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Functional relationships between estradiol and paternal care in male red-bellied lemurs, *Eulemur rubriventer*

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ABSTRACT

Fathers contribute substantially to infant care, yet the mechanisms facilitating paternal bonding and interactions with infants are not as well understood as they are in mothers. Several hormonal changes occur as males transition into parenthood, first in response to a partner's pregnancy, and next in response to interacting with the newborn. These changes may prepare fathers for parenting and help facilitate and maintain paternal care. Experimental studies with monkeys and rodents suggest that paternal care requires elevated estradiol levels, which increase when a male's partner is pregnant and are higher in fathers than non-fathers, but its role in the expression of paternal behaviors throughout infant development is unknown. To assess estradiol's role in paternal care, we analyzed the relationship between paternal estradiol metabolites and 1) offspring age, and 2) paternal care behavior (holding, carrying, huddling, playing, grooming), in wild, red-bellied lemurs (*Eulemur rubriventer*). We collected 146 fecal samples and 1597 h of behavioral data on 10 adult males who had newborn infants during the study. Estradiol metabolites increased four-fold in expectant males, and in new fathers they fluctuated and gradually decreased with time. Infant age, not paternal behavior, best predicted hormone levels in new fathers. These results suggest that hormonal changes occur in expectant males with facultative paternal care, but they do not support the hypothesis that estradiol is directly associated with the day-to-day expression of paternal care. Future research should explore estradiol's role in facilitating behaviors, including infant-directed attention and responsiveness, or preparing fathers for infant care generally.

1. Introduction

Infant care is arguably the most important requirement for mammals to survive and reproduce. Mother-infant bonding is critical for the survival of completely dependent offspring, and it is maintained through evolved behavioral and physiological processes. This early relationship helps shape infant development and can greatly impact health and survival (Gudsnuk and Champagne, 2011). During pregnancy, mothers undergo critical endocrine changes that are associated with the developing fetus, are important for birth and lactation, and enhance a mother's responsiveness to neonates (Numan and Insel, 2003). Early research on the neuroendocrinology of infant care focused on mothers

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because these same hormonal changes were unexpected in non-gestating individuals. Moreover, because mammalian paternal care is relatively rare, mothers were considered the default caregivers (Nelson, 2011; Saltzman and Maestripieri, 2011). It is now more broadly recognized that fathers can make substantial contributions to infant care, also serving as primary caregivers, and progress over the past 20 years has helped identify the hormonal mechanisms that facilitate paternal care (Storey et al., 2020).

Hormonal profiles and responsiveness to infants differ as males transition from non-parent, through expectant parent, to parent (Berg and Wynne-Edwards, 2001; Edelstein et al., 2015; Gettler et al., 2011b; Ziegler et al., 2004). Expectant human, tamarin monkey, and biparental rodent fathers undergo hormonal changes during gestation that appear to occur in response to their partner's pregnancy. During the last trimester and early postnatal period, cortisol, oxytocin, prolactin, and androgen levels have been found to change significantly in these species (Brown et al., 1995; Edelstein et al., 2015; Morris et al., 2021; Reburn and Wynne-Edwards, 1999; Saxbe et al., 2017; Storey et al., 2000; Ziegler et al., 2004). For example, prolactin, cortisol, and testosterone levels in men changed in coordination with their partners, and those with more sympathetic pregnancy symptoms such as fatigue and weight gain (Couvade symptoms, Clinton, 1986) had higher prolactin levels and lower testosterone levels; they also had greater responsiveness to infants (Storey et al., 2000). Hormonal changes at this time in biparental species have been shown to differ from closely related species with exclusive maternal care (Djungarian and Siberian hamsters, Reburn and Wynne-Edwards, 1999; siamangs and gibbons, Rafacz et al., 2012). These studies are consistent with the hypothesis that, in species with paternal care, changes in male physiology during their mate's gestation help them prepare for the arrival of their infants.

Various hormonal changes are also known to occur after offspring are born. These changes can help coordinate paternal behavior (Storey and Ziegler, 2015), initiating or maintaining parent-infant interactions. In humans and a range of other mammal species, changes in androgens, oxytocin, and prolactin are also associated with acute interactions such as playing, touching, and carrying infants (e.g., humans: Dixson and George, 1982; Feldman et al., 2010; Fleming et al., 2002; Gettler et al., 2011a; Gordon et al., 2017; Morris et al., 2021; common and Wied's marmosets: Mota and Sousa, 2000; Mota et al., 2006; Nunes et al., 2001). These hormone-behavior relationships suggest that paternal hormonal profiles in these species do not merely reflect their status as fathers, but also their performance and effectiveness as parents (Storey and Ziegler, 2015). Studies of brain-oxytocin-parenting relationships in different- and same-sex couples support this interpretation, with similarities across these axes among primary caregivers, whether they were mothers or biological or adoptive fathers (Abraham et al., 2014; Atzil et al., 2012). This research has been limited to a narrow range of species, including biparental rodents, cooperatively breeding marmosets and tamarins, and humans. Studies associating hormones and behavior in expectant and new fathers are often conducted under experimental conditions where specific behaviors are encouraged (e.g., play time in humans, Gettler et al., 2011a), and acute response to the testing conditions are analyzed at key points in time. Longitudinal, in situ observational studies with consistent sampling are needed to determine whether hormonal profile differences can explain sustained differences in parenting on an individual level, and how hormones are associated with the full, natural range of paternal behaviors.

Estradiol has not been studied extensively in males, but it is an estrogen steroid hormone that is associated with increased maternal sensitivity and responsiveness in several mammalian species (Numan and Insel, 2003; Rilling and Young, 2014), and is required for other hormones such as oxytocin and prolactin to induce maternal behavior (see Ziegler et al., 2009). Estradiol is potentially important for paternal care as well. Much of what we know of estradiol's role in paternal care is from a small set of experimental studies on a few species of captive, biparental rodents (Bales and Saltzman, 2016). Experimental castration combined with hormonal replacement and inhibition had no (or unclear) effects on paternal or alloparental behavior in Djungarian hamsters and prairie voles (Hume and Wynne-Edwards, 2006; Lei et al., 2010). However, exposure to estradiol (and androgens) during development in male prairie voles is critical for paternal behavior to occur later in life (see Bales and Saltzman, 2016). In fact, an experimental study in the California mouse determined that paternal behavior occurs only through the conversion of testosterone to estrogen in the brain, as paternal care was reinstated in castrated males by treatment with estrogen (Trainor and Marler, 2002, 2008). Administering exogenous estradiol to common marmoset fathers increased their response to infant distress calls (Ziegler and Sosa, 2016), suggesting that fathers with higher endogenous estradiol may be more responsive to infants. Increasing estradiol levels in expectant and new fathers (siamangs: Rafacz et al., 2012; marmosets and tamarins: Ziegler et al., 2004, 2009), and higher estradiol in fathers vs. non-fathers (humans: Berg and Wynne-Edwards, 2001), also suggest that elevated endogenous estradiol levels may facilitate paternal care. Estradiol may also inhibit aggression; all male Mongolian gerbils treated with estradiol implants decreased infant-directed aggression, while castrated and sham males did not (Martínez et al., 2015). Only one naturalistic study examined the relationship between estradiol and paternal behavior. Estradiol (and testosterone) in men did not vary with self-reports of frequency of playing, nor in response to interacting with their child for 30 min (Gettler et al., 2013). We are not aware of any studies that have associated paternal estradiol profiles with simultaneous, naturally occurring paternal care behavior with consistent, longitudinal sampling, which can help identify its dynamic relationship with multiple paternal behaviors throughout infant development.

Red-bellied lemurs (*Eulemur rubriventer*) are an excellent model system for investigating the relationship between paternal care and estradiol levels (Tecot et al., 2012, 2013; Tecot and Baden, 2018). First, red-bellied lemur and human fathers invest a comparable amount of time in their offspring; the paternal behavioral repertoire in this species is substantial (e.g., holding, grooming, carrying, and huddling and playing with infants); and paternal behavior is relatively easy to measure (Tecot and Baden, 2018). We are therefore able to associate estradiol levels with individual longitudinal behavioral data collected in a natural context. Second, red-bellied lemurs demonstrate natural variation in paternal care, enabling us to compare fathers who interact with their offspring to varying degrees (Tecot and Baden, 2018). Third, adult pairmates are very cohesive, within 5 m of each other the majority of the time (Overdorff and Tecot, 2006). Both individuals actively maintain their bond and they are affiliative and in close contact throughout the year (Overdorff and Tecot, 2006), including when females are gestating. Fourth, androgens are converted to estradiol by aromatization in the mammalian brain and can explain why testosterone promotes paternal behavior in the California mouse (Marler et al., 2003; Trainor and Marler, 2002); androgen levels in red-bellied lemurs are higher in fathers that spend more time in infant care, and specifically grooming and holding infants (Tecot and Baden, 2018). This finding leaves open the possibility that elevated androgens influence paternal care via conversion to estradiol.

We hypothesize that red-bellied lemur male estradiol (E2) levels are functionally associated with paternal care behavior. We investigate E2 as well as E2/androgen ratios because hormones may influence behavior based on their concentrations relative to other hormones (e.g., cortisol-dihydroepiandrosterone ratios better predict depression in humans than either hormone does alone, Michael et al., 2000). In birds, relative measures of E2 and androgens influence sexual differentiation (quail and zebra finches, Adkins-Regan et al., 2013) and have been investigated in relation to brooding behavior (Lupo et al., 1990). We predict that E2 levels and E2/androgen ratios: 1) increase in expectant males; 2) relate to future paternal behavior in expectant males, with higher E2 levels and E2/androgen ratios associated with more paternal care after infants are born; and 3) are positively associated with paternal behaviors

in new fathers.

2. Material and methods

2.1. Site and subjects

We collected data from red-bellied lemurs in Ranomafana National Park, Madagascar (located between 21° 02′ to 21° 25′ S and 47° 18′ to 47° 37′ E), using the Vatoharanana field site trail system (described in Tecot, 2008), from September 2013 through March 2015. This period spanned two reproductive seasons, with births occurring September through November (Tecot, 2010; unpublished data). While mating was not observed during the study, we estimated the mating season to be May through July, based on a gestation length of 126 days, or just over 4 months, in captive lemurs (Duke Lemur Center records). We observed 13 groups each consisting of at least one adult male, one adult female, and one infant born during the study. We identified individuals using size, pelage coloration, scars, group composition, territorial location, and other distinct markings, as well as photographs (described in Crouse et al., 2017). We assigned paternity based on group composition before and at the time of the infant's birth, as previous work suggests a relatively strict monogamous mating system, with rare exceptions (Jacobs et al., 2018; Merenlender, 1993). Our assignments are supported by preliminary genetic paternity analyses (Baden and Tecot, unpublished data).

We collected behavioral data from each male at least once per week during their mate's gestation period, through birth and infant development, until the period when infants are reportedly weaned (Wright, 1999). We recorded behavioral data on all group members including fathers using scan sampling and instantaneous recording at 5-min intervals (Altmann, 1974). We recorded fathers' activity (n = 1597 h of data), including the infant-focused behaviors huddle, hold, carry, play, and groom once infants were born.

We measured estradiol metabolites in fecal samples. We collected fecal samples from each father opportunistically during behavioral data collection, before 1200 h to control for circadian variation in hormone excretion. We only collected samples that were uncontaminated by urine or other individuals' feces. We dried all samples by a fire as described in Tecot and Baden (2018) and according to previously established methods (Brockman et al., 1998; Tecot, 2008), placed dried samples in Ziploc bags with desiccant, and kept them in dry storage conditions until transportation to the United States for analysis.

In total, we reliably collected fecal samples during the entire prenatal and postnatal periods from 7 of 13 males for which we also have corresponding behavioral data. During the postnatal period only, we reliably collected fecal samples and behavioral data for 10 of 13 males.

2.2. Estradiol extractions and assays

We extracted desiccated fecal samples (n = 136) following methods described in Tecot and Baden (2018). Briefly, we extracted 0.10 g of desiccated feces with 2.5 mL ethanol and 2.5 mL deionized water. We added 4 mL of ethyl acetate to 1 mL of extract. After vortexing and centrifuging the sample, we aspirated off the top layer, evaporated the sample under a stream of air in a water bath, and resuspended it in 1 mL ethanol. We assayed 50 μL of each sample in duplicate using Arbor Assays 17β-DetectX Estradiol Enzyme Immunoassay (EIA) kits (Ann Arbor, Michigan), according to kit instructions. The sensitivity of the kit is 39.6 pg/mL. The antibody cross-reacted 100 % with estradiol, 0.78 % with estrone, 0.22 % with 17α-estradiol, 0.11 % with 17α-ethynylestradiol, and <0.10 % with estrone sulfate, progesterone, testosterone, 5α-dihydroprogesterone, cortisol, and corticosterone. We determined the accuracy of the assay for red-bellied lemur estradiol metabolites by a spike-recovery analysis. Average estradiol recovery was near expected values (94.7 % ± 9.27 SEM). We found that serially diluted sample and estradiol standard slopes did not differ, $F(10,11) = 1.42$, $p = 0.26$. Coefficients of variation (CVs) for high and low pools, respectively, were 7.4 % and 12.3 % (inter-assay CVs) and 5.0 % and 3.6 % (intra-assay CVs).

2.3. Data analysis

2.3.1. Estradiol

We first tested the prediction (P1) that E2 and E2/androgen ratios increase in expectant males. We tested this prediction using data from all males for which we had hormone samples (n = 10). To standardize sampling intervals through time and across subjects, we calculated mean fecal estradiol levels (ng/g) for each male at 4-day intervals. Upon visual inspection of the data, E2 values appeared to vary nonlinearly with time (Fig. 1A). We therefore used generalized additive models (GAMs) in the 'mgcv' package of R (version 1.8–27, Wood, 2017). GAMs were fitted with a Gaussian distribution by using the restricted maximum likelihood (REML) algorithm because REML estimates are nearly unbiased in the presence of small data sets (Pinheiro and Bates, 2006). We investigated time-related changes in E2 levels during three periods: 1) the entire reproductive period (intervals −28 to 37); 2) during fetal development (intervals −28 to 0); and 3) during postnatal development (intervals 0 to 37). Interval 0 included the days immediately preceding and following birth; we therefore included data from interval 0 in both periods 2 and 3 because it straddled the pre- and postnatal periods. Since we did not witness every birth in the week that it occurred, interval 0 accounts for only two data points. We included 4-day interval as a smooth term, thereby allowing E2 values to vary nonlinearly with time. Further, for each male, we identified whether (Y/N) and when (by which 4-day interval) paternal E2 levels elevated relative to their own baseline levels ('Elevated_Own'), as well as to the average baseline levels of adult males in the population ('Elevated_Mean') (Table 1A).

Because previous studies have found correspondence between E2 and androgens (e.g., Ziegler et al., 2004), we further explored the relationship between paternal E2 and our previously published androgen values (Tecot and Baden, 2018), as well as the relationship between E2/androgen ratios and time, using the same three time periods described above (Table 1B; Figs. 1 & S1). While Sollberger and Ehlert (2016) caution against the use of hormone ratios in statistical analyses under certain conditions, our dataset does not suffer from the issues that they identified. Specifically, we have repeated measures; analyzed normalized data; used the same units of measurement for both hormones (ng/g); and analyze each hormone separately as well as in a ratio and thus maintain information that might be lost while only focusing on ratios alone.

Both E2 values and E2/androgen ratios were nonnormally distributed; however, QQ plots of residual E2 values (after controlling for time) were confirmed to be normal (Shapiro-Wilk, $W = 0.98$, $p = 0.35$). By contrast, residual E2/androgen ratios remained nonnormal, even after accounting for time. We therefore log-transformed E2/androgen ratios prior to further analysis. Both E2 values and E2/androgen ratio are reported as appropriate in the results.

2.3.2. Estradiol × paternal care

To evaluate the relationships between E2 levels, E2/androgen ratios and paternal behavior, we calculated the percentage of behavioral scans per 4-day interval in which each male was observed performing any form of paternal care (*%Paternal Care*), as well as the percentage of time he was engaged in each type of paternal care (huddle, hold, carry, play, and groom). Here we focused exclusively on behavioral data from the 2014–2015 dataset (n = 7 males). We then aligned behavioral and E2 and E2/androgen ratio data for each male by time interval. Further, we determined the total amount of paternal care each male provided during the course of our study (*Total Paternal Care*), calculated as the percentage of all behavioral scans in which each male was observed performing any form of paternal care, as well as their latency to care (*Latency*), calculated as the number of 4-day intervals following an infant's birth

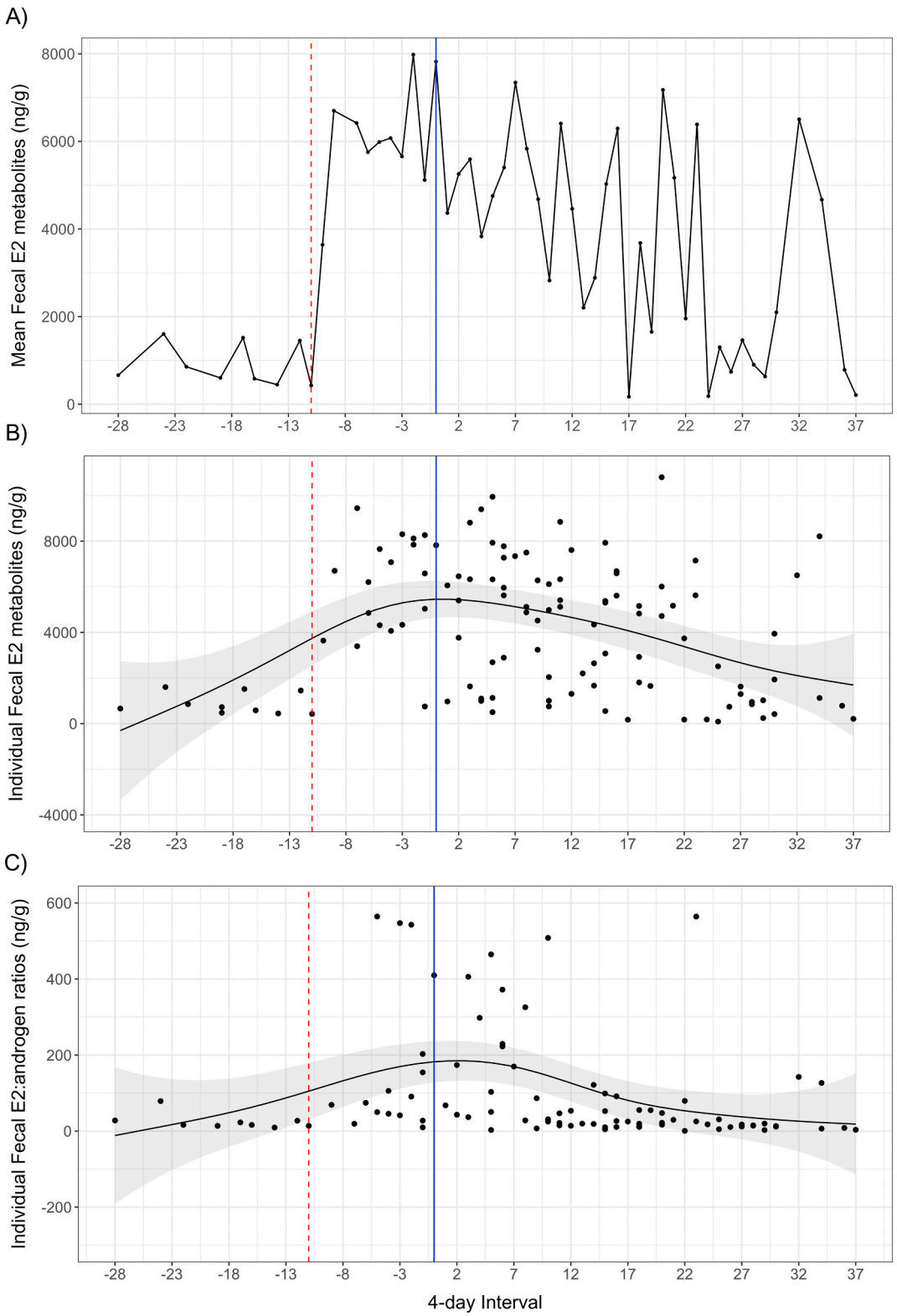

Fig. 1. A) Relationship between average male estradiol metabolites (E2) and time. Data include male E2 levels from both 2013–2014 (n = 5 males) and 2014–2015 (n = 7 males) breeding seasons (n = 10 individual males total), though this pattern held within each breeding season as well. Dashed red line indicates the time at which average male E2 levels elevated (interval −11); solid blue line indicates time of infant birth (interval 0). Note that for males that experienced elevated E2 concentrations, these levels remained elevated relative to baseline levels through post-natal development. B) Results from generalized additive model illustrating a significant relationship between male E2 and time (4-day interval) throughout the study period (Period 1: −28 to 37). C) Results from generalized additive model illustrating a significant relationship between male E2/androgen ratios and time (4-day interval) throughout the study period (Period 1: −28 to 37).

Table 1
Average E2 (A) and E2 to androgen ratios (B) by individual and reproductive season. Because of inconsistent and limited hormone and behavioral sampling in 2013–2014, we used only 2014–2015 data to build models relating E2 levels and E2/androgen ratios to individual measures of paternal care. Text in parentheses indicates imputed values calculated as the mean value of known measures for that variable in a given reproductive season. *indicates imputed values were generated using 2014–2015 data only, as baseline data were unavailable from the 2013–2014 season. Mean E2 metabolite level (in intervals −28 to −11) is used to infer baseline levels to which all other measures are compared. '–' indicates unknown, because baseline, prenatal, or postnatal data were unavailable for that individual.

A. Estradiol (E2)

							Elevated E2	
Male ID	Season	Mean baseline* (−28 to −11)	Mean prenatal (−9 to 0)	Mean postnatal (0 to 37)	Prenatal to own baseline (−9 to 0)	Prenatal to mean baseline (−9 to 0)	E2 %change (mean baseline to 0)	E2 %change (mean baseline to 37)
Cosine	2013–2014	(875.88)	4852.39	1765.89	–	Y	5.54	2.02
Helsinki	2013–2014	(875.88)	(5979.77)	2698.91	–	–	–	3.08
Rakoto	2013–2014	(875.88)	3640.72	2757.34	–	Y	4.16	3.15
Tolstoy	2013–2014	(875.88)	(5979.77)	1296.61	–	–	–	1.48
Vader	2013–2014	(875.88)	9446.21	1001.45	–	Y	10.78	1.14
Atody	2014–2015	1561.81	6528.16	(5714.85)	Y	Y	4.18	–
Helsinki	2014–2015	(875.88)	5191.91	4464.29	–	Y	5.93	5.10
Lane	2014–2015	(875.88)	751.71	7298.33	–	N	−0.86	8.33
Ned	2014–2015	530.86	7461.60	6086.89	Y	Y	14.06	11.47
Vader	2014–2015	1089.50	6752.56	6252.23	Y	Y	6.20	5.74
Zebra	2014–2015	655.32	6168.04	6080.38	Y	Y	9.41	9.28
Zephrix	2014–2015	428.49	(6257.52)	7487.34	–	–	–	17.47

B. Estradiol (E2) to androgen ratio

							Elevated E2/androgen ratio	
Male ID	Season	Mean baseline* (−28 to − 11)	Mean prenatal (−9 to 0)	Mean postnatal (0 to 37)	Prenatal to own baseline (−9 to 0)	Prenatal to mean baseline (−9 to 0)	E2/androgen ratio % change (mean baseline to 0)	E2/androgen ratio % change (mean baseline to 37)
Cosine	2013–2014	(24.02)	48.58	12.86	–	Y	2.02	−0.54
Helsinki	2013–2014	(24.02)	(56.47)	73.52	–	–	–	3.06
Rakoto	2013–2014	(24.02)	(56.47)	21.28	–	–	–	−0.89
Tolstoy	2013–2014	(24.02)	(56.47)	15.52	–	–	–	−0.65
Vader	2013–2014	(24.02)	64.36	25.68	–	Y	2.68	1.07
Atody	2014–2015	22.82	177.61	(147.01)	Y	Y	7.78	–
Helsinki	2014–2015	(24.02)	47.76	71.78	–	Y	1.99	2.99
Lane	2014–2015	(24.02)	9.75	434.19	–	N	−0.41	18.07
Ned	2014–2015	14.97	98.26	109.27	Y	Y	6.57	7.30
Vader	2014–2015	20.56	505.70	235.52	Y	Y	24.60	11.46
Zebra	2014–2015	17.88	100.08	113.30	Y	Y	5.60	6.34
Zephrix	2014–2015	13.81	(187.79)	94.99	–	–	–	6.88

before paternal care was observed.

To test our second prediction (P2), that E2 and/or E2/androgen ratios in expectant males (i.e., E2 levels during fetal development) are related to future paternal behavior, we ran Kendall's tau correlations relating *Total Paternal Care* to 1) average E2 or E2/androgen ratios during late fetal development (once male E2 has elevated; intervals −9 to 0), and 2) percent change in E2 or E2/androgen ratios from a male's baseline to infant birth (baseline to interval 0) (Table 1). Similarly, we evaluated whether either of these same two E2 values was related to a male's latency to exhibit paternal care (*Latency*). We adjusted significance for repeated tests using Bonferroni corrections.

Finally, to test our third prediction (P3) that E2 levels or E2/androgen ratios in new fathers (i.e., during intervals 0 to 37) are positively associated with paternal care behavior, we built generalized linear mixed-effect models using the lmer4 package (Bates et al., 2014) in R version 4.0.2 (R Core Team, 2020). Both mean E2 levels and E2/androgen ratios were significantly negatively linearly correlated with sampling interval during Period 3 (0 to 37; see Results below), so we included *Time* (i.e., 4-day interval) in every model. Additional fixed factors included a measure of paternal care behavior (*%Paternal Care*, *%Play*, *%Hold*, *%Huddle*, *%Carry*, or *%Groom*), and its interaction with *Time*. All models included male ID as a random effect.

We assessed models using an adjusted Akaike's Information Criterion (AICc) with the "dredge" function in the MuMIn package (Barton, 2020) to see which combination of effects best improved the model, and which variables had a significant impact on E2 concentrations (Bates et al., 2014; Tecot and Baden, 2018). We evaluated models using the change in AICc scores (ΔAICc) and Akaike weight value (*w*), with the 'best model' being that which had the lowest AICc score. Any model within 2 ΔAICc scores of the best model was considered equally good (reviewed in Symonds and Moussalli, 2011). We used likelihood tests to compare final models to a null model with no fixed effects to verify that our predictor variables improved model fit.

2.4. Ethics statement

This research was approved by the University of Arizona Institutional Animal Care and Use Committee (protocol 13-470) and Madagascar National Parks (055/15/MEEMEF/SG/DGF/DCB.SAP/ SCBSE), and adhered to the guidelines set forth by the American Society of Primatologists Principles for the Ethical Treatment of Non-Human Primates. It did not involve capture or handling of the study subjects, and study subjects were habituated or re-habituated prior to the start of field research.

3. Results

3.1. Estradiol

The GAM models indicated significant relationships between both fecal E2 and E2/androgen metabolite ratios across study periods (Fig. 1, Tables 2 & 3). In support of our first prediction (P1), fecal E2 and E2/

androgen metabolite ratios both increased significantly in expectant males during fetal development relative to baseline levels (Period 2, Intervals −24 to 0; Fig. 1B & C). Specifically, five of six males for which we had sufficient prenatal E2 and E2/androgen ratio data experienced at least a fourfold increase in E2 relative to the population baseline between 11 and 9 weeks pre-birth (intervals −11 to −9; Table 1; Fig. 1A). Levels remained elevated through parturition (interval 0), after which point E2 and E2/androgen metabolite ratios gradually decreased toward baseline (Period 3, Intervals 0 to 37; Fig. 1B & C; Tables 2 & 3). There was, however, considerable variation in individual patterns of E2 and E2/androgen ratios overall, as well as within periods (Fig. S1). Changes in E2 were unrelated to changes in fecal androgen metabolites, both overall (Period 1; $R^2 = 0.02158$, $F(1,95) = 2.095$, $\beta = 3.072$, $p = 0.1511$) and within pre- (Period 2; $R^2 = 0.045$, $F(1,23) = 1.077$, $\beta = 13.39$, $p = 0.310$) and post-birth time periods (Period 3; $R^2 = 0.022$, $F(1,69) = 1.553$, $\beta = 2.738$, $p = 0.217$) (Table 4, Fig. S2).

3.2. Estradiol × paternal care

Contrary to our second prediction (P2), neither E2 levels nor E2/androgen ratios in expectant males were related to their subsequent paternal care. This held true using average hormone levels (between intervals −9 to 0), binomial measures (elevated relative to own baseline, Y/N; elevated relative to mean population baseline, Y/N), and percent change from baseline (baseline to interval 0) (Table 5). Males did not vary in their latency to care, precluding the need for further analysis.

Finally, the best predictor of *Total Paternal Care* in new fathers (P3) included *Time* (4-day interval) ($p < 0.001$) (Table 5). This was true whether we included (A) Elevated E2 (Y/N), (B) E2, or (C) E2/androgen ratios as fixed effects. In all cases, the models including *Time* significantly outperformed the null model, and no other models performed equally as well. When considering individual care behaviors (*Carry*, *Groom*, *Hold*, *Huddle*, *Play*), even time was not a significant predictor; the best models for individual behaviors were always the null.

Table 2
Results of generalized additive models showing the relationship between E2 and time (4-day interval).

Period 1: Overall (−28 to 37)						
Linear effects	β	SE	t	p-Value	R^2	Deviance explained (%)
Intercept	4116.6	241.8	17.02	<0.001	0.204	
Smooth terms	edf	rf	F	p-Value		23
Time (4-day interval)	3.624	4.524	6.392	<0.001		

Period 2: Pre-natal (−28 to 0)						
Linear effects	β	SE	t	p-Value	R^2	Deviance explained (%)
Intercept	3896.9	403.9	9.647	<0.001	0.558	
Smooth terms	edf	rf	F	p-Value		59.4
Time (4-day interval)	1.929	2.415	12.65	<0.001		

Period 3: Post-natal (0 to 37)						
Linear effects	β	SE	t	p-Value	R^2	Deviance explained (%)
Intercept	4115	289	14.24	<0.001	0.12	
Smooth terms	edf	rf	F	p-Value		13.1
Time (4-day interval)	1.001	1.001	12.48	<0.001		

Abbreviations: β, parameter estimates; SE, standard error; t: t-value; edf, effective degrees of freedom; rf, reference degrees of freedom.

Table 3
Results of generalized additive models showing the relationship between E2/androgen ratios and time (4-day interval).

Period 1: Overall (−28 to 37)						
Linear effects	β	SE	t	p-Value	R^2	Deviance explained (%)
Intercept	105.76	15.57	6.791	<0.001	0.139	
Smooth terms	edf	rf	F	p-Value		17.1
Time (4-day interval)	3.495	4.364	3.816	<0.001		

Period 2: Pre-natal (−28 to 0)						
Linear effects	β	SE	t	p-Value	R^2	Deviance explained (%)
Intercept	111.41	32.49	3.429	<0.001	0.113	
Smooth terms	edf	rf	F	p-Value		15
Time (4-day interval)	1	1	4.048	<0.001		

Period 3: Post-natal (0 to 37)						
Linear effects	β	SE	t	p-Value	R^2	Deviance explained (%)
Intercept	99.48	17.86	5.569	<0.001	0.126	
Smooth terms	edf	rf	F	p-Value		14.4
Time (4-day interval)	1.507	1.858	5.155	<0.001		

Abbreviations: β, parameter estimates; SE, standard error; t: t-value; edf, effective degrees of freedom; rf, reference degrees of freedom.

Table 4
There was no relationship between either E2 levels or E2/androgen ratios in expectant males and their future levels of paternal care. Significance values adjusted using Bonferroni corrections.

A. Estradiol (E2)		
	Kendall's tau	p-value
Mean E2 (−9 to 0)	0.1428571	0.7726
E2, Elevated relative to own baseline	–	–
E2, Elevated relative to mean baseline	−0.1572427	0.6219
% change in E2 from baseline	0.2380952	0.5619

B. Estradiol (E2) to androgen ratio		
	Kendall's tau	p-value
Mean E2.A (−9 to 0)	0.3289758	0.3418
% change in E2.A from baseline	0.3333333	0.3813

'–' data were insufficient to test this relationship.

4. Discussion

Male red-bellied lemurs showed marked, prenatal elevations in fecal estradiol and estradiol/androgen metabolites. In the postnatal period, estradiol levels fluctuated and declined over time. These findings extend our research on hormonal changes in expectant and new fathers to a species with facultative paternal care. This same prenatal pattern has been observed in biparental callitrichine primates (Ziegler et al., 2004). Though the source of estradiol in red-bellied lemurs is not known, elevated prenatal estradiol levels may result from an increase in androgens and subsequent aromatization to estrogens. This conversion process has been shown to elicit paternal behavior in monogamous

Table 5
Conditional average from LMMs for repeated measures in predicting Total Paternal Care provided by males.

A. Elevated E2 (Y/N) – conditional average

Predictors	Estimate	SE	Adjusted SE	z value	Pr(>|z|)
(Intercept)	7.5541	10.2017	10.4687	0.722	0.470548
Elevated E2 (Y/N)	9.4177	10.1083	10.3931	0.906	0.364858
4-day interval	1.0332	0.3002	0.3086	3.348	0.000815*

B. Mean estradiol (E2) – conditional average

Predictors	Estimate	SE	Adjusted SE	z value	Pr(>|z|)
(Intercept)	24.1320	17.7546	18.0941	1.334	0.182
Average E2	−0.3693	2.4321	2.4790	0.149	0.882
4-day interval	1.2383	0.2561	0.2610	4.744	2.1e−06*

C. E2 to androgen ratio – conditional average

Predictors	Estimate	SE	Adjusted SE	z value	Pr(>|z|)
(Intercept)	21.4016	10.3048	10.4974	2.039	0.0415*
E2.androgen	0.7700	1.7965	1.8311	0.421	0.6741
4-day interval	1.2570	0.2574	0.2623	4.792	1.7e06*

* Indicates statistically significant effects.

California mice during the post-natal period (Trainor and Marler, 2002), and pre-natal elevations were hypothesized to prepare males for future infant care (e.g., Ziegler et al., 2004). There is no indication that pre-natal elevations or postnatal profiles were associated with the expression of paternal care behaviors measured in our study. While our study did not support this hypothesis, it is possible that these changes set the stage for paternal behavior and motivation, and that other factors influence its expression.

4.1. Temporal patterns

Paternal estradiol and estradiol/androgen ratio levels in this study were low in the first and second trimesters of their mate's pregnancy, and elevated in the last trimester. During the postnatal period, estradiol levels continued to reach very high levels, but they fluctuated a great deal. They gradually declined through time, but rarely fell as low as baseline levels until 3 months post-partum, which corresponds with the end of paternal infant carrying in this study (infant carrying was not observed after day 107). In women, salivary estradiol levels are also their highest at the end of their pregnancy and then plummet after they give birth, when they expel the placenta and thus remove that source of estradiol (Berg and Wynne-Edwards, 2002; Edelstein et al., 2015). The patterns in males are less clear. In men, only one study measured pre- and post-natal levels together, and they found no significant changes (Berg and Wynne-Edwards, 2002). However, one sample was analyzed at each timepoint, and the assay was unable to detect and differentiate low levels present in male samples, as indicated by the low number of samples in which estradiol was detectable (Berg and Wynne-Edwards, 2002). Therefore, estradiol profiles in expectant men are still unknown. In hamster species with exclusive maternal care and biparental care, serum estradiol levels elevate in expectant fathers to the range observed in females, though these changes were significant only in the *uniparental* species (Schum and Wynne-Edwards, 2005). In contrast, urinary estradiol levels in biparental, expectant Wied's black tufted-ear marmoset males are similar to what we observed in red-bellied lemurs, with higher estradiol levels four weeks prepartum vs. postpartum (Nunes et al., 2000).

While a case could be made for the benefits of lower estradiol for paternal care (see Edelstein et al., 2015), we based our prediction for elevated estradiol levels on the temporal patterns observed in females (Berg and Wynne-Edwards, 2001; Edelstein et al., 2015), the association between maternal responsiveness, sensitivity, and estradiol (Numan and Insel, 2003; Rilling and Young, 2014), the fact that fathers have higher estradiol vs. nonfathers in multiple species (Berg and Wynne-Edwards, 2001; Ziegler et al., 2004), and the elevated pre-partum levels observed in biparental marmosets (Nunes et al., 2000). Our results add to the number of studies that find elevated estradiol levels in a species with facultative biparental care. The small number of studies exploring prenatal estradiol levels in males makes it difficult to determine whether there is a prenatal paternal estradiol profile, and whether individual variation impacts paternal outcomes. This question will be informed by additional studies constructing longitudinal profiles with consistent sampling across the pre- and post-partum period, in both biparental and uniparental species.

4.2. Hormone-behavior relationships

Despite a pronounced elevation in expectant males, we found no indication that prenatal estradiol or estradiol/androgen ratios impacted the total amount of time devoted to future paternal care. Ziegler et al. (2004) found that cotton-top tamarin males similarly elevate estradiol levels during their mate's gestation, and suggested that it could prepare males to care for infants as soon as they're born. In this study, most males (86 %; 6 of 7 males in 2014–2015) initiated care by one week of infant age, so pre-partum elevations may similarly prepare males for paternal care shortly after infants are born. However, there was variation in the timing of paternal care onset (mean = 1.29 weeks, range = 0 to 4 weeks, n = 7). In male rats, estradiol decreases the latency to respond to foster infants from 5 to 2 days (Samuels and Bridges, 1983). We did not find sufficient variation in the timing of paternal care onset to determine whether prenatal estradiol levels were related to latency to care, likely due to our weekly sampling regime and our relatively small number of study subjects. This question requires further research that would benefit from more frequent sampling.

Increasing estradiol levels in expectant and new fathers (this study; Rafacz et al., 2012; Ziegler et al., 2004), and higher estradiol in fathers vs. non-fathers (Berg and Wynne-Edwards, 2001), could be explained by body fat differences (Gettler et al., 2014). In non-pregnant people, estradiol is primarily produced in the ovaries and testes via conversion from testosterone, but it is also produced in the adrenal glands and peripheral tissues, including adipose tissue (Nelson and Bulun, 2001). Overweight and obesity in the United States and Europe are more likely to occur in fathers than non-fathers (Laroche et al., 2007; Weng et al., 2004), and fathers may gain weight if they experience sympathetic pregnancy (Couvade Syndrome, Clinton, 1986), a response even found in expectant male cotton-top tamarins and common marmosets (Ziegler et al., 2006). Because estrogens are produced in part by adipose tissue, changes in paternal estradiol levels may not necessarily facilitate paternal care, as they may serve other functions, and/or reflect other correlated changes at that time. These concurrent changes in estradiol and weight may indicate that estradiol levels in expectant males are unrelated to paternal care behavior or the timing of its onset per se, and better reflect changes in weight gain in preparation for the energetic costs of infant care (Gettler et al., 2013). However, it is possible that prepartum estradiol elevations are a component of Couvade Syndrome, which in itself can prepare males for fatherhood, and has been associated with males' level of involvement in their partner's pregnancy (Clinton, 1986).

In new fathers, estradiol can facilitate paternal care in some species. In common marmoset fathers, treatment with low dose estradiol increased responsiveness of fathers to infant distress calls (Ziegler and Sosa, 2016). This acute response was not present in non-fathers, indicating that exogenous estradiol may not be sufficient to stimulate paternal care in non-fathers, but that it might facilitate paternal motivation in males who have already been primed in some way (Ziegler and Sosa, 2016), perhaps during their mate's gestation. In this study, long-term, endogenous estradiol profiles in red-bellied lemur fathers were not associated with any of the paternal behaviors that we measured,

though it is possible that the elevations above baseline that we observed facilitate ongoing parental motivation. Interestingly, Ziegler and Sosa (2016) found that low dose, but not high dose, estradiol stimulated paternal responsiveness, reflecting the complex actions that hormones can have at varying levels of administration (and circulation).

It is unclear what the fluctuating estradiol levels in the postnatal period reflect. In men, estradiol levels were not associated with self-reported play frequency or 30 min play bouts, which the authors note is consistent with the idea that estradiol changes are associated with body fat rather than paternal care (Gettler et al., 2013). If estradiol merely reflects adiposity, we would not expect such pronounced fluctuations, but rather a steady decline, after infants are born.

4.3. Hormone interactions

We calculated estradiol/androgen metabolite ratios to account for the possibility that they are interdependent and have combined effects on behavior. We previously found that in red-bellied lemurs, paternal androgen levels were negatively associated with infant carrying, and positively associated with infant huddling, grooming, and overall infant care (Tecot and Baden, 2018). Androgens are converted to estrogens through aromatization, and ratios of androgen and estradiol levels in serum have been used as a measure of this reaction (e.g., Franik et al., 2019). While fecal steroid metabolites may not similarly reflect this process, our results support a role for androgen conversion to estradiol during a mate's gestation. Importantly, hormones do not act alone; multiple hormones may have synergistic or antagonistic effects on behavior, and behavior can feed back onto multiple hormones to impact their secretion (Trumble et al., 2015; Lotz et al., 2022). Estradiol/androgen metabolite levels elevated in expectant males, but we did not find support for their combined role in the expression of paternal care behavior. In our study we calculated estradiol/androgen ratios from samples that were collected concurrently for each hormone. Hormonal changes can also have downstream effects on other hormones. For example, estradiol is necessary to stimulate the secretion of important maternal hormones including oxytocin (Ochedalski et al., 2007; Pedersen and Prange, 1979), prolactin, and progesterone (see Ziegler et al., 2009). In fact, estradiol stimulates prolactin which appears to have a priming function on the brain necessary for maternal care, and prolactin in turn decreases estradiol (Schradin and Anzenberger, 1999; see Ziegler et al., 2009). These non-linear, asynchronous relationships could explain why we did not find strong relationships between estradiol and paternal care. They could also help explain the role of elevated estradiol levels in expectant males.

5. Conclusions

Using profiles to explore hormonal changes over time in red-bellied lemur males, we have found that estradiol levels increase in expectant males (this study), that glucocorticoid levels increase mid-gestation and just after infants are born (Tecot, 2008), and that androgen levels vary with paternal behavior (Tecot and Baden, 2018). Our results are consistent with the hypothesis that fathers' hormonal changes during the prenatal period are responsive to their partners and prime fathers for care that begins shortly after parturition, and hormonal changes during the postnatal period are responsive to infants. Additional research could explore whether estradiol changes during the prenatal period are coordinated with other hormonal changes and have a priming effect on future fathers, and whether they are associated with changes in body mass. While we did not find evidence that estradiol levels predicted the total amount of paternal behavior each male provided, nor that it was related to specific paternal behaviors, there are several remaining questions regarding the role of estradiol in paternal care. For example, are estradiol levels associated with latency in responsiveness to infants and the initiation of care (vs. engagement in infant-initiated activities)? Can low-level elevations above baseline, such as those observed in this study, help maintain paternal care despite not explaining differences in the frequency of care? This research adds to our growing understanding of paternal hormonal profiles and their role in the pre- and postnatal periods, and highlights the importance of all individuals comprising the social environments of mothers and their offspring, before and after infants are born.

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Declaration of competing interest

None.

Data availability

Data are available upon request to the corresponding author.

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