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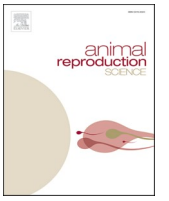

Prenatal transportation stress did not impact ovarian follicle count for three generations of female Brahman offspring

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ABSTRACT

As prenatal transportation stress altered behavior and adrenal glucocorticoid secretion of calves, we hypothesized that prenatal transportation stress would decrease ovarian reserve size and negatively impact female offspring fertility. The impact of prenatal transportation stress on ovarian follicle numbers in female offspring for three generations was studied. Brahman cows were transported for 2 h on day 60 ± 5, 80 ± 5, 100 ± 5, 120 ± 5, and 140 ± 5 of gestation. Ovaries were collected from offspring of transported or non-transported dams at multiple ages. Primordial, primary, secondary, and antral follicles were histologically analyzed. Antral follicle numbers were determined by ultrasound in a subset of offspring. Numbers of primordial, primary, secondary, and antral follicles were analyzed using the MIXED procedure, while the CORR procedure of SAS was used to determine the correlation between follicles observed by ultrasonography and histology. There were no differences ($P > 0.05$) in the number of primordial, primary, secondary, antral, or total follicles observed histologically due to treatment. Younger females had significantly greater numbers of follicles than older females ($P < 0.0001$). Antral follicles tended to be correlated with total histological ovarian follicles ($P = 0.10$). There was no difference in the number of antral follicles observed at ultrasound due to treatment ($P = 0.3147$), or generation ($P = 0.6005$) when controlling for age at observation. These results show that short-term transportation stress during early- to mid-gestation did not impact fertility as measured by ovarian follicle numbers in female Brahman offspring for three generations.

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1. Introduction

Fetal growth, development, and postnatal performance are processes influenced genetically, epigenetically, and environmentally (Wu et al., 2006). Primordial follicle formation occurs during early gestation, and fetal calf ovaries contain growing and antral follicles by late gestation (Fortune, 2003). All follicular stages are present around 170–190 days of gestation (Burkhart et al., 2010). Fixed primordial follicle number at birth (Erickson, 1966a) is associated with mature ovarian antral follicle number (Ireland et al., 2008). Antral follicle number is important to beef production as it is associated with reproductive efficiency (Cushman et al., 2009; Ireland et al., 2008; Martinez et al., 2016; McNeel and Cushman, 2015; Mossa et al., 2012; Starbuck-Clemmer et al., 2007). Antral follicle number is highly repeatable within individual cows, indicating potential use as a fertility biomarker (Burns et al., 2005).

Environmental and behavioral stressors impact reproductive factors (Dobson and Smith, 2000; Moberg, 1991; von Borell et al., 2007; Welsh et al., 1999); Wolfenson et al., 2000). The generational impact of maternal heat stress and undernutrition have been studied extensively. Dairy heifers from heat stressed dams in late gestation were lighter at birth, required more services to conception, were less likely to reach their first lactation and produced less milk in their first lactation compared to heifers from dams unexposed to heat stress (Monteiro et al., 2016). Akbarinejad et al. (2017) reported that maternal heat stress was detrimental to female offspring fertility when experienced in the second and third trimesters in dairy cattle. Alternatively, first trimester maternal undernutrition resulted in calves born with fewer antral follicles before and after puberty despite having similar birth weights, pubertal ages, and maximum follicular diameters compared to calves from control dams (Mossa et al., 2013).

The impact of transportation stress on fetal development and post-natal performance is less understood. Previous studies have reported greater circulating cortisol concentrations (Lay et al., 1997b; Littlejohn et al., 2016) and decreased cortisol clearance rate (Lay et al., 1997b) in calves from dams transported at five timepoints throughout early- and mid-gestation compared to calves born from control dams . Fetal calves from transported dams had heavier pituitary glands while having similar adrenal gland weights and plasma concentrations of adrenocorticotropin and cortisol compared to those from control dams (Lay et al., 1997a). Prenatal transportation stress resulted in calves born with different DNA methylation patterns compared to calves born to control dams (Baker et al., 2020; Littlejohn et al., 2018). Prenatally stressed heifer calves had hypomethylated and hypermethylated regions corresponding to genes involved in hypothalamic-pituitary-adrenocortical (HPA) axis regulation and pituitary function (Baker et al., 2020).

These results indicate that the HPA axis is altered in calves from transported dams, but it is unclear how these alterations influence hypothalamus or pituitary gland function, or if they impact folliculogenesis. It was hypothesized that prenatal transportation stress would decrease ovarian reserve size and negatively impact female offspring fertility. This hypothesis was tested by assessing the impact of prenatal transportation stress on female offspring fertility measured by ovarian follicle count.

2. Materials and methods

Animals included in Replicates 1 and 3 of this experiment were born in 2012 to females included in the analysis of Price et al. (2015) reporting maternal response to repeated transportation. Additionally, animals in Replicates 1, 2, and 3 are cohorts of animals included in the experiments of Littlejohn et al., (2016, 2018) and Baker et al. (2020) reporting changes to temperament, circulating serum cortisol, and genome-wide DNA methylation patterns due to prenatal transportation. It is, therefore, evident that the progeny involved in the current study are developing *in utero* in an environment that is physiologically different, influencing post-natal development and physiology.

2.1. Experimental Design

Pregnant Brahman cows were assigned to one of two treatment groups based on methods described by Price et al. (2015). Cows were transported by truck and trailer for 2 h on days 60 ± 5, 80 ± 5, 100 ± 5, 120 ± 5, and 140 ± 5 of gestation or pastured and not transported in 2011 and 2018. Female offspring of transported (Stressed, $n = 26$) or non-transported (Control, $n = 23$) dams were slaughtered at approximately 1825 days of age (2017, Replicate 1, $n = 14$, Stressed: $n = 7$, Control: $n = 7$), 25 days of age (2019, Replicate 2, $n = 15$, Stressed: $n = 7$, Control: $n = 8$) or ovariectomized at approximately 2920 days of age (2020, Replicate 3, $n = 20$, Stressed: $n = 12$, Control: $n = 8$).

2.2. Histology

In Replicate 1, tissues were embedded and sectioned at Texas A&M College of Veterinary Medicine and Biomedical Sciences, College Station, TX, while tissues from Replicates 2 and 3 were embedded and sectioned at US Meat Animal Research Center, Clay Center, NE. Histological analysis of ovarian follicular populations was performed as previously described (Cushman et al., 2019; Tenley et al., 2019).

In Replicate 1, ovaries were collected at slaughter, and the ovary contralateral to the corpus luteum (if present) was measured and weighed before a section (<10 mm) of ovarian cortex was fixed in formalin, then embedded in paraffin and serially sectioned at eight μm. Sections were placed on slides and stained with hematoxylin and picric methyl blue. Numbers of total, primordial, primary, secondary, and antral follicles were determined per section every 40th section.

In Replicates 2 and 3, whole ovaries (contralateral to the corpus luteum if present) were collected at euthanasia (Replicate 2) or ovariectomy (Replicate 3), fixed in formalin, embedded in paraffin, and serially sectioned at six μm. Five sections (at least 60 μm apart)

were placed on slides, stained with hematoxylin and eosin and numbers of total, primordial, primary, secondary, and antral follicles were determined per section.

In all replicates, ovarian follicle counts were determined by a single technician blind to treatment and only follicles with an oocyte containing a nucleus were counted. Primordial follicles were defined as those containing an oocyte surrounded by a single layer of flattened granulosa cells. Primary follicles were defined as those containing an oocyte surrounded by a single layer of cuboidal granulosa cells. Secondary follicles were defined as those containing an oocyte surrounded by two to six layers of cuboidal granulosa cells with no antrum. Antral follicles were defined as those containing an oocyte surrounded by six or more layers of cuboidal granulosa cells, a fully formed theca interna and zona pellucida, and an antrum. Total numbers of follicles per section were determined by adding the number of primordial, primary, secondary, and antral follicles per section. A single primordial, primary, secondary, antral, and total follicle count was determined by calculating an average of the five sections counted. Additionally, ovarian length (mm) and width (mm) were determined as measured on slides and averaged for a single ovarian length (mm) and width (mm) measurement per animal. To allow for consistent measurement and analysis across all replicates, ovarian area (mm^2) was determined by multiplying average length (mm) and width (mm) for each ovary and follicles per mm^2 was analyzed. Proportions of primordial, primary, secondary, and antral follicles were calculated as proportions of the total number of follicles per mm^2 of ovarian tissue.

2.3. Ultrasonography

To determine the relationship between follicles observed by ultrasonography and histology, cows in Replicate 3 ($n = 20$, Stressed: $n = 12$, Control: $n = 8$) were subjected to 3D ultrasonography (GE Voluson I: SonoAVC, Boston, MA) to determine total antral follicle number 61 days prior to collection of ovaries at ovariectomy and histological analysis of ovarian follicle number. Cows were not synchronized prior to ultrasonography, and thus, observation of antral follicle number is not specific to any particular day of the estrous cycle.

2.4. Impact of prenatal transportation stress through three generations

To analyze the potential impact of prenatal transportation stress on the fertility of future generations, cows ($n = 44$, Stressed: $n = 28$, Control: $n = 16$) were grouped by generations removed from prenatal transportation stress. Female offspring ($n = 20$) born to transported (Stressed: $n = 12$) and non-transported (Control: $n = 8$) dams were grouped into the first generation. Female offspring born to first generation animals ($n = 18$; Stressed: $n = 14$ and Control: $n = 4$) were grouped into the second generation, and female offspring born to second generation animals ($n = 6$; Stressed: $n = 2$ and Control: $n = 4$) were grouped into the third generation. All females were subjected to 3D ultrasonography (GE Voluson I: SonoAVC, Boston, MA) in year 2020 or 2021 to determine total antral follicle number at various ages (1 year: $n = 9$, 2 years: $n = 4$, 3 years: $n = 4$, 5 years: $n = 4$, 6 years: $n = 3$, 8 years: $n = 20$).

2.5. Statistical analysis

Data were checked for heterogeneity of variance using Hartley's test (Cushman et al., 2002) and normality using distribution of Pearson's residuals. Due to significant heterogeneity, data were log transformed for statistical analysis and reversed transformed for presentation purposes.

Ovarian follicle count for primordial, primary, secondary, antral, and total follicles per mm^2 was analyzed using the MIXED procedure of SAS (version 9.4; SAS Inst. Inc., Cary, NC, USA). The statistical model included treatment (Stressed or Control), age (25, 825, and 2920 days) and their interaction (treatment by age) as fixed effects. The CORR procedure of SAS was used to determine the correlation between follicles observed by 3D ultrasonography and at histological analysis. Differences in antral follicle number determined by 3D ultrasonography were analyzed using the MIXED procedure of SAS with treatment (Stressed or Control), generation (1, 2, or 3), and their interaction (treatment by generation) as fixed effects, as well as age at ultrasound as a covariate. Statistical

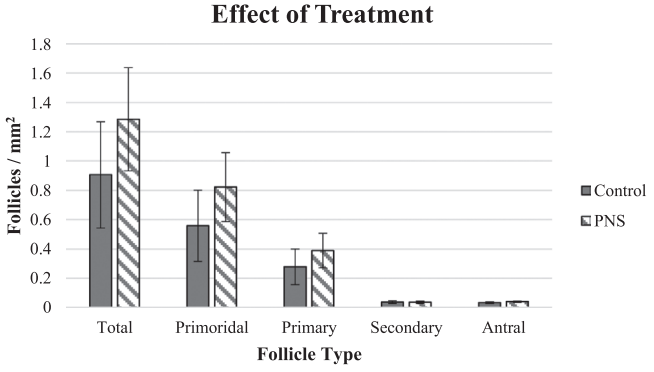

Fig. 1. Numbers of each follicle type per mm^2 of ovarian tissue from control or prenatally stressed offspring. No differences were observed due to treatment ($P > 0.10$).

significance was identified at $P \leq 0.05$ and statistical tendency as identified at $0.05 < P \leq 0.10$. Pairwise comparisons were analyzed when a main effect was statistically significant.

3. Results

There was no significant interaction of treatment and age for primordial ($P = 0.36$), primary ($P = 0.84$), secondary ($P = 0.59$), antral ($P = 0.60$), or total ($P = 0.64$) follicles per mm^2. There were no differences in the number of primordial ($P = 0.66$; S = 0.82 ± 0.24/mm^2, C = 0.56 ± 0.24/mm^2), primary ($P = 0.98$; S = 0.39 ± 0.12/mm^2, C = 0.28 ± 0.12/mm^2), secondary ($P = 0.27$; S = 0.04 ± 0.01/mm^2, C = 0.04 ± 0.01/mm^2), antral ($P = 0.79$; S = 0.04 ± 0.01/mm^2, C = 0.03 ± 0.01/mm^2), or total ($P = 0.84$; S = 1.29 ± 0.35/mm^2, C = 0.91 ± 0.36/mm^2) follicles per mm^2 of ovarian tissue due to treatment (Fig. 1). Age significantly impacted ovarian follicle count per mm^2 of ovarian tissue ($P < 0.0001$, Table 1), such that females in Replicate 2 (25 days) had greater numbers of primordial, primary, secondary, antral, and total follicles compared to females in Replicate 1 (1825 days) and Replicate 3 (2920 days). While there was no difference in numbers of primordial ($P = 0.51$), and secondary ($P = 0.54$) follicles per mm^2 of ovarian tissue, females in Replicate 1 (1825 days) had greater numbers of primary ($P = 0.01$; 1825 = 0.04 ± 0.16/mm^2, 2920 = 0.02 ± 0.13/mm^2), antral ($P < 0.01$; 1825 = 0.03 ± 0.01/mm^2, 2920 = 0.02 ± 0.01/mm^2), and total ($P = 0.05$; 1825 = 0.12 ± 0.47/mm^2, 2920 = 0.08 ± 0.40/mm^2) follicles per mm^2 of ovarian tissue compared to females in Replicate 3 (2920 days). Antral follicle numbers determined by 3D ultrasonography tended to be correlated with total ovarian follicle numbers determined by histological analysis in Replicate 3 ($n = 20$, r = 0.6735, $P = 0.10$).

There was no significant interaction of treatment and age on percentages of primordial ($P = 0.19$), primary ($P = 0.45$), secondary ($P = 0.50$), and antral ($P = 0.50$) follicles per mm^2 of ovarian tissue. Proportions of primordial ($P = 0.76$; S = 40.68 ± 3.57 %, C = 42.24 ± 3.69 %), primary ($P = 0.51$; S = 31.67 ± 2.06 %, C = 26.69 ± 2.13 %), secondary ($P = 0.15$; S = 5.42 ± 1.14 %, C = 7.82 ± 1.17 %), and antral ($P = 0.63$; S = 22.23 ± 2.87 %, C = 20.25 ± 2.96 %) follicles per mm^2 of ovarian tissue were not different between treatments (Fig. 2). Age significantly impacted proportions of primordial ($P < 0.0001$), secondary ($P = 0.03$), and antral ($P < 0.0001$) follicles per mm^2 of ovarian tissue. The proportion of primordial follicles in females in Replicates 1 and 3 was less than the proportion of primordial follicles per mm^2 of ovarian tissue in females in Replicate 2, while the proportion of antral follicles in females in Replicates 1 and 3 was greater than the proportion of antral follicles per mm^2 of ovarian tissue in females in Replicate 2 (Table 2). Additionally, females in Replicate 3 had a greater proportion of secondary follicles per mm^2 of ovarian tissue compared to females in Replicates 1 and 2 (Table 2).

There was no significant interaction of treatment and generation ($P = 0.13$) considering the number of antral follicles. Controlling for age at ultrasound examination, there was no difference in the number of antral follicles determined by 3D ultrasonography due to treatment ($P = 0.3147$), or generation ($P = 0.6005$).

4. Discussion

The present study demonstrates that prenatal transportation stress culminating over five events from early- to mid-gestation does not impact any stage of follicular development in female Brahman offspring, regardless of offspring age or the number of generations removed from prenatal stress at the time of analysis. Age significantly impacted the number of primordial, primary, secondary, antral, and total ovarian follicles per mm^2 of ovarian tissue. These results in regard to a relationship between ovarian follicle numbers and cow age support the results of previous research such that numbers of primordial as well as growing follicles decrease with cow age (Erickson, 1966a).

The results of the present study regarding the effects of prenatal stress differ from those reported by several others, and what may have been anticipated in the present study. In mice, prenatally stressed female offspring had fewer primordial, primary, and secondary (Barra et al., 2014), as well as antral follicles (García-Vargas et al., 2019) compared to female offspring that were not prenatally stressed. These studies differed in a variety of ways from the present study; however, as chronic cold stress was implemented in late-gestation (García-Vargas et al., 2019) or throughout the entirety of gestation (Barra et al., 2014) in a rodent model. In cattle, Mossa et al. (2013) reported that restricting pregnant beef heifers to 60 % of their maintenance energy requirement during the first 110 days of gestation decreased antral follicle numbers in female offspring. Additionally, gestating dairy cows exposed to heat stress in the second, or third trimesters gave birth to female offspring with decreased concentrations of anti-Mullerian hormone [indicative of fewer

Table 1
Mean numbers of follicles (per mm^2) for each age category.

	Age (d)			*P* - value
Follicle category	25 Mean ± SE	1825 Mean ± SE	2920 Mean ± SE	
Primordial	1.99 ± 0.30^a	0.04 ± 0.31^b	0.04 ± 0.27^b	< 0.0001
Primary	0.94 ± 0.15^a	0.04 ± 0.16^b	0.02 ± 0.13^b	< 0.0001
Secondary	0.10 ± 0.01^a	0.01 ± 0.01^b	0.01 ± 0.01^b	< 0.0001
Antral	0.06 ± 0.01^a	0.03 ± 0.01^b	0.02 ± 0.01^b	< 0.0001
Total	3.09 ± 0.45^a	0.12 ± 0.47^b	0.08 ± 0.40^b	< 0.0001

^{ab}Means without a common superscript differ within a row by the given *P*-value.

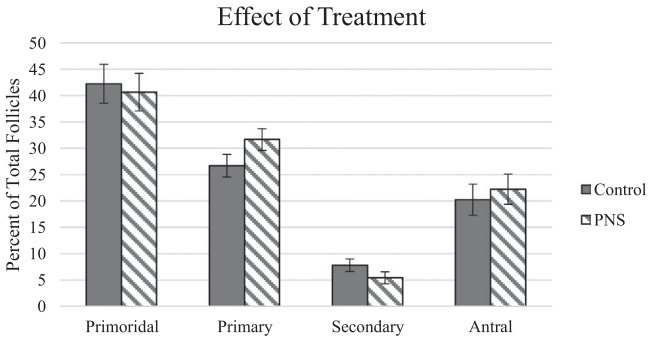

Fig. 2. Proportion of each follicle type (%) in control and prenatally stressed offspring. No differences were observed due to treatment ($P > 0.10$).

Table 2
Proportion of each follicle type (%) for each age category.

	Age (d)			
	25	1825	2920	*P* - value
% of Total Follicles	Mean ± SE	Mean ± SE	Mean ± SE	
Primordial	59.93 ± 4.57[a]	28.93 ± 4.72[b]	35.52 ± 4.03[b]	< 0.0001
Primary	31.09 ± 2.64	33.75 ± 2.72	27.21 ± 2.33	0.19
Secondary	4.70 ± 1.45[a]	5.61 ± 1.50[a]	9.55 ± 1.28[b]	0.03
Antral	4.29 ± 3.66[a]	31.70 ± 3.78[b]	27.73 ± 3.23[b]	< 0.0001

[ab]Means without a common superscript differ within a row by the given *P*-value.

ovarian follicles (Ireland et al., 2008)] compared to female offspring that had not been exposed to prenatal heat stress (Akbarinejad et al., 2017). Dairy cows experiencing high environmental temperatures in early gestation gave birth to heifers with smaller ovarian reserves (lower AFC and AMH concentrations) compared to heifers born to cows experiencing lower temperatures in early gestation (Succu et al., 2020). Evans et al. (2012) reported that pregnant cows in a diseased state produced female offspring with decreased circulating concentrations of AMH as well. The results of the present study may differ from previous reports of relationships between prenatal stressors and ovarian follicle numbers in female bovine offspring due to the nature, duration, and intensity of the stressor. Methods used to induce stress in pregnant dams by both García-Vargas et al. (2019) and the present study increased circulating concentrations of corticosteroids (Lay et al., 1996), indicating activation of the HPA axis and a successful stress response in the gestating female. Nevertheless, it is not known how the responses to heat, nutrient restriction, and transportation stress compare in a similar species. For example, while heat stress and stress induced by a disease state can differ in chronic and acute nature, both have similar modes of alteration to fertility, as they both impact the HPG axis in dairy cattle (Roth and Wolfenson, 2016). Additionally, the response to and impact of similar types of stressors likely differ from species to species.

While many studies focusing on prenatal transportation stress have reported alterations to the HPA axis in cattle (Baker et al., 2020; Lay et al., 1997a, 1997b; Littlejohn et al., 2016, 2018), the impact to the hypothalamic-pituitary-gonadal (HPG) axis is less known. There are many interactions between the HPA and HPG axes; however, as glucocorticoids produced by the adrenal gland in response to stress are inhibitory to gonadotropin-releasing hormone (GnRH) at the hypothalamus level, as well as to luteinizing hormone (LH) and follicle stimulating hormone (FSH) at the pituitary level (reviewed by (Welsh et al., 1999 and Whirledge and Cidlowski, 2010). While prenatal transportation stress changed methylation patterns in genes related to abnormal hypothalamic and pituitary function (Baker et al., 2020), the present study suggests that any possible changes to the function of the hypothalamus and pituitary gland due to prenatal stress were not inhibitory to follicular development and growth. Knowing that calves born to dams who had been transported during gestation had greater circulating cortisol concentrations and a decreased rate of cortisol clearance, the reproductive efficiency of females exposed to prenatal transportation stress may still be negatively impacted through the inhibitory actions of cortisol on reproduction (Dobson and Smith, 2000; Moberg, 1991; Whirledge and Cidlowski, 2010). While there was no difference in reproductive potential, with regard to the number of ovarian follicles present, reproduction may still be impacted by changes in the steroidogenic capacity of follicular cells, and follicular fluid composition due to the increase in circulating cortisol. Previous studies that cultured bovine cells have reported stimulatory effects of cortisol on the number of granulosa and theca cells as well as progesterone production, while high doses of cortisol have been reported to decrease the number of LH receptors and inhibit estradiol production in granulosa cells (Kawate et al., 1993; Spicer and Chamberlain, 1998).

To our knowledge, this is the first study to analyze the transgenerational impact of prenatal transportation stress on ovarian follicle numbers in three subsequent generations of cattle. While environmental stress factors have provoked transgenerational inheritance in rodents (Franklin et al., 2011; Yao et al., 2014), the present study indicates no transgenerational impact of prenatal transportation stress on ovarian follicle populations. While different methylation patterns were observed in leukocytes of Brahman heifers due to transportation stress (Baker et al., 2020), perhaps the same patterns are not imprinted and observed in germline cells and therefore, not

passed to future generations. Furthermore, the number of primordial oocytes undergo a massive increase followed by an extensive decrease during prenatal development, such that the population decreases from approximately 2 million to 100 000 by day 170 of gestation (Erickson, 1966b). It may be feasible that germ cells impacted by potential changes in methylation due to stress are simply lost in the rapid decline due to apoptosis of primordial germ cells. Additionally, while pregnant dams were transported during key timepoints of oogonial mitosis and meiosis in the present study, the primordial germ cells had already migrated and were established in an undifferentiated gonad (Erickson, 1966b). Primordial germ cells in the developing fetus therefore would have already gone through epigenetic reprogramming and potential methylation patterns induced by transportation stress that were not imprinted may be lost (Huber et al., 2020; Ispada et al., 2018; Seisenberger et al., 2013).

5. Conclusions

In conclusion, short-term transportation of pregnant cows during early- to mid-gestation is not detrimental to fertility, as measured by ovarian follicle count, of female offspring in multiple generations. These data suggest that management practices such as trailering pregnant cows in instances of inadequate feed resources or at the time of sale do not impact the reproduction potential of female offspring by altering ovarian follicular populations.

CRediT authorship contribution statement

Lacey K. Quail: Analysis, Writing – original draft. **Ronald D. Randel:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing. **Thomas H Welsh, Jr.:** Conceptualization, Methodology, Writing – review & editing. **Robert A. Cushman:** Methodology, Writing – review & editing. **Hannah K. Yake:** Methodology, Writing – review & editing. **Rui A. d'Orey Branco:** Methodology, Writing – review & editing. **Donald A. Neuendorff:** Methodology, Writing – review & editing. **George A. Perry:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Competing Interests Statement

Robert A. Cushman is an Editor of Animal Reproduction Science, but was blinded from the peer review process for this paper.

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