end-of-cycle for all 3 reproductive groups, hence – in contrast to the study of Willis et al. [25] – not offering potential as a discriminating marker for pregnancy.

No clear differences are demonstrated between singleton and twin pregnancies, although higher mean fold change values have been observed in singletons compared to twins during AL3 (e.g. around the timing of attachment) and AL6 (around the timing of birth) (Fig. 7C).

3.2.5. Fecal output

Fecal output is an easy to record metabolic marker that can disclose information about how far a female giant panda has evolved into the luteal phase (see also 3.1). Typically, fecal output remains at high levels during the CLD phase and starts to decrease at the end of AL1, beginning of AL2 (D-37 to D-33), i.e. when progestogen numbers start to climb rapidly (Fig. 3A). Thirty days prior to birth, a significant decrease can be observed, consistent with a 30–50% drop in fecal output compared to 1 week before. Typically, when individual profiles are observed, a rather steep decline continues until D-28/D-27, followed by a plateauing with decreasing trend at lower levels for pseudopregnant and non-birth cycles (Fig. 3). Pregnant cycles resume to decline distinctly with the onset of AL4 (Fig 9 A). During AL4, lowest absolute and mean fold change fecal output numbers were observed for pregnant profiles (Fig. 8 A&B; Table 2). In contrast, at the start of AL4, values stabilize at higher levels for pseudopregnant and non-birth cycles compared to pregnant cycles (Fig. 8A).

Overall, mean fold change fecal output numbers are significantly lower in pregnant cycles from AL3 onwards until birth (and after) (Fig. 8 A&B). This means that daily records of fecal output cannot only help in timing the most likely interval for birth but may also give indications on whether or not a female giant panda is experiencing a true pregnancy.

With the exclusion of AL4, and not taking into account the averaged lower fecal output in pregnant cycles, both averaged fecal output profiles behave very similar between pregnant and pseudopregnant cycles (mirroring reduced intake of bamboo during the AL) (Fig. 8A). The same is largely true for non-birth cycles for which fecal output levels show to be very similar to those of pseudopregnant cycles in the last three weeks of the cycle (Fig. 8A).

Based on absolute numbers, it is also relevant to highlight that pregnant females seem to show a higher fecal output during the CLD phase and AL1 (Fig. 8A versus Supplementary figure 10). This significant difference indicates females invest in higher resource intake during the CLD as a preparation for exponential fetal development after diapause. Potentially, this is initiated by feto-maternal signaling. Other metabolic markers, detectable in non-invasive sample matrices, may therefore be good candidates as potential future pregnancy markers.

4. Conclusions

According to our study results, giant pandas seem to show similar endocrine mechanisms during late pregnancy compared to other species, with increasing fetal glucocorticoids initiating parallel changes in the estrogen and subsequent $PGF_{2\alpha}$ metabolism. Whether the latter shifts are predominantly mediated by feto-placental or ovarian factors still needs to be unraveled. Nevertheless, these profile changes are pregnancy-specific in the final 2 weeks prior to birth. For progestogens, clearly from luteal origin for at least the majority of the AL phase, our study demonstrates higher mean fold changes for almost the complete luteal phase in pregnant cycles. In addition, progestogen values decrease slower in pregnant cycles compared to pseudopregnant and non-birth cycles in the final 2 weeks prior to birth, until occurrence of the second luteolytic PGFM spike. This means that the USpG-corrected progestogen profile is discriminative in case of pregnancy, reflecting pregnancy health, particularly in the second half of pregnancy.

It is important to highlight that our results obtained after normalization with USpG disclosed different information compared to creatinine-corrected datasets. Three endocrine markers were now identified with a clear pregnancy-specific signature, offering a toolset to assist in confirming and monitoring a healthy pregnancy in the final crucial stage of the cycle. Nevertheless, when no resources or samples are available for close real-time endocrine monitoring, fecal output numbers, particularly low during pregnant cycles from AL4 onwards, may also support a tentative pregnancy diagnosis. Monitoring the daily fecal output has shown to be extremely helpful in estimating the reproductive status of the females as well as in predicting the day of birth.

With a post-diapause development of only 42 days, preceded by a long period of diapause and the occurrence of pseudopregnancy, giant panda pregnancy diagnosis and monitoring remains truly a challenging process. The search for biomarkers of early pregnancy, e.g. during diapause, therefore continues and should consider the relevance of alternative non-invasive markers such as those involved in metabolism.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.therwi.2023.100063.

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