

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

Michael J. Byron^{1,2,*}, Diana C. Koester³, Katie L. Edwards^{1,4}, Paul E. Mozdziak², Charlotte E. Farin², Adrienne E. Crosier¹

¹Center for Species Survival, Smithsonian Conservation Biology Institute, Front Royal, VA

²College of Agriculture and Life Sciences, North Carolina State University, Raleigh, NC

³Conservation and Science Department, Cleveland Metroparks Zoo, Cleveland, OH

⁴North of England Zoological Society, Chester Zoo, Upton-by-Chester, United Kingdom

* Corresponding author

Email: mjbyron@ncsu.edu

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 ± 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 ³H-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 μ 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 μ 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 μ L of each sample was added to a well. 200 μ 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 NaH_2PO_4 , 3.5 M Na_2HPO_4 , 0.3 M NaCl, H_2O ; 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 17β -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 β -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 μ L. Human recombinant IgJ (Abcam #140727) was used as a positive control at 16.67 μ 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 ± 0.29 mg/mL), non-
197 pregnant luteal phases (6.24 ± 0.23 mg/mL), and pre-breeding (5.69 ± 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 (μ g/g dry feces) | Progesterone metabolites (μ g/g dry feces) |
|----------------------------------|--|--|
| <i>Pregnant Luteal Phase</i> | 0.32 \pm 0.06 | 28.66 \pm 6.49 ^a |
| <i>Non-Pregnant Luteal Phase</i> | 0.28 \pm 0.05 | 43.92 \pm 12.68 ^a |
| <i>Pre-Breeding</i> | 0.33 \pm 0.07 | 0.92 \pm 0.20 ^b |

210 ^{a,b}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 ± 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 ± 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 ± 0.07 , $n=10$) compared to females experiencing a non-pregnant luteal phase (0.69 ± 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 ± 0.03 , $n=9$) compared to females experiencing a non-
245 pregnant luteal phase (0.69 ± 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_{2α}
334 (PGF_{2α}) by the placenta during week 8 post-breeding, as levels of a fecal PGF_{2α} 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_{2α} 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.

369 **Acknowledgments**

370 This study was conducted under a consortium agreement of the Conservation Centers for Species
371 Survival (C2S2), a formal partnership that shares unique resources to improve the biological
372 understanding and management of endangered species, especially those that require space,
373 natural group sizes, and scientific research. The authors would like to thank the Smithsonian
374 National Zoological Park, Fossil Rim Wildlife Center, Wildlife Safari, White Oak Conservation,
375 The Wilds, Cincinnati Zoo, Birmingham Zoo, Denver Zoo, Columbus Zoo, Caldwell Zoo, and
376 Omaha Zoo for participation in this study. We would also like to thank Amber Dedrick, Adri
377 Kopp, Jaelyn Carrant, and Kelly Mickael for technical support.

378 **References**

- 379 1. Durant SM, Mitchell N, Groom R, Pettorelli N, Ipavec A, Jacobson AP, et al. The global
380 decline of cheetah *Acinonyx jubatus* and what it means for conservation. PNAS. 2017;114(3):
381 528-533.
- 382 2. Brown JL, Wildt DE, Wielebnowski N, Goodrowe KL, Graham LH, Wells S, et al.
383 Reproductive activity in captive female cheetahs (*Acinonyx jubatus*) assessed by faecal
384 steroids. Journal of Reproduction and Fertility. 1996;106(2): 337-346.
- 385 3. Denker HW, Eng LA, Hamner CE. Studies on the early development and implantation in the
386 cat. II. Implantation: proteinases. Anatomy and embryology. 1978;154(1): 39-54.
- 387 4. Leiser R, Koob B. Development and characteristics of placentation in a carnivore, the
388 domestic cat. The Journal of experimental zoology. 1993;266(6): 642-656.
- 389 5. Wildt DE, Brown JL, Bush M, Barone MA, Cooper KA, Grisham J, et al. Reproductive
390 status of cheetahs (*Acinonyx jubatus*) in North American Zoos: The benefits of physiological
391 surveys for strategic planning. Zoo Biology. 1993;12(1): 45-80.
- 392 6. Burke HB. Predicting clinical outcomes using molecular biomarkers. Biomarkers in Cancer.
393 2016;8.
- 394 7. Kuribayashi T, Shimizu M, Shimada T, Honjyo T, Yamamoto Y, Kuba K, et al. Alpha 1-acid
395 glycoprotein (AAG) levels in healthy and pregnant beagle dogs. Experimental animals.
396 2003;52(5): 377.
- 397 8. Bauman JE, Clifford DL, Asa CS. Pregnancy diagnosis in wild canids using a commercially
398 available relaxin assay. Zoo Biology. 2008;27(5): 406-413.

- 399 9. Curry E, Stoops MA, Roth TL. Non-invasive detection of candidate pregnancy protein
400 biomarkers in the feces of captive polar bears (*Ursus maritimus*). *Theriogenology*.
401 2012;78(2): 308-314.
- 402 10. Koester DC, Wildt DE, Maly M, Comizzoli P, Crosier AE. Non-invasive identification of
403 protein biomarkers for early pregnancy diagnosis in the cheetah (*Acinonyx jubatus*). *PLoS*
404 *One*. 2017;12(12): e0188575.
- 405 11. Brandtzaeg P. Immunohistochemical characterization of intracellular J-chain and binding site
406 for secretory component (SC) in human immunoglobulin (Ig)-producing cells. *Molecular*
407 *Immunology*. 1983;20(9): 941-966.
- 408 12. Halpern MS, Koshland ME. Noval subunit in secretory IgA. *Nature*. 1970;228(5278): 1276.
- 409 13. Johansen F, Braathen R, Brandtzaeg P. The J chain is essential for polymeric Ig receptor-
410 mediated epithelial transport of IgA. *The Journal of Immunology*. 2001;167(9): 5185-5192.
- 411 14. Howard JG, Roth TL, Byers AP, Swanson WF, Wildt DE. Sensitivity to exogenous
412 gonadotropins for ovulation induction and laparoscopic artificial insemination in the cheetah
413 and clouded leopard. *Biology of Reproduction*. 1997;56(4): 1059.
- 414 15. Pelican KM, Wildt DE, Pukazhenti B, Howard J. Ovarian control for assisted reproduction
415 in the domestic cat and wild felids. *Theriogenology*. 2006;66(1): 37-48.
- 416 16. Brown JL, Wasser SK, Wildt DE, Graham LH. Comparative aspects of steroid hormone
417 metabolism and ovarian activity in felids, measured noninvasively in feces. *Biology of*
418 *Reproduction*. 1994;51(4): 776-786.
- 419 17. R Core Team. R: A language and environment for statistical computing. R Foundation for
420 Statistical Computing, Vienna, Austria. 2016. URL <https://www.R-project.org/>.

- 421 18. Crosier AE, Comizzoli P, Baker T, Davidson A, Munson L, Howard J, et al. Increasing age
422 influences uterine integrity, but not ovarian function or oocyte quality, in the cheetah
423 (*Acinonyx jubatus*). *Biology of reproduction*. 2011;85(2): 243.
- 424 19. Welinder C, Ekblad L. Coomassie Staining as Loading Control in Western Blot Analysis.
425 *Journal of Proteome Research*. 2011;10(3): 1416-1419.
- 426 20. Marker LL, Dickman AJ, Jeo RM, Mills MGL, Macdonald DW. Demography of the
427 Namibian cheetah, *Acinonyx jubatus jubatus*. *Biological Conservation*. 2003;114(3): 413-
428 425.
- 429 21. O'Brien SJ, Johnson WE, Driscoll CA, Dobrynin P, Marker L. Conservation genetics of the
430 cheetah: lessons learned and new opportunities. *The Journal of heredity*. 2017;108(6): 671-
431 677.
- 432 22. Wildt DE, O'Brien SJ, Howard JG, Caro TM, Roelke ME, Brown JL, et al. Similarity in
433 ejaculate-endocrine characteristics in captive versus free-ranging cheetahs of two subspecies.
434 *Biology of Reproduction*. 1987;36(2): 351.
- 435 23. Crosier AE, Marker L, Howard J, Pukazhenti BS, Henghali JN, Wildt DE. Ejaculate traits in
436 the Namibian cheetah (*Acinonyx jubatus*): influence of age, season and captivity.
437 *Reproduction, Fertility and Development*. 2007;19(2): 370-382.
- 438 24. Laurenson MK, Caro TM, Borner M. Female cheetah reproduction. *National Geographic*
439 *Research & Exploration*. 1992;8(1): 64-75.
- 440 25. Kelly MJ, Laurenson MK, FitzGibbon CD, Collins DA, Durant SM, Frame GW, et al.
441 Demography of the Serengeti cheetah (*Acinonyx jubatus*) population: the first 25 years.
442 *Journal of Zoology*. 1998;244(4): 473-488.

- 443 26. Johansson M, Bromfield JJ, Jasper MJ, Robertson SA. Semen activates the female immune
444 response during early pregnancy in mice. *Immunology*. 2004;112(2): 290-300.
- 445 27. Robertson SA, Sharkey DJ. The role of semen in induction of maternal immune tolerance to
446 pregnancy. *Seminars in Immunology*. 2001;13(4): 243-254.
- 447 28. Monteiro RC. Immunoglobulin A as an anti-inflammatory agent. *Clinical & Experimental*
448 *Immunology*. 2014;178: 108-110.
- 449 29. Tsutsui T, Suzuki Y, Toyonaga M, Oba H, Mizutani T, Hori T. The role of the ovary for the
450 maintenance of pregnancy in cats. *Reproduction in domestic animals*. 2009;44 Suppl 2(s2):
451 120-124.
- 452 30. Siemieniuch MJ, Jursza E, Szostek AZ, Skarzynski DJ, Boos A, Kowalewski MP.
453 Steroidogenic capacity of the placenta as a supplemental source of progesterone during
454 pregnancy in domestic cats. *Reproductive biology and endocrinology*. 2012;10(1): 89.
- 455 31. Siiteri PK, Stites DP. Immunologic and endocrine interrelationships in pregnancy. *Biology of*
456 *Reproduction*. 1982;26(1): 1-14.
- 457 32. Szekeres-Bartho J. Immunological relationship between the mother and the fetus.
458 *International Reviews of Immunology*. 2002;21(6): 471-495.
- 459 33. Dehnhard M, Finkenwirth C, Crosier A, Penfold L, Ringleb J, Jewgenow K. Using PGFM
460 (13,14-dihydro-15-keto-prostaglandin F2 α) as a non-invasive pregnancy marker for felids.
461 *Theriogenology*. 2012;77(6): 1088-1099.
- 462 34. Senger PL. Pathways to pregnancy and parturition. 2nd rev. ed. Pullmann, Wash: Current
463 Conceptions; 2005.

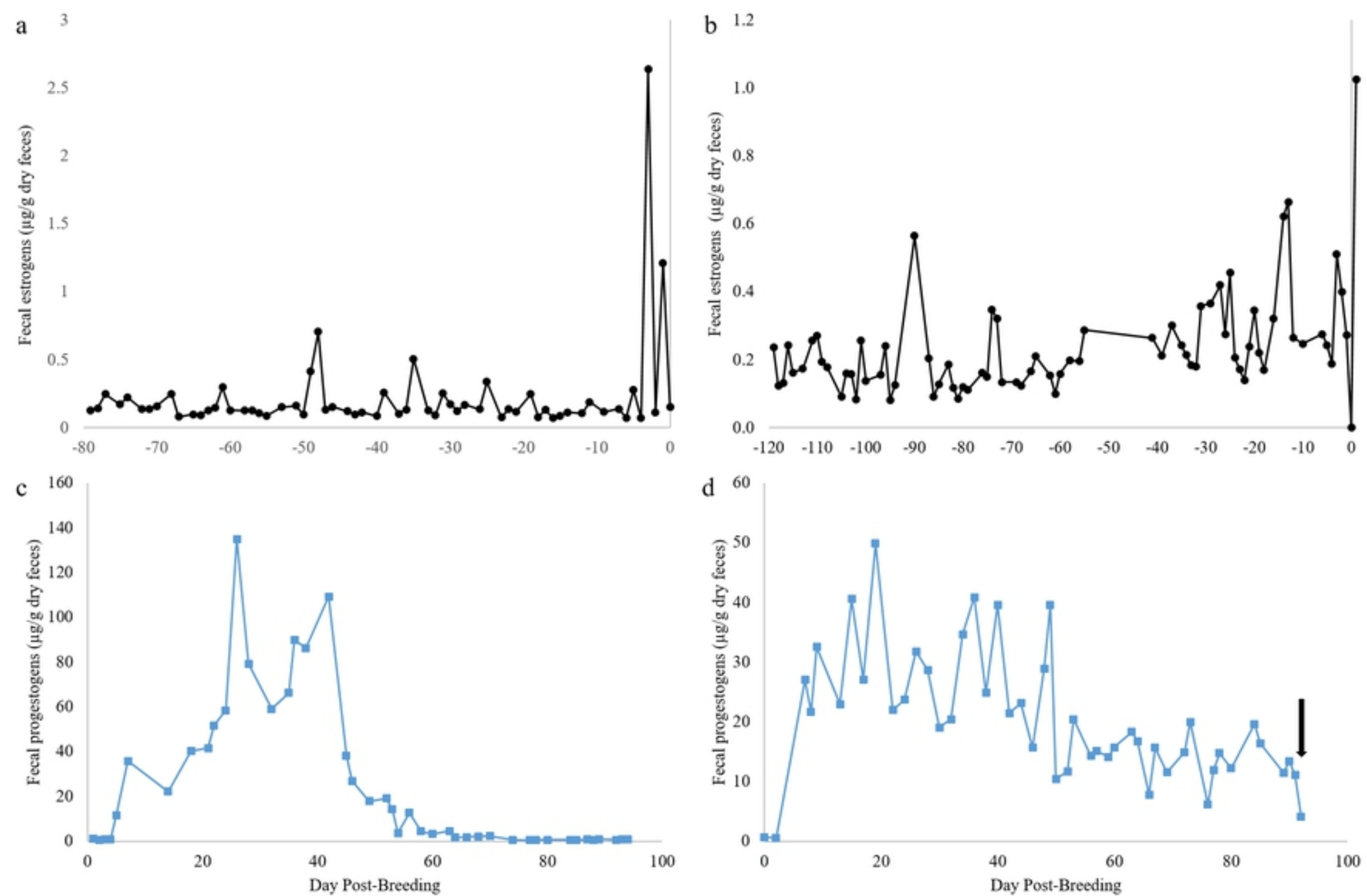


Figure 1

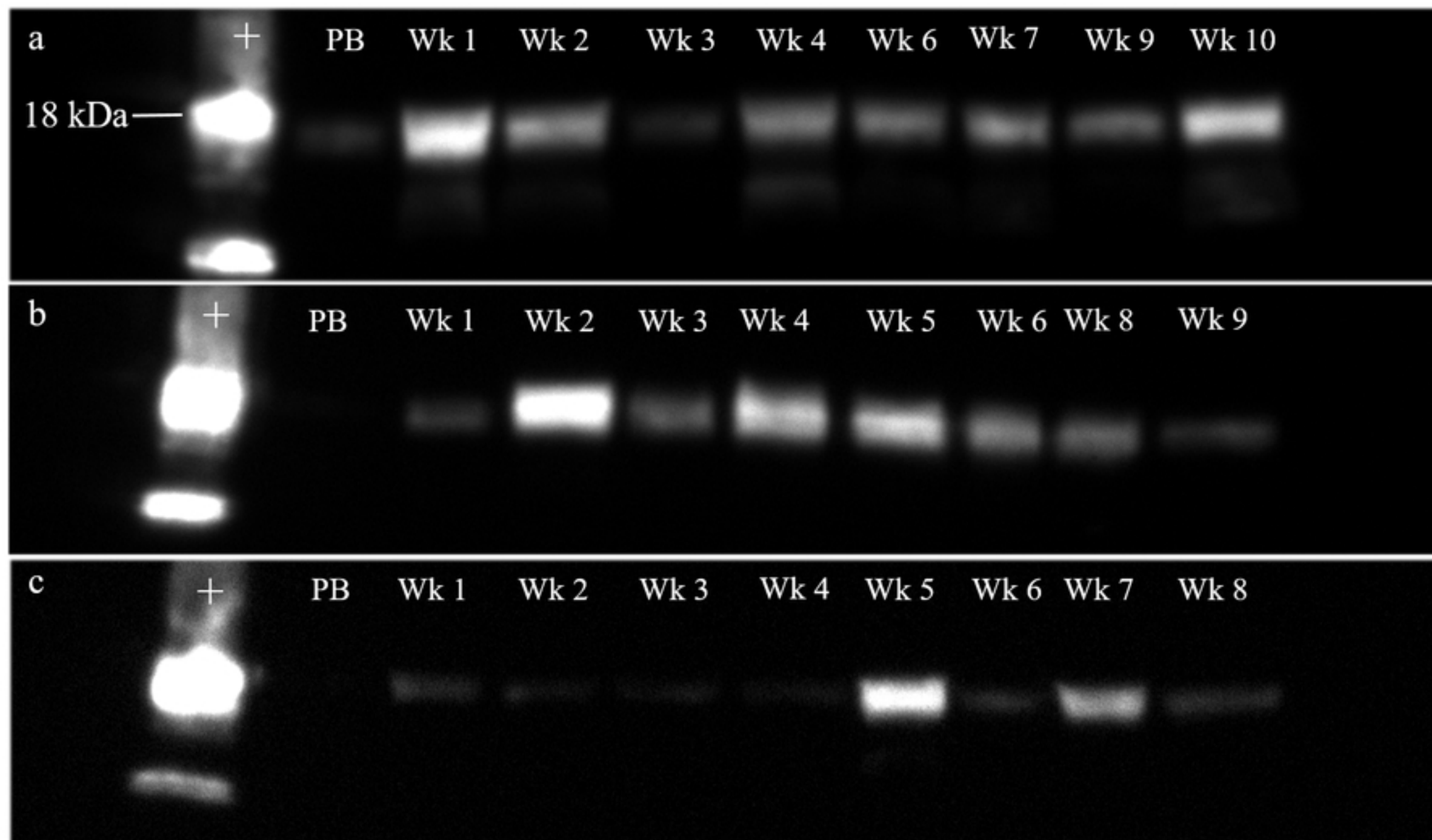


Figure 2

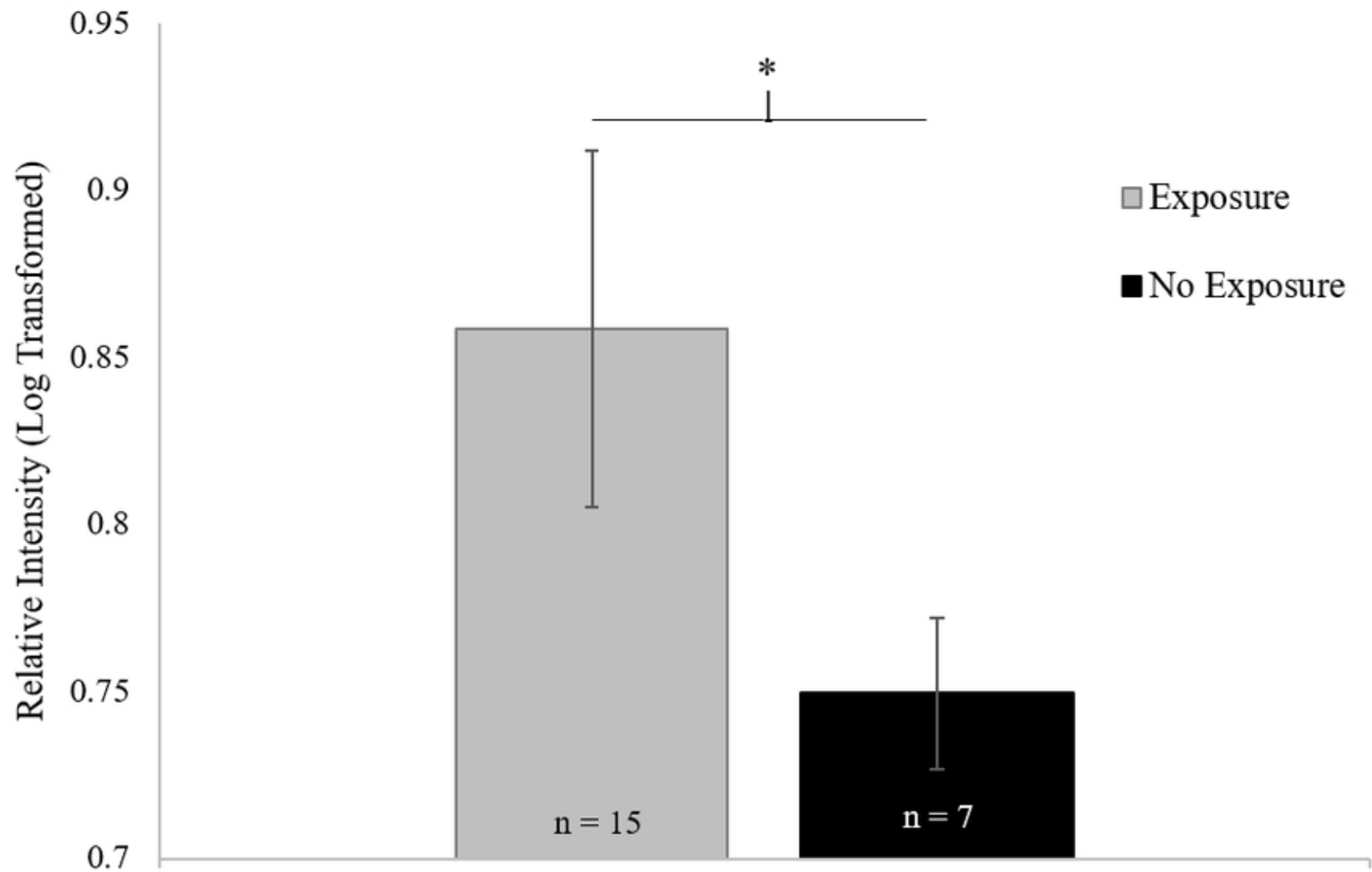


Figure 3

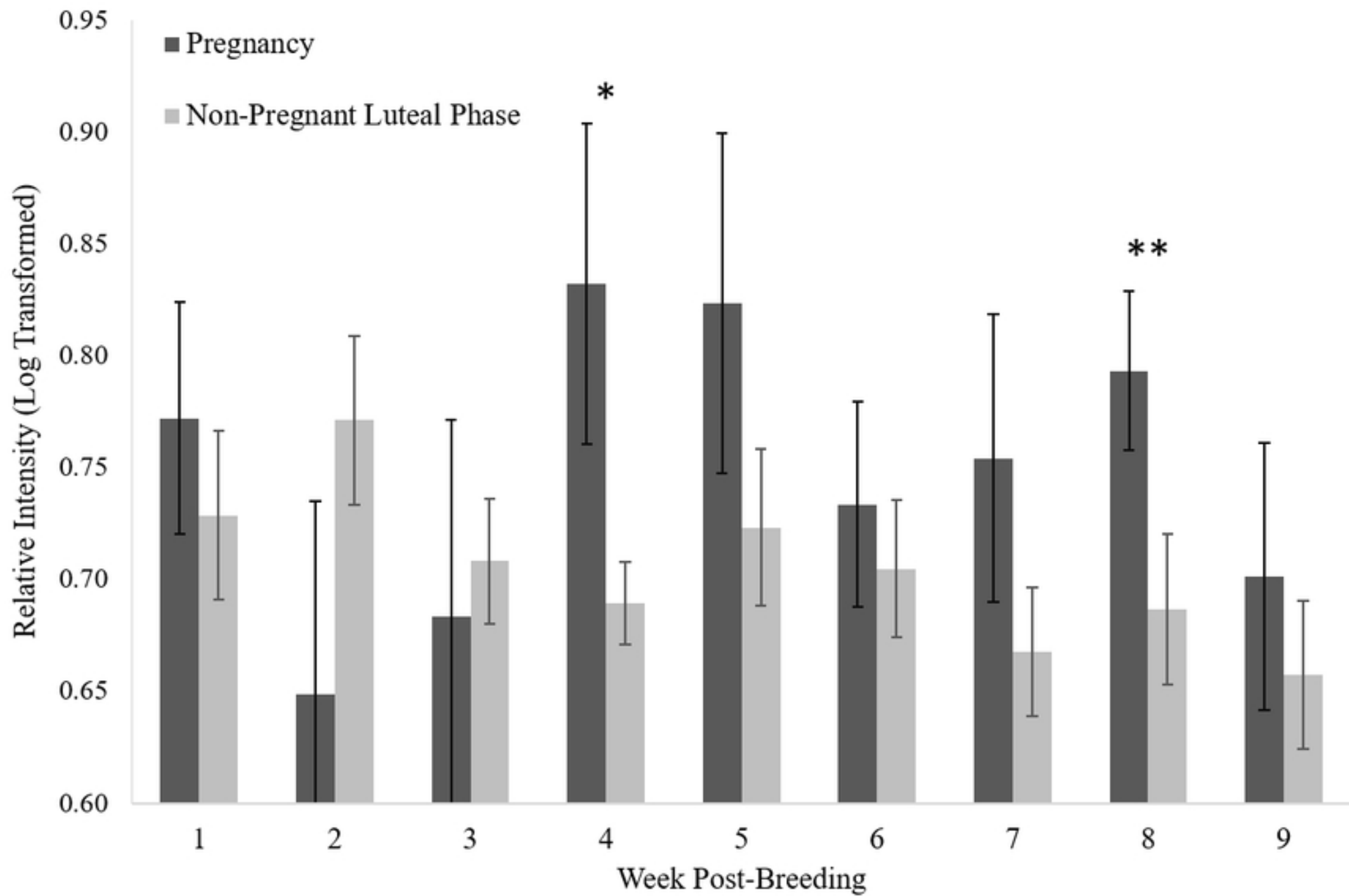


Figure 4

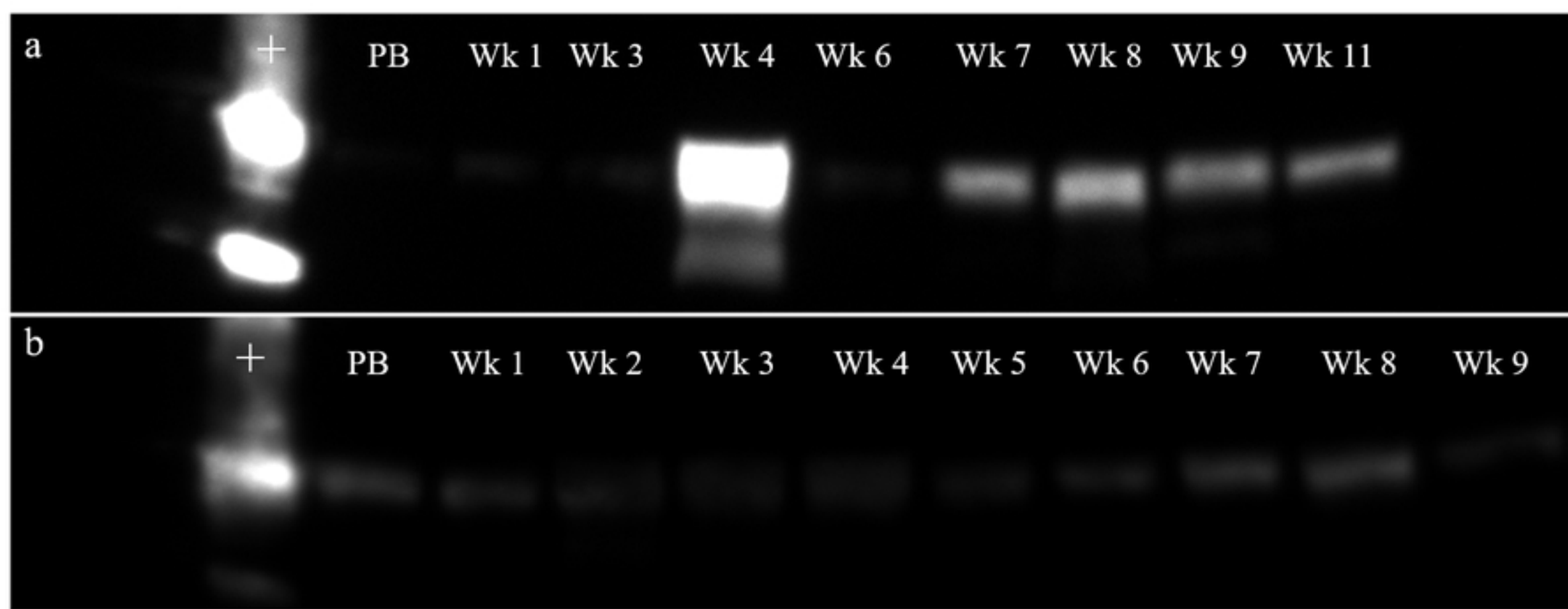


Figure 5