Non-invasive hormonal characterization of the ovarian cycle, pregnancy, and seasonal anestrus of the female addra gazelle (*Nanger dama ruficollis*)

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23 **Abstract**

24 Non-invasive fecal hormone metabolite monitoring was used to characterize the
25 estrous cycle, pregnancy, and seasonal anestrus of the critically endangered addra gazelle
26 (*Nanger dama ruficollis*). With less than 250 animals remaining in the wild and ~168
27 individuals managed in captivity, it is crucial to maintain sustainable populations.
28 Progestogen and estrogen profiles were obtained from analysis of fecal samples collected
29 approximately every other day, within varying intervals, over the course of 7 years (n = 8
30 adult females). Average estrous cycle length was 19.5 ± 0.4 days (range, 14-26 days),
31 with a luteal phase length of 14.6 ± 1.2 days (range, 10-16 days), and an inter-luteal
32 period of 5.2 ± 1.4 days (range, 2-7 days). Mean gestation length for six pregnancies was
33 200.7 ± 0.4 days (range, 200-202 days). Fecal progestogens increased at 12 weeks of
34 gestation and remained elevated until parturition. Addra gazelle females exhibited a
35 period of seasonal anestrus with consistently low progestogen concentrations and no
36 cyclic activity from about September to March. Analysis of reproductive and climate
37 records demonstrated a peak in U.S. births that coincided with maximal rainfall in the
38 native habitat of the addra gazelle. Results show that estrous cycle, luteal phase, and
39 inter-luteal phase lengths in addra are similar to those observed in other gazelle species,
40 however, to our knowledge, this is the first study to demonstrate seasonal anestrus in the
41 Nanger genus.

42

43 **Keywords:** Seasonality; Photoperiod; Hormone; Anestrus; Endangered species; Antelope

44 **Introduction**

45 Once widespread throughout the grasslands and savanna of northern Africa, dama
46 gazelle (*Nanger dama*) populations have dramatically plummeted in recent history. The
47 species is categorized as critically endangered by the IUCN, and remaining populations
48 are fragmented and limited to small areas in Chad, Mali, and Niger [1]. The dama gazelle
49 species is classified into three subspecies: addra (*N. d. ruficollis*), mohor (*N. d. mhorr*)
50 and dama (*N. d. dama*), which differ in geographical distribution and coat color [1, 2]. *In*
51 *situ* and *ex situ* populations are not self-sustaining and individuals managed in captivity
52 are dispersed across North America in small herds, hindering effective genetic
53 management [3]. Spatial segregation of remaining wild populations increases possibility
54 of inbreeding and concomitant decline in reproductive success [4, 5, 6]. Species survival
55 is also threatened by poaching and habitat degradation [2, 7, 8]; habitat quality is
56 predicted to deteriorate further as the climate continues to change [9, 10, 11]. Maintaining
57 sustainable populations of threatened non-domestic species is challenging. The
58 development and utilization of assisted reproductive technologies (ARTs) may facilitate
59 genetic management and enhance offspring production, but this approach relies heavily
60 upon a thorough understanding of fundamental reproductive biology of the species [12,
61 13, 14, 15, 16]. There is currently no specific information on the reproductive biology of
62 the female addra gazelle.

63 Reproductive processes and their regulatory mechanisms appear to be generally
64 conserved across mammalian species. Studies in domestic species or closely related
65 species can provide insight, however, estrous cycle duration, and timing of ovulation can
66 differ among species and may impact reproductive success, therefore the usefulness of

heterologous studies is limited [6, 15, 17]. Endocrine monitoring can offer insight into an individual's physiology and welfare state, as well as provide information regarding reproductive characteristics. In contrast to non-invasive hormone monitoring, stress associated with blood sampling may alter physiology including concentrations of hormone metabolites, which can skew results [18, 19]. Monitoring metabolites in feces is a useful alternative and requires little to no interaction with the animals, limiting stressful encounters, and its use has been validated for a number of species [15, 20, 21, 22]. Analysis of fecal progesterone metabolites has been used to monitor pregnancy, seasonality, and puberty in a variety of species including bovids, felids, and primates, among others [13, 16, 22, 23, 24]. Our goals were to use non-invasive fecal hormone monitoring to characterize ovarian steroid patterns during the estrous cycle and pregnancy, and to investigate incidence of reproductive seasonality in captive female addra gazelle.

Materials and Methods

All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Animals

Female addra gazelles (*N. d. ruficollis*, n = 8, 1-9 years of age) were maintained in herds of four with one herd managed at the Smithsonian's National Zoological Park (SNZP), Washington D.C. (38°55'N, 77°2'W), and the other at the Smithsonian Conservation Biology Institute (SCBI), Front Royal, VA (38°53'N, 78°9'W). Those housed at SNZP had access to alfalfa hay and fresh water ad libitum and were fed ~1 kg Mazuri ADF-25 herbivore pellets/animal/day (PMI Nutrition International, LLC, St.

90 Louis, MO). The herd had access to two stalls (5.0 m x 3.5 m and 4.4 m x 3.5 m) and two
91 holding yards (9.1 m x 9.1 m) overnight. Animals were on exhibit approximately 8 h/day.
92 A male calf born September 2012 was separated from the herd in May 2013. Females had
93 visual and fence-to-fence contact, and occasionally shared exhibit space with a male
94 scimitar-horned oryx (*Oryx dammah dammah*) prior to September 2013. Female #114263
95 was removed from the study in late 2009 due to poor health.

96 The herd maintained at SCBI received 1 kg Zeigler ADF-25 pellets/animal/day
97 (Zeigler Bros, Inc., Gardners, PA) with ad libitum access to alfalfa hay and water and
98 occasional supplementation with bamboo shoots. The herd was maintained in a 21.3 m x
99 9.1 m holding yard with access to a stall (9.1 m x 21.3 m), and was not on public exhibit.
100 Three male calves were kept with the female herd from their birth in February 2014 until
101 September 2014. The females had olfactory and auditory contact with an intact adult
102 male addra gazelle and a herd of scimitar-horned oryx of both sexes. All procedures were
103 approved by the Institutional Animal Care and Use Committee of SCBI.

104

105 *Fecal Sample Collection and Hormone Extraction*

106 Individuals were identified by colored ear-tags, and when defecation was
107 observed between 9 and 10 am, fresh fecal samples were collected. Sample collection
108 occurred approximately every other day during portions of 2008-2009, 2011-2014 and
109 2015 at SNZP, and 2014-2015 at SCBI (Table 1). All samples were stored without
110 preservative at -20°C until lyophilized.

111 Feces were lyophilized (VirTis Ultra 35XL, SP Scientific, Warminster, PA),
112 crushed to a powder, and 0.1 g of each sample was extracted using a modified version of

113 methodologies developed to extract gonadal steroids from felid and baboon feces [23, 24]
114 and validated for scimitar-horned oryx feces [25]. Briefly, crushed fecal matter was
115 mixed with 5.0 ml of 90% ethanol and continuously agitated for 30 min. After
116 centrifugation (1,800 x g; 20 min), supernatant was poured off and an additional 5.0 ml of
117 90% ethanol was added to the pellet. This mixture was agitated for 30 s, centrifuged
118 (1,800 x g; 15 min), and the supernatant was combined with the supernatant from the first
119 round of centrifugation. Extracts were dried overnight, under a steady stream of air, then
120 dissolved in 1.0 ml of methanol, dried for approximately 1 h, and reconstituted in 1.0 ml
121 dilution buffer (BSA-free phosphate buffer: 0.2 M NaH₂PO₄ + 0.2 M Na₂HPO₄ + 0.14 M
122 NaCl; pH = 7.0).

123 To evaluate procedural efficiency, ~20,000 dpm (100 µl) ³H-progesterone (New
124 England Nuclear, Perkin Elmer, Boston, MA) was added to each fecal sample before
125 extraction. Final concentrations were corrected for any losses; samples with an extraction
126 efficiency less than 65% were re-extracted. Fecal extracts were diluted with dilution
127 buffer at 1:50 or 1:800-1:2500 during pregnancy for the progesterone, and 1:50 for the
128 estradiol assays.

129

130 *Fecal Estrogen Metabolite Analysis*

131 Fecal estrogen metabolites were analyzed using a single-antibody estradiol
132 enzyme immunoassay (EIA) that used a polyclonal antibody (1:10,000; R4972; C.
133 Munro, University of California, Davis, CA) raised in rabbits against 17β-estradiol,
134 which cross-reacts with 17β-estradiol 100%, estrone 3.33%, and < 0.01% with estrone
135 sulfate, progesterone, testosterone, cortisol, and corticosterone [23]. Antibody (50 µl;

1:10,000) was added to each well of a 96-well microtiter plate (Nunc-Immuno, Maxisorp; Fisher Scientific, Pittsburgh, PA) and incubated for 12 h (4°C). The plate was washed five times with wash solution (0.05% Tween 20, #P1379, Sigma Aldrich; in 0.15 M NaCl solution), excess liquid was blotted, and 50 µl assay buffer (0.2 M NaH₂PO₄ + 0.2 M Na₂HPO₄ + 0.14 M NaCl + 0.1% BSA; pH = 7.0) was immediately added to each well. Diluted fecal extracts, in duplicate, and estradiol standards, in triplicate, (20 µl; 97.5 – 25,000 pg/ml; Sigma Diagnostics, St. Louis, MO) were added to the plate followed by 50 µl estradiol-17β-horseradish peroxidase (1:50,000; C. Munro). The plate was incubated at room temperature (RT) for 2 h in the dark, washed five times with wash solution, and then blotted dry by repeatedly tapping the plate against an absorbent paper with the well side down. A TMB (3,3', 5,5'-tetramethylbenzidine; Moss Inc., Pasadena, MD) chromagen solution was added, and optical densities measured using a microplate reader (Dynex MRX, Chantilly, VA) at 405 nm (reference of 540 nm). Sensitivity of the assay at maximum binding was 1.95 pg/well. The intra-assay coefficient of variation was <10% (n = 24 assays) and the inter-assay coefficient of variation for two internal controls was 8.6% (mean binding: 24.2%) and 8.7% (mean binding: 69.0%). The assay was validated using serially diluted pooled fecal extracts that demonstrated displacement curves parallel to those of standard hormone preparations. In addition, recovery of standard added to fecal extract demonstrated significant recovery ($P < 0.05$).

155

156 *Fecal Progestogen Metabolite Analysis*

157 A double antibody progesterone EIA was utilized to measure progestogen
158 metabolites in extracted feces, and relied on a monoclonal primary antibody (1:50,000;

159 CL425; Quidel Co., San Diego, CA) produced against 4-pregnen-11-ol-3, 20-dione
160 hemisuccinate: bovine serum albumin with cross-reactions as described in Graham et al.
161 [26].

162 The secondary antibody, a goat anti-mouse IgG (Arbor Assays, Ann Arbor, MI),
163 was diluted (10 µg/ml) in 20X coating buffer (Arbor Assays) and 150 µl was added to
164 each well of a 96-well microtiter plate, followed by incubation for 15-24 h at RT. The
165 solution was removed, replaced with 250 µl 10X blocking solution (Arbor Assays), and
166 incubated at RT for 15-24 h. Following incubation, blocking solution was removed and
167 plates were dried at RT in a Dry Keeper (humidity <20%; Sanplatecorp, Osaka, Japan),
168 with loose desiccant placed in the bottom. Plates were heat sealed in a foil bag with a 1.0
169 g desiccant packet, and stored at 4°C until use.

170 For fecal extract analysis, the plate was loaded in duplicate with 50 µl of
171 progesterone standard (15.6 to 4000 pg/ml; Sigma Diagnostics) and diluted fecal extracts,
172 followed by 25 µl of progesterone-3CMO-horseradish peroxidase conjugate (1:110,000;
173 C. Munro). This was followed by 25 µl primary antibody added to all wells except for the
174 non-specific binding control wells. The plate was incubated at 23°C for 2 h then washed
175 five times and incubated with 100 µl TMB for 30 min at RT. A microplate reader (Dynex
176 MRX) was used to measure optical density at 405 nm. Sensitivity of the assay at
177 maximum binding was 0.78 pg/well. Inter- and intra-assay coefficients of variation (CVs)
178 were 8.9% (31.2% mean binding) and 8.3% (69.8% mean binding) respectively (n=69).
179 The assay was validated by demonstrating parallelism between serial dilutions of pooled
180 fecal extracts and the standard curve. The recovery of exogenous hormone added to fecal
181 extract was significant ($P < 0.05$). Progestogen concentrations increased in verified

182 pregnancies similar to the timing and pattern observed in mohor gazelles [17], further
183 demonstrating biological relevance.

184

185 *Birth Record Analysis*

186 Birth records, from March 1967 to August 2015, were obtained from the AZA
187 North American Regional Studbook for Addra Gazelle [3]. Due to possible influence of
188 management on monthly birth frequency outcomes, data were modeled against a
189 population of addra gazelle known to have unrestricted breeding opportunities [White
190 Oak Conservation Center (WOCC), Yulee, Florida]. Monthly birth numbers were used to
191 evaluate if seasonality exists in addra gazelle populations outside of those managed at the
192 SNZP and SCBI.

193 Temperature and rainfall averages (from 1909 to 2012) for Ouadi-Rimé-Ouadi
194 Achim, Chad (15°30'N, 19°40'E), Tamensa, Mali (18°6'N, 3°49'E), and Termit and Tin
195 Toumma, Niger (16°23'N, 11°39'E) (i.e., current and historical range of addra gazelle) [2,
196 27], Yulee, FL (WOCC), and the United States (U.S.), were obtained from the World
197 Bank Group Climate Change Knowledge Portal
198 (<http://sdwebx.worldbank.org/climateportal/index.cfm>). Average day length for
199 Washington D.C. (SNZP and SCBI), WOCC, and Chad, Mali and Niger (Africa) were
200 obtained from the University of Lincoln Nebraska's Daylight Hours Explorer
201 (astro.unl.edu/classaction/animations/coordsmotion/daylighthoursexplorer.html and
202 accuracy verified through the Astronomical Applications Department of the U.S. Navy
203 (aa.usno.navy.mil/ata/docs/Dur_OneYear.php). Studbook, weather data, and day length
204 were analyzed to evaluate the influence of potential modulators of seasonality.

205

206 *Statistical Analysis*

207 Progesterone and estrogen metabolite concentrations were expressed as $\mu\text{g/g}$ feces
208 and analyzed using standard descriptive statistics, including mean and standard error
209 (SEM). A mean baseline that excluded all values greater than the overall mean plus 1.5
210 standard deviations (SD; progesterone) or 2 SD (estrogen) was calculated. Fecal steroid
211 concentrations were considered elevated when they exceeded the individual's baseline
212 plus 1 SD, for two or more days [28]. The non-pregnant luteal phase was defined as two
213 consecutive progesterone values greater than the baseline plus 1 SD. Estrous cycle
214 duration was calculated from the start of one luteal phase to the start of the next ($n = 61$
215 cycles; 8 females). Matings were observed and pregnancy length calculated based on date
216 of parturition. Weekly averages of progesterone and estrogen concentrations were
217 calculated for each individual pregnancy ($n = 6$ pregnancies in 4 females), followed by
218 calculation of overall and baseline means. Concentrations during gestation were
219 considered elevated when values exceeded 2 SD of the baseline for at least 15
220 consecutive days for progesterone (the approximate length of a luteal phase) and 2
221 consecutive days for estrogen. Anestrus was defined as >1 month of progesterone values
222 continuously at or below baseline. Only profiles uninterrupted by a pregnancy or a lack
223 of sampling were considered in mean calculations of anestrus ($n = 4$ profiles in 4 females;
224 1 profile/female).

225 Temperature, rainfall, and day length for Chad, Mali, and Niger (1909-2012 for
226 temperature and rainfall) were averaged and data referred to as 'Africa'. Pearson product-
227 moment correlation coefficient was calculated to determine the degree of relationship

228 between birth numbers, monthly rainfall, and temperature. Average day length was used
229 in comparisons with birth frequencies and time of anestrus. Conception data was
230 estimated by subtracting 200 d from birth month. All analyses were conducted using
231 SPSS for Windows (Version 23; IBM Corp. Armonk, NY).

232

233 **Results**

234 All females displayed a cyclical pattern of progestogen excretion demonstrating
235 two phases of the reproductive cycle: a non-pregnant luteal phase (NPLP; 2.96 ± 0.17
236 $\mu\text{g/g}$; range, 0.35-13.12 $\mu\text{g/g}$) and an inter-luteal phase (0.51 ± 0.02 $\mu\text{g/g}$; range, 0.16-
237 0.92 $\mu\text{g/g}$) (Figure 1). Estrous cycle length was 19.5 ± 0.4 d (range, 14-26 d; n = 61
238 cycles) (Table 1). The luteal phase was 14.6 ± 1.2 d (range, 10-16 d), and the inter-luteal
239 period was 5.2 ± 1.4 d (range, 2-7 d) in duration. Fecal estrogen metabolite
240 concentrations (0.43 ± 0.02 $\mu\text{g/g}$; range: 0.17-0.73 $\mu\text{g/g}$) generally remained at baseline
241 (Figure 1), with no detectable cyclic pattern, and were not useful for predicting time of
242 ovulation or cycle length. Six pregnancies monitored in four females demonstrated fecal
243 progestogen concentrations at or near non-pregnant luteal levels until approximately 11-
244 12 weeks of gestation, after which concentrations of nearly five-fold NPLP levels ($9.67 \pm$
245 0.53 $\mu\text{g/g}$; range, 2.58-28.57 $\mu\text{g/g}$) were sustained until a few days after parturition
246 (Figure 2). Fecal estrogen concentrations (1.07 ± 0.05 $\mu\text{g/g}$; range, 0.32-2.44 $\mu\text{g/g}$)
247 remained at baseline until week 19, when levels increased 2 SD above baseline ($1.07 \pm$
248 0.05 $\mu\text{g/g}$; range, 0.32-2.44 $\mu\text{g/g}$), and were sustained until parturition (Figure 2). Mean
249 pregnancy length was 200.7 ± 0.4 d (range, 200-202 d) and all pregnancies resulted in a
250 single calf. Sampling did not occur past parturition in two pregnancies, and two females

251 began cycling within a month of pregnancy. Parturition coincided with the start of
252 anestrus in the remaining two pregnancies.

253 All females ($n = 7$) displayed a period of anestrus within the span of September to
254 March, averaging 157.5 ± 5.9 d (range, 140-165 d) (Table 2, Figure 3, and Figure 4). In
255 12 of 13 profiles, progestogen concentrations (0.31 ± 0.007 $\mu\text{g/g}$; range, 0.10-0.89 $\mu\text{g/g}$)
256 were consistently below cyclic baseline levels. The exception was female #114265, who
257 continued to cycle throughout 2011-2012, although she did exhibit anestrus during 2008-
258 2009, 2012-2013, and 2014-2015.

259 The North American addra gazelle studbook reported 2,036 births between March
260 1967 and August 2015; 411, of which, occurred at WOCC. Addra gazelle calves were
261 born in each month of the year (Table 3 and Figure 5), with 876 births (43.0 %) occurring
262 between July and September. Birth frequencies at WOCC mirrored birth numbers
263 reported in the studbook: 161 (39.2%) between July and September. The number of
264 monthly births at WOCC were strongly correlated to U.S. birth numbers ($r = 0.947$).

265 Addra gazelle birth frequency mirrored rainfall patterns in Africa; the greatest
266 percentage of births (July-September; U.S.: 43.0%; WOCC: 39.2%) coincided with the
267 greatest amount of rainfall (126.6 mm; 94.7%; Table 3). Addra gazelle birth frequency
268 was highly correlated to rainfall in corresponding areas (WOCC birth numbers to Yulee
269 rainfall, $r = 0.811$; U.S. birth numbers to U.S. rainfall, $r = 0.802$). The correlations were
270 stronger when birth numbers (WOCC and U.S.) were compared to Africa rainfall ($r =$
271 0.925 and $r = 0.918$, respectively). The lowest percentage of U.S. births (297 births;
272 14.6%) coincided with the period of least rainfall (December to February; Africa: 0.5
273 mm, <0.01%) and lowest temperatures (Africa: $21.8 \pm 0.7^\circ\text{C}$). Temperature was found to

274 be moderately correlated with birth numbers ($r < 0.55$), for all possible comparisons.
275 Anestrus at SNZP and SCBI overlapped with the months when average day length was
276 shortest (November to February) for SNZP and SCBI (9.9 ± 0.2 h day light) and Africa
277 (11.2 ± 0.1 h; Table 3). The beginning of the average anestrus period (September –
278 October) occurred when day length began to decrease below 12 h in all locations, and
279 cyclicity generally resumed in March, when day length approached 12 h. Day length was
280 correlated ($r = 0.99$) among all locations.

281

282 **Discussion**

283 Validation of non-invasive fecal hormone monitoring in the addra gazelle allowed
284 for characterization of the estrous cycle, gestation, and seasonal anestrus in this species.
285 Though cycle and pregnancy duration and profiles mirrored data previously published for
286 the mohor gazelle, to our knowledge, this is the first study to demonstrate seasonality
287 through hormone monitoring in any member of the Nanger genus. Seasonality has been
288 proposed to exist in wild dama gazelle populations, and preliminary studies have reported
289 that birth peaks occur at times when food is most abundant [10]. The analysis of
290 reproductive and climate records collected across North America and African range
291 countries demonstrated a seasonal birth peak in captive populations that coincides with
292 maximal rainfall in the historical range. Our finding of seasonality in captive addra
293 gazelle suggests that it is an evolutionary mechanism that may persist even after
294 translocation to a new climate zone. The data gathered in this study will aid the
295 development of ART techniques for this species, which will facilitate captive breeding.

296 Based on fecal progestogens, the estrous cycle of the addra gazelle (19.5 ± 0.4 d)
297 was similar in length to that of the mohor gazelle subspecies (18.6 ± 0.3 d) [17] and other
298 related Bovidae species [29, 30, 31, 32, 33, 34, 35]. Progestogen concentrations were also
299 similar between addra gazelle (luteal phase mean: $2.96 \mu\text{g/g}$; inter-luteal phase mean:
300 $0.51 \mu\text{g/g}$) and mohor gazelle ($2.06 \mu\text{g/g}$ and $0.30 \mu\text{g/g}$) [17], throughout the estrous
301 cycle. An increase in fecal estrogens did not precede a rise in progestogens, as expected.
302 Detection of preovulatory estrogen surges to predict timing of estrus and ovulation has
303 been reported in species like the domestic horse [36], scimitar-horned oryx [25] and
304 Indian rhinoceros (*Rhinoceros unicornis*) [37]. However, similar to our study, a lack of
305 predictable cyclic increases in fecal estrogen concentrations have been reported for
306 species including mohor gazelle [17], moose (*Alces alces*) [38] and black and white
307 rhinoceros (*Diceros bicornis* and *Ceratotherium simum*, respectively) [39]. It is possible
308 that limited estrogen excretion via feces occurs in addra; steroid metabolism studies to
309 elucidate route and forms of estradiol excretion have not been conducted in any gazelle
310 species.

311 Addra gazelles exhibited a pregnancy length of ~200 d, resulting in a single calf,
312 similar to reports of wild dama and captive mohor gazelles [2, 17, 40]. Progestogen levels
313 remained consistently low during the first 12 weeks of pregnancy, similar to
314 concentrations detected during the NPLP. Concentrations significantly increased at 12
315 weeks, to a mean of $9.67 \mu\text{g/g}$, until a couple of days following parturition; this was
316 expected based on reports in the mohor gazelle, which also exhibited a rise to a mean
317 value of $23.56 \mu\text{g/g}$, occurring at week 10 of gestation [17]. Fecal estrogens also
318 increased significantly during the latter part of gestation, likely in preparation for

319 parturition, similar to that observed in a number of other ungulate species [41, 42, 43].
320 Addra gazelle, similar to mohor gazelle [17] do not appear to experience lactational
321 anestrus as two females resumed cyclicity immediately following pregnancy. Of the other
322 four analyzed pregnancies, two were not sampled past parturition and two coincided with
323 anestrus, and so could not contribute to this hypothesis.

324 To our knowledge this is the first study demonstrating an annual anestrus in the
325 Nanger genus; an earlier study of mohor gazelles was less than a year in duration [17].
326 Seasonality likely evolved as a response to predictable environmental variations in day
327 length or photoperiod for species indigenous to temperate or arctic regions [10, 44, 45].
328 Day length changes cue timing of mating and typically result in births occurring at a time
329 when food is most abundant, and therefore when conditions are most favorable for
330 offspring survival [44, 46, 47]. Nutrition is typically managed in captive settings while
331 food availability can fluctuate for wild individuals, potentially impacting reproductive
332 activity. As such, seasonality can differ between wild and captive populations of the same
333 species [10, 33]. In regions where there is little variation in photoperiod, such as the
334 native habitat of the addra gazelle, reproductive seasonality may have also evolved in
335 response to other modulators including (but not limited to) resource competition,
336 predation pressure, temperature and rainfall fluctuations [45, 48, 49].

337 In the present study, all seven addra females displayed an annual anestrus during a
338 time of decreased photoperiod in both captive and historical ranges. Amplitude of annual
339 day length changes varied greatly among ranges from 1.8 h (Africa) and 3.8 h (WOCC)
340 to 5.2 h (D.C.). However, females generally entered anestrus when day length decreased
341 below 12 h (October: 11.6 h SNZP, 11.9 h Africa) and cyclicity typically resumed by

342 March when average day length approached 12 h (11.2 h SNZP, 11.7 h Africa). It
343 appears that day length may play a role in regulating reproductive seasonality in this
344 species, and that 12 h may be a crucial threshold.

345 The semi-arid climate of the Sahelo-Saharan region, historical range of the addra
346 gazelle, experiences minimal rainfall and falls within tropic and sub-tropic zones. Peak
347 rainfall in this region occurs between the months of July to September, mirroring peak
348 birth frequency of addra gazelle managed in North America [10, 50]. Given the timing of
349 anestrus in this species, seasonality may have evolved as a strategy for births to occur at a
350 time when rainfall was at a maximum and therefore, a time of optimal food availability
351 for the lactating mother. Births in North America occurred throughout the year and one of
352 the study females continued to cycle throughout the typical anestrus period of 2012,
353 suggesting anestrus is not strict in this species. Dama gazelles have been described as
354 having a preferred season as opposed to being firmly seasonal [10], and influencing
355 factors are not fully understood. Some tropical species have been shown to respond to
356 photoperiod changes, though the responses appear to vary widely [48]. Eld's deer (*Cervus*
357 *eldi thamin*) are strongly seasonal in native habitat, a characteristic that persists even
358 when moved to a temperate zone, whereas sable antelope display seasonality in the wild,
359 but not when managed in captivity in temperate regions [33, 51]. The addra's historical
360 range and managed habitat share similar climatic patterns, despite different day length
361 and rainfall fluctuation amplitudes, and the females appear to maintain seasonality,
362 similar to the Eld's deer [48, 51].

363 Anestrus or reproductive seasonality has been demonstrated in a variety of
364 ungulate species. However, there is significant variability in seasonality timing and

365 occurrence between related species, especially those indigenous to tropical regions [48,
366 49]. The scimitar-horned oryx, a species sympatric to the addra gazelle, displays anestrus
367 from March to June [31]. Differing gestation lengths, 6.6 months in the addra gazelle and
368 8 to 8.5 months in the scimitar-horned oryx, and differences in average size of the
369 animals, may partially explain the disparity in timing of anestrus in these two species
370 [48]. Factors influencing timing of seasonality may impact onset of seasonality
371 differently in these species [48]. To fully understand these mechanisms, further
372 investigation is required.

373 In conclusion, monitoring fecal hormones is a useful method for assessing ovarian
374 cycle activity and gestational changes during the reproductive lifetime of the addra
375 gazelle. The discovery of reproductive seasonality for female addra gazelle, suggests that
376 reproductive status should be closely monitored before attempting to breed or implement
377 ARTs. The primary triggers of seasonality in this species are unclear, although rainfall
378 and photoperiod appear to be highly influential. Data stemming from this characterization
379 of the estrous cycle, pregnancy, and seasonality will aid in the optimization of captive
380 management and provide valuable information critical to future studies analyzing the
381 impact of environmental changes on reproductive success.

382

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393

394

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545 **Figure Legends:**

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547 **Figure 1.** Fecal progesterone (solid line) and estradiol (dashed line) concentrations ($\mu\text{g/g}$
548 feces) in a female addra gazelle during a 3-month period. NPLP = non-pregnant luteal
549 phase.

550

551 **Figure 2.** Weekly mean ($\pm$ SEM) fecal progesterone (solid line) and estradiol (dashed line)
552 metabolite concentrations ($\mu\text{g/g}$) during six pregnancies in four addra gazelle females.
553 Arrow indicates approximate date of parturition.

554

555 **Figure 3.** Fecal progesterone concentrations ($\mu\text{g/g}$ feces) during anestrus periods in
556 seven addra gazelle females. Mean estrus cycle progesterone baseline indicated by
557 dashed line.

558

559 **Figure 4.** One example of addra gazelle fecal progesterone concentrations spanning 1 and
560 $\frac{1}{2}$ years to include pregnancy, seasonal anestrus, and resumption of cycling. Dashed line
561 indicates baseline.

562

563 **Figure 5.** Percentage of addra gazelle births per month (1967-2015) for White Oak
564 Conservation Center in Yulee, FL and the United States, represented in the context of
565 average rainfall in the historical range, United States, and Yulee, FL.

566

Table 1. Date of birth, sample collection dates, reproductive status during specific collection periods, estrous cycle range and average length based on fecal progesterone concentrations (n = 61 cycles of 8 females; 5 – 15 cycles/female) in individual Addra gazelles managed at the Smithsonian National Zoological Park (SNZP) and Smithsonian Conservation Biology Institute (SCBI).

A = anestrus; P = pregnant; C = cycling

Table 2. Anestrus length in seven Addra gazelles based on fecal progestogen concentrations.

Animal ID	Dates	Anestrus Days
114265	04-Nov-08 to 14-Feb-09	103 *
	31-Aug-12 to 11-Feb-13	165 §
	08-Nov-14 to 17-Jan-15	71 ^
114378	28-Sep-11 to 05-Feb-12	140 §
	06-Sep-12 to 16-Feb-13	164 §
114465	28-Sep-11 to 07-Mar-12	161 §
	15-Oct-12 to 28-Feb-13	136 *
	05-Nov-14 to 06-Mar-15	122 ^
114964	09-Nov-14 to 03-Mar-15	114 ^
114965	11-Nov-14 to 22-Feb-15	103 ^
114966	09-Nov-14 to 10-Feb-15	93 ^
114967	11-Nov-14 to 17-Feb-15	98 ^
Overall mean $\pm$ SEM		157.5 $\pm$ 5.9
* pregnant immediately prior ^ no samples collected in month prior to earliest date § used in calculation of mean		

Table 3

Table 3. Addra gazelle birth records (1967-2015) summarized from the Addra gazelle studbook for White Oak Conservation (WOCC; Yulee, FL) and the United States (U.S.), mean day length for Smithsonian National Zoological Park (SNZP; Washington D.C.) and mean monthly rainfall data (1909-2012) for Yulee, FL, the U.S., and the Addra gazelle historical range in Africa. Values were color-coded using a gray-scale: darkest gray indicates the lowest value and white indicates the highest value for that column.

	Birth #		Day Length (h)			Rainfall (mm)		
	WOCC	U.S.	D.C.	WOCC	Africa	WOCC	U.S.	Africa
January	23	86	9.4	10.1	11.1	86.2	47.5	0.1
February	31	106	10.2	10.6	11.3	85.5	43.3	0.4
March	25	144	11.2	11.4	11.7	105.2	50.7	0.1
April	26	144	12.5	12.4	12.2	71	49.3	0.1
May	28	108	13.6	13.2	12.6	91.4	58.1	0.7
June	39	180	14.5	13.8	12.9	158.1	63.6	5.6
July	44	266	14.6	13.9	12.9	172.5	65.5	38.9
August	71	360	13.9	13.4	12.7	162.1	65.8	73.3
September	46	250	12.8	12.6	12.3	151.5	61.2	14.4
October	32	162	11.6	11.7	11.9	84.9	51.5	0.1
November	21	125	10.4	10.8	11.4	59.7	49.6	0.0
December	25	102	9.6	10.2	11.1	78.4	51.4	0.0

Figure 1

Figure 2

Figure 2.

Figure 3

Figure 4

Figure 4.

Figure 5