

**Immunoglobulin J chain as a non-invasive indicator of pregnancy in the cheetah (*Acinonyx jubatus*)**

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## 1 **Abstract**

2           The North American cheetah population serves as a reservoir for the species, and acts as  
3 a research population to help understand the unique biology of the species. Little is known about  
4 the intrauterine physiology of the cheetah, including embryo differentiation, implantation, and  
5 the development of the placenta. After mating, cheetah females frequently experience (30-65%  
6 of matings) a non-pregnant luteal phase where progesterone metabolite levels match those found  
7 in pregnant females for the first ~55 days of gestation, but parturition does not occur.  
8 Immunoglobulin J chain (IgJ) is a molecule that is involved in the activation of the secretory  
9 immune response and has been found to be indicative of pregnancy in the cheetah using fecal  
10 monitoring. In this study, western blotting was employed to track IgJ abundance in pooled  
11 weekly fecal samples following natural breeding or exogenous stimulation to ovulate, and IgJ  
12 levels were compared between individuals undergoing a pregnant (n = 12) and non-pregnant (n =  
13 19) luteal phase. It was revealed that IgJ abundance was increased in pregnant females compared  
14 to non-pregnant females at week 4 and week 8 post-breeding, indicating the potential modulation  
15 of maternal immunity in response to sensitive events such as implantation and the increased  
16 secretory activity of the placenta. IgJ levels also tended to be higher early after breeding in  
17 females that were bred naturally with intact males compared to exogenously stimulated females  
18 with no exposure to seminal plasma, indicating the promotion of maternal tolerance to seminal  
19 antigens present upon embryonic implantation. Monitoring fecal IgJ may be a potential method  
20 to determine gestational status in the cheetah and will aid future conservation efforts of the  
21 species.

## 22 **Introduction**

23           The cheetah (*Acinonyx jubatus*) is listed as vulnerable by the International Union for  
24 Conservation of Nature (IUCN), with an estimated population of ~7100 individuals in the wild,  
25 and numbers continuously decreasing due to habitat fragmentation and human conflict [1].  
26 Because of the threats to wild cheetahs, an *ex situ* population is critical to serve as an insurance  
27 population should the wild cheetah's numbers diminish further. The *ex situ* population can serve  
28 as a reservoir for the species and could potentially be used for reintroduction efforts in the future.  
29 The insurance population is also invaluable for research purposes, allowing for studies that  
30 cannot be conducted on *in situ* populations due to the scarcity of the species in the wild.

31           The cheetah is an induced ovulator, meaning that mating or exogenous hormones are  
32 necessary for ovulation to occur [2]. While the reproductive events of the domestic cat have been  
33 studied in depth [3, 4], little is known about the intrauterine physiology following breeding in  
34 wild felids, including the timing of events such as embryo differentiation, implantation, and  
35 placentation. Interestingly, cheetahs in human care often encounter reproductive challenges that  
36 their wild counterparts do not, as many breedings among cheetahs in human care are  
37 unsuccessful. "Pseudopregnancy," or a non-pregnant luteal phase, has occurred in up to 30% to  
38 60% of matings in North American zoos over recent years (2013-2018; Crosier, personal  
39 communication). In these unsuccessful matings, ovulation is confirmed by detectable rises in  
40 progesterone metabolites in feces, serum or urine [2, 5]. The concentration of these metabolites is  
41 elevated for approximately 55 days, and during this time the hormonal profile of a non-pregnant  
42 individual is indistinguishable from a pregnant individual. The high prevalence of a non-  
43 pregnant luteal phase after breeding in cheetahs under human care has greatly reduced the

44 reproductive potential of the *ex situ* population and has contributed to the challenge of reaching  
45 sustainability due to the impact on the genetic diversity of the population.

46         Recent advances in mass spectrometry and other proteomic analyses have led to the study  
47 of excreted biomarkers as diagnostic or treatment tools in a clinical setting [6]. The production of  
48 some biomarkers has been shown to be affected by reproductive events, and certain biomarkers  
49 have been found to indicate physiological status such as pregnancy in the domestic dog [7] and  
50 several wild canid species [8]. Recently, methods have been developed for the identification of  
51 fecal biomarkers of pregnancy in the polar bear [9], and another study in the cheetah identified  
52 fecal biomarkers with potential roles in early pregnancy establishment using commercially  
53 available antibodies [10]. Koester and colleagues identified a novel biomarker immunoglobulin J  
54 chain (IgJ), with increased levels in pregnant individuals and were able to distinguish between  
55 pregnant and non-pregnant cheetahs in the 4 weeks following breeding. IgJ is a small  
56 polypeptide that serves to regulate polymer formation of Immunoglobulin A (IgA) and  
57 Immunoglobulin M (IgM), modulating the secretory activity of these molecules [11, 12]. IgJ  
58 functions to provide high levels of avidity to IgA and IgM and facilitates their exocrine transfer  
59 to mucosal surfaces [13]. The secretory immunoglobulins that IgJ helps to activate are integral in  
60 the response to foreign antigens at surfaces such as the endometrium, and IgJ expression is likely  
61 modulated by the unique physiological status of pregnancy. Placental factors that are absent in  
62 females undergoing a non-pregnant luteal phase may act to modulate the maternal immune  
63 response and affect IgJ abundance, allowing for IgJ monitoring as a method for distinguishing  
64 between the gravid and non-gravid states in the cheetah. The objective of the current study was  
65 to evaluate the temporal patterns of fecal IgJ abundance over the course of pregnancy in the  
66 cheetah to determine the timing of intrauterine events that result in either a successful pregnancy

67 or a non-pregnant luteal phase. Changes in IgJ abundance may indicate maternal immune  
68 modulation and could reveal certain events such as implantation and placental development that  
69 occur during the establishment of pregnancy early after breeding.

70

## 71 **Materials and methods**

### 72 **Animals**

73 This study was conducted according to the recommendations in the Guide for the Care  
74 and Use of Laboratory Animals of the National Institutes of Health. The female cheetahs  
75 included in this study (n=17 individuals) were all housed at accredited Association of Zoos and  
76 Aquariums (AZA) institutions within the United States. All subjects were born *ex situ* and  
77 managed according to the guidelines developed by the Cheetah Species Survival Plan (SSP). The  
78 animals included in the study were female adults from 2 to 12 years of age (mean  $\pm$  standard  
79 error of the mean (SEM) =  $6.0 \pm 0.6$  y). The animals in this study were fed a diet of commercial  
80 beef or horse-based meat product (Central Nebraska Packing, Inc., Milliken Meat Products Ltd,  
81 or Carnivore Diet 10; Natural Balance Pet Foods Inc., Pacoima, CA) a minimum of five days per  
82 week, with supplements that included whole rabbit, beef and horse bone, or organ meat. Water  
83 was available *ad libitum*.

84 Fecal samples were collected opportunistically from females that were naturally bred  
85 according to SSP breeding management recommendations and from females receiving  
86 exogenous gonadotropins to stimulate ovulation. Exogenous gonadotropin therapy was  
87 conducted under IACUC approval for separate projects according to previously published  
88 methods [14, 15], and included the stimulation of follicular development (with equine chorionic  
89 gonadotropin), followed by stimulation of ovulation (with human chorionic gonadotropin or

90 porcine luteinizing hormone). Pregnancy was confirmed by the birth of offspring, and non-  
91 pregnant luteal phase was confirmed by an increase in progesterone metabolite concentration  
92 after either natural breeding with no cubs produced or exogenous gonadotropin administration.

### 93 **Sample collection and preparation**

94 Fecal samples were collected non-invasively from enclosures approximately 3-4 times  
95 weekly. Only fresh samples (deposited within 24 h) were chosen. Samples, about 50g in size,  
96 were collected in individual plastic bags and immediately stored in a -20°C freezer. Individual  
97 fecal samples were then lyophilized (VirTis, 35L Ultra Super XL-70, Gardiner, NY), crushed,  
98 and transferred to individually labeled tubes. Reproductive cyclicity and ovulation were  
99 confirmed by steroid hormone metabolite analysis. Fecal samples underwent a steroid hormone  
100 metabolite extraction protocol according to previously published methods [16, 10]. Extraction  
101 efficiency was determined by the addition of radiolabeled <sup>3</sup>H-progesterone prior to shaking  
102 extraction. The mean extraction efficiency ( $\pm$ SEM) was found to be 73.6%  $\pm$  0.2% for all  
103 samples.

104 Weekly pooled fecal samples of 0.5g were created by combining approximately 0.125g  
105 of four individual samples in a 15 mL centrifuge tube. Individual samples from day 1-7 post-  
106 breeding were used to create the pooled sample for week 1. Individual samples from day 8-14  
107 post-breeding were used to create the pooled sample for week 2, etc. Total protein was  
108 subsequently extracted from pooled samples as follows. 6 mL of 0.1 M phosphate buffered saline  
109 (0.138 M NaCl, 0.0027 M KCl; pH, 7.4) with protease inhibitor (1:1000) was added to the  
110 pooled fecal sample, and the mixture was shaken for 30 min and centrifuged at 4600 x g for 30  
111 min. The supernatant was filtered using a 0.22  $\mu$ m syringe driven filter unit (Millipore Sigma),  
112 and the proteins were then precipitated from the supernatant using a 60% ammonium sulfate

113 saturation. The ammonium sulfate solution was shaken for 30 min and centrifuged at 7000 x g  
114 for 30 min. The protein extract pellet was collected and resuspended in 400  $\mu$ L of phosphate  
115 buffered saline with protease inhibitor. This protein extract solution was then desalted using a 3  
116 kDa Millipore spin column (Amicon Ultra-0.5) and centrifugation at 7400 x g. All extraction  
117 steps were performed at 4°C. Extracted samples were then run on a Bradford assay (Bio-Rad  
118 Protein Assay, Hercules, CA) to determine total protein concentration. Briefly, standards for the  
119 assay were created by serial dilution at 0.388 mg/mL to 0.012 mg/mL. Fecal protein samples  
120 were diluted to 1:30, and 10  $\mu$ L of each sample was added to a well. 200  $\mu$ L of Bio-Rad Quick  
121 Start™ Bradford Dye Reagent was added to each well, and after 5 minutes protein  
122 concentrations were determined using a Molecular Devices Filtermax F5 plate reader.  
123 Differences in steroid hormone and total protein concentrations between pregnant and non-  
124 pregnant groups were determined using a Student's T-test in R (version 3.3.2) [17], with  
125 differences considered significant at  $P < 0.05$ .

## 126 **Steroid hormone metabolite analysis**

127 Steroid hormone neat extracts were diluted 1:20 to 1:16,000 in phosphate buffer (2.2 M  
128  $\text{NaH}_2\text{PO}_4$ , 3.5 M  $\text{Na}_2\text{HPO}_4$ , 0.3 M NaCl,  $\text{H}_2\text{O}$ ; pH, 7.0) and were run for analysis on enzyme  
129 immunoassay (EIA). Estrogen metabolites in diluted fecal extracts were used to determine  
130 reproductive cyclicity using an estradiol EIA that has been validated for use in the cheetah [18].  
131 Briefly, for samples collected before 2015, a polyclonal anti-estradiol antibody (R4972; C.  
132 Munro, University of California, Davis, CA) was added to a 96-well microtiter plate and  
133 incubated for 12 h. Diluted samples, standards, and peroxidase-enzyme conjugated  $17\beta$ -estradiol  
134 were added, and the plate was incubated for 2 h at 23°C. Unbound components were washed off,  
135 and an ABTS chromogen solution (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) was

136 added as a substrate. Optical densities of each well on the plate were determined using a  
137 microplate reader (Molecular Devices Filtermax F5, reading filter 405 nm, reference filter 540  
138 nm). Estrogen metabolite concentrations for samples collected from 2015-2018 were determined  
139 using a revised protocol for the estradiol EIA [18]. Briefly, diluted samples, standards,  
140 peroxidase enzyme conjugated 17 $\beta$ -estradiol, and antibody (R4972; C. Munro, University of  
141 California, Davis, CA) were added to a pre-coated goat-anti-rabbit IgG plate, and the plate was  
142 incubated for 2 h at 23°C. Unbound components were washed off, a TMB chromogen solution  
143 (3,3', 5,5'-tetramethylbenzidine) was added as a substrate, and the reaction was halted with  
144 addition of 1N HCL. Optical densities of each well on the plate were determined using a  
145 microplate reader (Molecular Devices Filtermax F5, reading filter 405 nm, reference filter 540  
146 nm). Inter-assay variation was monitored through the use of two internal controls, and  
147 coefficients of variation for all samples in duplicate were <10%.

148 Progesterone metabolites were used to determine ovulation and the presence of a luteal  
149 phase. Concentrations were determined using a progesterone EIA that has been validated for use  
150 in the cheetah [18], using a monoclonal progesterone antibody (no. CL425, Quidel Co., San  
151 Diego, CA), and an associated peroxidase-enzyme conjugated to progesterone (C. Munro,  
152 University of California, Davis, CA). Plates were prepared and run using the same procedure as  
153 the estradiol assay, with samples run using the revised protocol and pre-coated goat-anti-mouse  
154 IgG plates beginning in 2012. Internal controls were used to monitor inter-assay variation, and  
155 coefficients for samples in duplicate were <10%. In both cases, fecal hormone metabolite  
156 concentrations and profiles were comparable across the two EIA protocols.

## 157 **Western blotting and protein quantification**



158 Total protein samples were diluted to 2 mg/mL in MilliQ water to a final volume of 30  
159  $\mu$ L. Human recombinant IgJ (Abcam #140727) was used as a positive control at 16.67  $\mu$ g/mL.  
160 Samples were then separated by SDS-PAGE, transferred to a PVDF membrane, blocked with 5%  
161 milk, and incubated overnight at 4°C with a primary antibody (Aviva Systems Biology  
162 ARP55440\_P050) diluted 1:1000 in 1% milk. This polyclonal antibody was developed in a  
163 rabbit against human recombinant IgJ and was previously found to be reactive to cheetah IgJ in  
164 western blot [10]. The membrane was then incubated with a secondary antibody (Cell Signaling  
165 Technology, Anti-Rabbit IgG HRP-linked antibody, #7074S) diluted 1:2500 in 1% milk, and  
166 then incubated with a chemiluminescent substrate (Bio-Rad, Clarity Max Western ECL  
167 Substrate, #1705062). Membranes were imaged on a G:Box Chemi XRQ (Syngene). Coomassie  
168 staining and image analysis of total protein were conducted in order to serve as a loading control  
169 [10, 19].

170 Intensity of IgJ abundance was determined using GeneSys Spot Blot analysis of the band  
171 occurring within each lane at 18 kDa for each weekly pooled sample. GeneSys Total Lane  
172 analysis for each sample of the Coomassie image was used to determine the total protein in the  
173 sample as a loading control. A ratio of IgJ intensity to Coomassie intensity was calculated for  
174 each pooled sample, as well as for the positive control. Relative intensity for each pooled sample  
175 was calculated by dividing the ratio for each pooled sample by the ratio of the positive control, in  
176 order to control for inter-blot variation. A pre-breeding relative intensity value specific to each  
177 individual luteal phase was then subtracted from each weekly post-breeding relative intensity  
178 value to control for pre-breeding IgJ levels. If relative intensity values were not normally  
179 distributed, then the data was subjected to a log transformation. After transformation, 15 of 18  
180 groups were verified for normality using a Shapiro-Wilk test in R (version 3.3.2) [17].

181 Differences in IgJ intensity between pregnant and non-pregnant groups were determined using  
182 Student's T-test or Mann-Whitney U-Test in R (version 3.3.2) depending on normality of the  
183 group, with differences considered significant at  $P < 0.05$ , and differences considered a tendency  
184 at  $0.05 \leq P < 0.09$ .

185

## 186 **Results**

### 187 **Fecal steroid metabolite and total protein concentrations**

188 Fecal estrogen metabolite profiles confirmed the cyclicity of monitored females.  
189 Examples of the estrogen metabolite concentrations of cycling females prior to natural breeding  
190 can be seen in Fig 1a and 1b. Fecal progesterone metabolite concentrations were significantly  
191 higher ( $P < 0.01$ ) during pregnancy and non-pregnant luteal phase than during a pre-breeding  
192 period (Table 1), confirming the presence of a luteal phase after breeding. Fecal progesterone  
193 profiles of pregnant females can be distinguished from non-pregnant luteal phase females after  
194 around 55 days post-breeding, when progesterone concentrations of non-pregnant females drop  
195 (Fig 1c), while pregnant females have extended progesterone excretion until parturition (Fig 1d).  
196 Mean total protein concentration of sample extracts from pregnancies ( $5.93 \pm 0.29$  mg/mL), non-  
197 pregnant luteal phases ( $6.24 \pm 0.23$  mg/mL), and pre-breeding ( $5.69 \pm 0.57$  mg/mL) were not  
198 significantly different ( $P > 0.05$ ).

199  
200 **Fig 1. Cheetah estrogen and progesterone fecal metabolite profiles.** (a) Estrogen fecal  
201 metabolite profile of a cycling female prior to natural breeding and successful pregnancy. (b)  
202 Estrogen fecal metabolite profile of a cycling female prior to natural breeding and non-pregnant  
203 luteal phase (Axes are different scales). (c) Progesterone fecal metabolite profile of a non-  
204 pregnant luteal phase after natural breeding. Progesterone metabolite concentrations return to pre-  
205 ovulatory levels around ~55 days post-breeding. (d) Progesterone fecal metabolite profile of a  
206 pregnant female after natural breeding. Progesterone fecal metabolite concentrations remain  
207 elevated until parturition (Parturition indicated by arrow) (Axes are different scales).

208

209 **Table 1. Mean ( $\pm$ SEM) cheetah estrogen and progesterone fecal metabolite concentrations.**

	Estrogen metabolites ( $\mu$ g/g dry feces)	Progesterone metabolites ( $\mu$ g/g dry feces)
<i>Pregnant Luteal Phase</i>	0.32 $\pm$ 0.06	28.66 $\pm$ 6.49 <sup>a</sup>
<i>Non-Pregnant Luteal Phase</i>	0.28 $\pm$ 0.05	43.92 $\pm$ 12.68 <sup>a</sup>
<i>Pre-Breeding</i>	0.33 $\pm$ 0.07	0.92 $\pm$ 0.20 <sup>b</sup>

210 <sup>a,b</sup>Values in the same column with different superscripts differ at P < 0.05.

## 211 **Post-breeding IgJ response in females exposed to seminal plasma** 212 **during natural breeding**

213 Detection of IgJ by western blotting with the use of a commercially available antibody  
214 was confirmed by the use of a positive control. IgJ was confirmed in the positive control and in  
215 fecal samples at a molecular weight of ~18 kD (Fig 2). Females that were bred naturally by intact

216 males with semen deposition, including both successful pregnancies and non-pregnant luteal  
217 phases, tended to have significantly higher peak IgJ levels ( $P = 0.076$ , mean  $\pm$  SEM =  $0.86 \pm 0.05$ )  
218 in the 2 weeks immediately following breeding compared to exogenously stimulated females that  
219 did not have exposure to seminal plasma ( $0.75 \pm 0.02$ ) (Fig 3). An example of a post-breeding  
220 response can be seen in Fig 2a, with high IgJ abundance in week 1 and week 2 following the  
221 female's first breeding and exposure to seminal plasma. A response from a second female can be  
222 seen in Fig 2b, with high IgJ abundance in week 2 following natural breeding and exposure to  
223 seminal plasma. In females that were exogenously stimulated to ovulate without exposure to  
224 seminal plasma, no increase in IgJ was seen in the two weeks following breeding (Fig 2c).

225

226

227 **Figure 2. IgJ response immediately following breeding in the cheetah.** (a) A female (#6593)  
228 that was bred naturally with exposure to seminal plasma had high IgJ levels in week 1 and week  
229 2 post-breeding. (b) A female (#6462) that was bred naturally with exposure to seminal plasma  
230 had high IgJ levels in week 2 post-breeding. (c) A female (#8957) with no exposure to seminal  
231 plasma following exogenous stimulation to ovulate did not experience an increase in IgJ levels in  
232 weeks 1 or 2 following breeding. Positive control is denoted by “+.” Pre-breeding sample is  
233 denoted by “PB.”

234

235 **Fig 3. Effect of exposure to seminal plasma on IgJ abundance in cheetahs.** Females that were  
236 bred naturally by intact males and exposed to seminal plasma ( $n = 15$ ) tended to have higher IgJ  
237 levels in the two weeks following breeding compared to females exogenously stimulated to  
238 ovulate with no exposure to seminal plasma ( $n = 7$ ,  $P = 0.076$ ).

239

## 240 **Temporal tracking of IgJ abundance**

241 Pregnant females tended to have higher IgJ levels ( $P = 0.081$ ) in week 4 post-breeding  
242 ( $0.83 \pm 0.07$ ,  $n=10$ ) compared to females experiencing a non-pregnant luteal phase ( $0.69 \pm 0.02$ ,  
243  $n=18$ ) (Fig 4). Pregnant females were also found to have significantly higher IgJ levels ( $P <$   
244  $0.05$ ) in week 8 post-breeding ( $0.79 \pm 0.03$ ,  $n=9$ ) compared to females experiencing a non-  
245 pregnant luteal phase ( $0.69 \pm 0.03$ ,  $n=15$ ). IgJ abundance was not different between the two  
246 groups in weeks 1, 2, 3, 5, 6, 7, or 9 ( $P > 0.1$ ) (Fig 4). An example of a pregnancy can be seen in  
247 Fig 5a, with high IgJ levels in Week 4 and following Week 7 post-breeding. An example of a  
248 non-pregnant luteal phase can be seen in Fig 5b, with low IgJ levels at or near pre-breeding  
249 levels throughout the sample period.

250

251 **Fig 4. Mean ( $\pm$  SEM) relative intensity of IgJ following breeding in the cheetah.** IgJ  
252 abundance tended to be higher ( $P = 0.081$  \*) in pregnant females ( $n = 12$ ) compared to those  
253 experiencing a non-pregnant luteal phase ( $n = 19$ ) at week 4 post-breeding. IgJ abundance was  
254 significantly higher ( $P < 0.05$  \*\*) for pregnant females compared to during a non-pregnant luteal  
255 phase at week 8 post-breeding.

256

257 **Fig 5. Comparison of pregnant and non-pregnant luteal phase in the cheetah.** (a) IgJ was  
258 increased in week 4 and following week 7 post-breeding during pregnancy (Female #6593). (b)  
259 IgJ remained at or near pre-breeding levels throughout the 9 week sample period during a non-  
260 pregnant luteal phase (Female #6339). Positive Control is denoted by “+.” Pre-breeding sample  
261 is denoted by “PB.”

## 262 **Discussion**

263           The reproductive biology of the cheetah has been studied for decades, with great  
264 advances in the understanding of this species both *in situ* and *ex situ*. However, cheetahs in the  
265 wild are in decline, as habitat fragmentation and human conflict have reduced the natural range  
266 of the species [1, 20]. Because of the vulnerable status of the cheetah, it has become an important  
267 goal of conservationists to create an *ex situ* insurance population. However, creating a  
268 sustainable *ex situ* population with the goal of improving genetic diversity and ensuring future  
269 health and adaptability of the species has become a challenge, as many cheetahs struggle to  
270 successfully reproduce in human care. The low genetic diversity of the species as a whole, and  
271 high levels of inbreeding depression, have contributed to many health and reproductive issues  
272 that affect the cheetah, including the impairment of genes mediating immune defenses [21], low  
273 fecundity, and the poor semen quality of males both in captivity and in the wild [22, 23]. While  
274 wild cheetahs appear to face similar obstacles in terms of inbreeding depression and low genetic  
275 diversity, they are able to reproduce with greater success than cheetahs under human care [24,  
276 25], indicating the possibility that *ex situ* environmental factors may be having a negative effect  
277 on reproductive capacity. Of importance to animal managers is understanding the relatively high  
278 incidence of non-pregnant luteal phase that occurs in the *ex situ* population, whether this  
279 represents failure to conceive or early embryo loss.

280           Temporal tracking of IgJ abundance over the first 9 weeks post-breeding provides insight  
281 into the intrauterine events that occur after the success or failure to establish pregnancy in the  
282 cheetah. Females in our study demonstrated an elevation of IgJ after natural breeding with an  
283 intact male and seminal exposure compared to exogenous stimulation for ovulation with no  
284 seminal exposure. One explanation is that a secretory immune response was stimulated by the

285 presence of seminal plasma in the reproductive tract which, as a foreign substance interacting  
286 with a mucosal surface, could induce an upregulation in IgJ. An immune response to semen has  
287 been documented previously in mice, as lymphocyte synthesis and cytokine activation is  
288 triggered in response to the constituents of seminal plasma [26]. The immune response to semen  
289 may help to promote active maternal tolerance of paternal antigens of the fetus at the  
290 implantation site [26, 27], preventing rejection of the fetus. Upon successful breeding and  
291 exposure to semen, it is possible that the females in this study were experiencing a subsequent  
292 activation of the secretory immune response in order to promote tolerance of paternal antigens  
293 upon implantation of the fetus and invasion of the endometrium. In contrast to naturally bred  
294 females, all eight females that were exogenously stimulated to ovulate demonstrated IgJ levels  
295 that were at or near pre-breeding levels in the first two weeks post-breeding, indicating that the  
296 IgJ response is not due to ovulation, and that natural breeding and successful deposition of semen  
297 are needed to see this immune response.

298         The timing of early intrauterine events, including implantation and the development of  
299 the placenta, is unknown in wild felids. In the domestic cat, fertilization takes place in the  
300 oviduct up to 48 h after ovulation, and implantation occurs at day 13-14 post-breeding [3]. It is  
301 possible that implantation in the cheetah occurs at a different time as the domestic cat (day 13-14  
302 of ~65 d gestation), at a proportional point in the longer ~93 day gestation of the cheetah (day  
303 19-21). In the present study, IgJ levels tended to be elevated in pregnant cheetahs compared to  
304 non-pregnant cheetahs during the pooled fourth week after breeding (day 22-28). Increased IgJ  
305 levels during the fourth week post-breeding could indicate an activated secretory immune  
306 response to the invasion of the endometrium by the embryo following implantation. IgJ regulates  
307 the polymer formation of IgA and is a crucial component of the secretory form, facilitating the

308 exocrine secretion of the molecule. Increased IgA secretion likely alters dendritic cell function,  
309 leading to the promotion of regulatory T cell expansion and inhibiting the release of  
310 inflammatory cytokines, preventing an inflammatory response to paternal antigens present on the  
311 fetal trophoblast [28]. These findings suggest that implantation could occur directly before week  
312 4 post-breeding (day 19-21), as a sustained secretory immune response at the endometrium likely  
313 continues and intensifies as the implanted embryo continues to develop during the fourth week.

314         Pregnancy in the cheetah first becomes distinguishable from a non-pregnant luteal phase  
315 using progesterone metabolite monitoring at ~day 55 post-breeding, as progesterone levels in fecal  
316 samples drop to pre-ovulatory levels in non-pregnant individuals. It can be theorized that around  
317 this time in non-pregnant individuals a regression of the corpora lutea (CL) occurs which results  
318 in a subsequent decrease in progesterone production, followed by a plateau at lower basal levels.  
319 It is unknown whether luteal regression occurs in pregnant females as well, or if the CL is  
320 maintained until parturition. Recent studies have suggested that the placenta is a source of  
321 progesterone production in the domestic cat [29, 30], and acts to supplement luteal progesterone  
322 later in gestation. A placental source of progesterone secretion is likely in the cheetah and may  
323 explain the observed increase in IgJ levels at week 8 (day 50-56) post-breeding. An increased  
324 and sustained secretion of progesterone from the fetal-maternal barrier at day 50-56 in gestation  
325 may modulate secretory immunity, causing IgJ levels to be increased in pregnant individuals  
326 compared to non-pregnant individuals. Local placental synthesis of progesterone may act as an  
327 immunosuppressive factor at the site of embryonic implantation during both murine and human  
328 gestation [31]. Progesterone suppresses T lymphocyte proliferation and inhibits natural killer cell  
329 activity at the maternal-fetal interface [32], decreasing the activity of these immune molecules to  
330 protect the semi-allogeneic fetus and preventing an inflammatory immune reaction. Because the



331 endometrium is a mucosal surface, it is likely that secretory immunity is modulated as well,  
332 promoting tolerance of the fetus and allowing for non-inflammatory neutralization of foreign  
333 pathogens. Secretory immunity may also be modulated by the secretion of Prostaglandin F<sub>2α</sub>  
334 (PGF<sub>2α</sub>) by the placenta during week 8 post-breeding, as levels of a fecal PGF<sub>2α</sub> metabolite  
335 (PGFM) were found to be elevated in the pregnant cheetah beginning at day 48 post-breeding  
336 and increased through parturition [33]. While PGF<sub>2α</sub> is known to have a strong luteolytic effect  
337 in many species [34], suggesting a possible impact on immunity, the action of this molecule is  
338 unknown in felids and the impact on IgJ levels has not yet been determined.

339         It is possible that the presence of external immune stressors, which potentially could alter  
340 the production of systemic IgJ, could influence the results and interpretation of our experiment.  
341 IgJ is a protein that is upregulated in response to an activation of the secretory immune system,  
342 which is present in all mucosal surfaces of the body in order to protect from foreign pathogens.  
343 An immune challenge to a mucosal surface that is independent of pregnancy could increase  
344 expression of IgJ, resulting in high IgJ values in our assay that do not correspond to intrauterine  
345 events and could affect the accuracy of the assay. Inter-individual variability in IgJ expression  
346 was controlled for here by assessing relative changes in concentration from pre-breeding levels  
347 within each individual. A future goal is to extend the Western analysis to a reliable benchtop  
348 enzyme-linked immunosorbent assay (ELISA) for measuring fecal IgJ levels after breeding.  
349 Daily quantification using this method would improve the accuracy and efficiency of IgJ  
350 monitoring and may be able to reveal moments of immune challenge that impact establishment  
351 of pregnancy with greater precision. The occurrence of a non-pregnant luteal phase is common in  
352 other species, indicating the potential for a felid-wide or carnivore-wide assay for determining  
353 pregnancy using IgJ monitoring.

354           In summary, this study detailed IgJ levels throughout the first 9 weeks of pregnancy and  
355 non-pregnant luteal phase in the cheetah. Females that were bred naturally and exposed to  
356 seminal plasma tended to have a higher immediate IgJ response than females that were  
357 exogenously stimulated to ovulate with no seminal exposure, indicating an immune response to  
358 the constituents of seminal plasma that may have the effect of promoting maternal tolerance of  
359 fetal tissue upon implantation. There was a tendency towards increased IgJ abundance at week 4  
360 post-breeding in pregnant cheetahs compared to non-pregnant cheetahs, indicating an activation  
361 of the secretory immune system in response to implantation and the invasion of the maternal  
362 endometrium by the fetal trophoblast. A significant increase in IgJ abundance was also found in  
363 week 8 post-breeding in pregnant cheetahs compared to non-pregnant cheetahs. Taken together,  
364 these data support the suggestion that the window of implantation in the cheetah is between 19-  
365 21 days post-breeding, and that the placenta is a source of extragonadal progesterone during the  
366 third trimester. These findings will help to improve *ex situ* management of the species, and  
367 further research will continue to advance the understanding of cheetah reproductive physiology  
368 following breeding, aiding future conservation efforts for the species.

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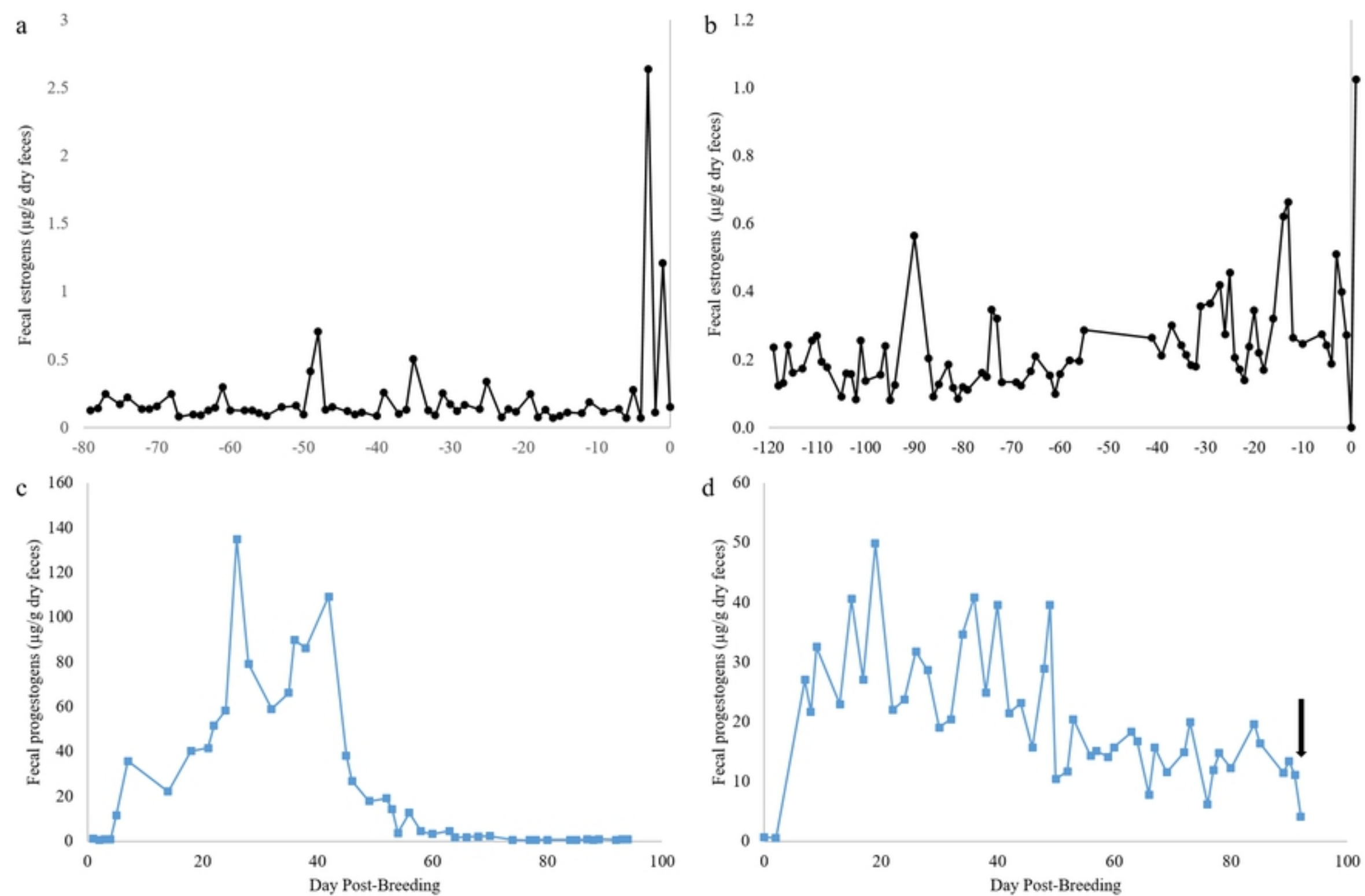


Figure 1



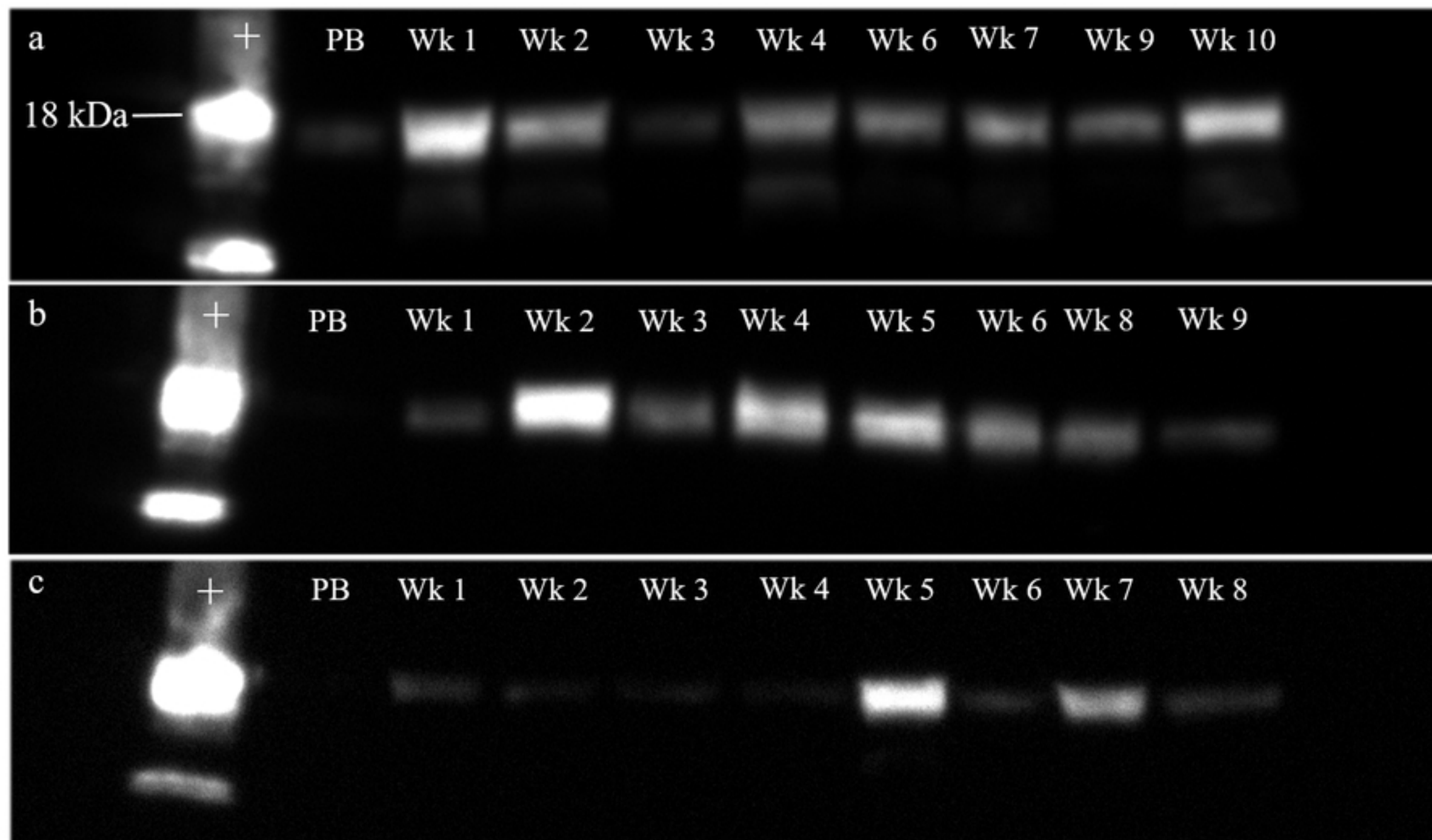


Figure 2

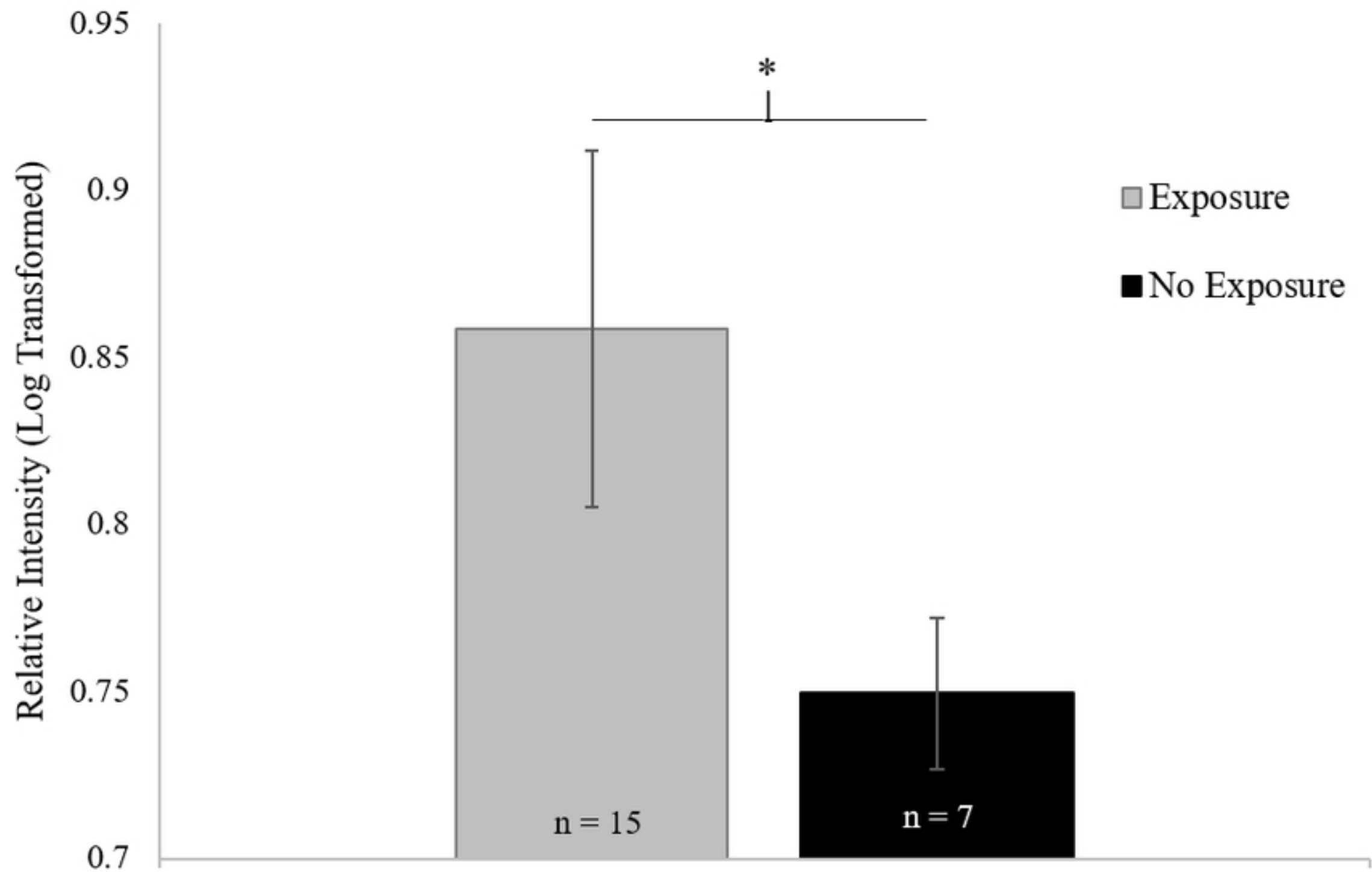


Figure 3

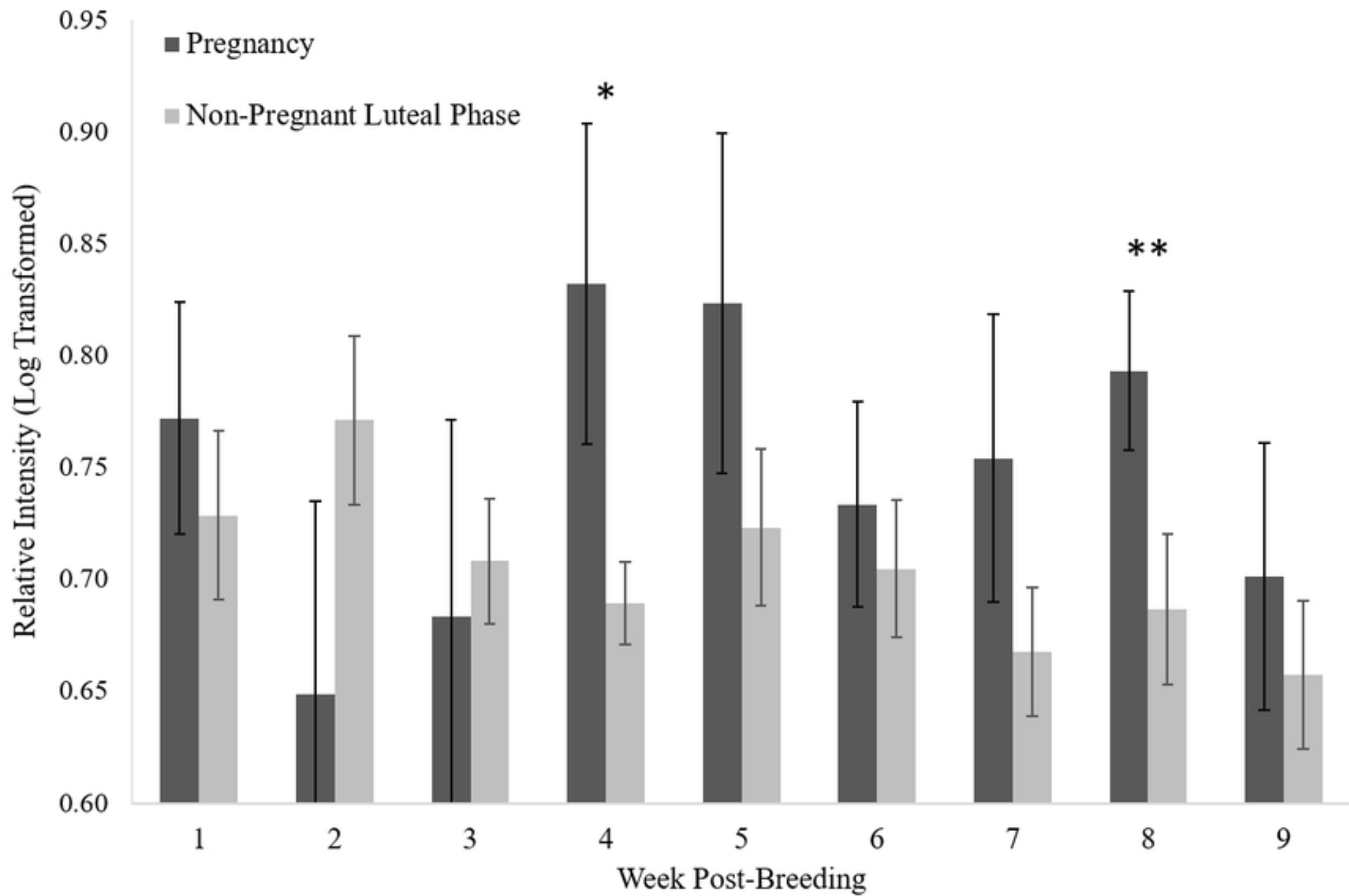


Figure 4

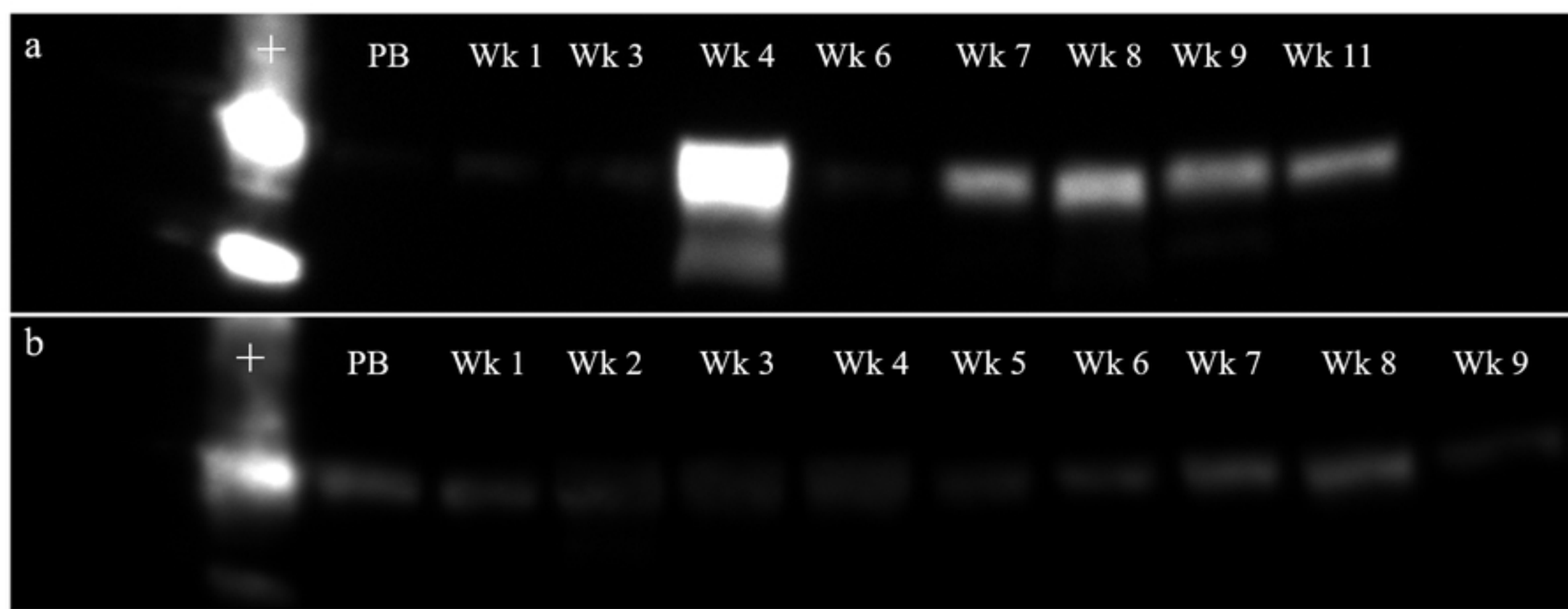


Figure 5