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Abstract: In this study the estrous cycle of the aoudad has been analyzed and characterized for the first time, using non-invasive methods for tracking reproductive cyclicity. The duration of the estrous cycle is 23 days (range 16-32 days), with a luteal phase of 17 days (range 12-27 days) and an interluteal phase of 6 days (range 3-14 days). The estrous cycle did not differ between females, but it was affected by the time of the year. Intra-individual variation of the cycle was observed in one out of the nine individuals. The average hormone concentration values, the estrogen:progestogen ratio, as well as their minimum and maximum values for each interluteal and luteal phases of the estrous cycle, are shown. Inter-individual differences found in these values were basically associated with age. Females tended to start their cycle when in the presence of an adult male. Anoestrus was observed in study females except for the oldest (14 years old). Age and anoestrus onset were correlated, with younger females starting earlier than the older ones. This study reveals that Ammotragus reproductive biology is more similar to that of Capra than Ovis, except for some endocrinological features.

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Characterization of the estrous cycle and reproductive traits of the aoudad (*Ammotragus lervia*) in captivity.

Short title: Aoudad oestrus cycle

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Abstract

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Key words. *Ammotragus lervia*, anoestrus, fecal steroids, estrous cycles, Aoudad

1. Introduction

The aoudad or Barbary sheep (*Ammotragus lervia*) is a caprid formerly widespread in all mountainous areas of North Africa, from the Atlantic to the Red Sea coasts and, from the Mediterranean coast in the north to the south of the Saharan desert [1-5] Classified as vulnerable (VU C1) by the IUCN red list [6], their native populations, as happens with other ungulate species in North Africa, are decreasing and facing a high risk of extinction in the wild. Paradoxically though, and due to hunting interests, the species has been introduced out of their native range to some regions where it is successfully breeding and spreading: Spain [7,8], USA [1,9] and Mexico (F. Gonzalez Saldivar, pers. com,).

As observed in other ungulate species, the taxonomy of the aoudad is controversial and up to six subspecies have been described, almost entirely through morphological characteristics [10]. In order to preserve the Western Sahara aoudad population, originally ascribed to the subspecies *sahariensis* (*Ammotragus lervia sahariensis*, Rothschild, 1913), a captive breeding program started in 1975 at “La hoya” Experimental Field Station (Estación Experimental de Zonas Aridas, Spanish Research Council-CSIC) in Almería, southeast of Spain [11,12].

Taxonomically, the genus *Ammotragus* has been claimed to be either an ancestor of *Capra* spp. and *Ovis* spp. or an intermediate phylogenetic stage between both genera [13]. In fact, the aoudad shares morphological, physiological and behavioral traits with goats and sheep [4,10,14,15]. In terms of their reproductive biology, goats and sheep are closely related (quite similar estrous cycles, gestation length, twinning), although there are significant physiological differences related to the endocrinology of gestation [16].

The gestation period of the aoudad ranges between 150-160 days [4,12] being quite similar to several breeds of wild and domestic goats and sheep [17-21]; however, the precise duration of their ovarian cycles is unknown. In captivity, the aoudad is relatively precocious, reaching sexual maturity around 415 days for males and 270 days for females [22], and births may take place all year round [12,22] particularly when resources are not limiting and where seasonal variations are relatively moderate [22]. Most wild species of *Capra* and *Ovis* genera that live in seasonal climates are seasonally polyestrous, with a period of rut in autumn and births in spring [17,18]; however, even under captive conditions, the aoudad shows a peak of births in spring (March-April) [12,22]. Like goats and sheep, the aoudad often has twins [12,22] and although it shares with the former genera the same type of placentation, the endocrinological support of the gestation of the aoudad is more similar to that of sheep than goats [16]. Moreover, whereas live offspring of aoudad male x nanny goat crosses have been reported, those of aoudad male x domestic ewes are not viable [23-26].

Fecal steroid determination has been widely used for analysis of reproductive status of many Bovid species, both in captive and wild conditions [27]. Fecal steroid techniques have a series of advantages; they are non-invasive and allow the analysis of long series of data. Changes in the level of circulating reproductive hormones elicit changes in sexual behaviour that reflect the reproductive physiology of individual animals [28,29]; alternatively, the quality and intensity of the repertory of behaviours can be used to infer the reproductive status of individual females.

Using these procedures, we have carried out a study in order to describe and characterize, for the first time, the ovarian cycle of the aoudad. This study will provide additional data on reproductive biology between three taxonomically related geni: *Ammotragus*, *Ovis* and *Capra*. The final purpose of this study is to provide practical information for the improvement of “ex situ” and “in situ” conservation actions through more suitable breeding management practices, as well as to manage exotic introduced populations of the aoudad around the world.

2. Material and Methods

2.1 Animals and sample collection

The aoudad study population is maintained in captivity at the “la hoya” Experimental Field Station (Estación Experimental de Zonas Aridas) in Almería (36º 45´N, 3º 00´W), on the

Mediterranean coast of southeastern Spain, one of the warmest and most arid areas in Europe. The mean annual temperature is 18ºC and the average rainfall is 237 mm [30]. This aoudad population has successfully bred in captivity since 1975, when the founder individuals were shipped from Western Saharan [22].

Nine adult females (more than 2 years-old) were selected for the study. Normal day-to-day management procedures were described by Alados et al [11]. Captive aoudads were fed with commercial pellets, barley and fresh alfalfa; moreover, they received a daily ration of straw. Water was always available *ad libitum*.

Of the nine selected adult females, one (STD 302) belonged to a f 2-3 year-old cohort, four were 3-4 years of age. (STDs 297, 299, 300, 301), three were 4-5 years of age. (STDs 293, 294, 295) and the eldest one was 14 years old. (STD 211). During the study, and for management breeding reasons, all females were kept apart from the males to avoid reproduction; only one (STD 211) had given birth previously. In order to carry out longitudinal estrous cycle monitoring, selected females were penned with other females in the presence of a vasectomized adult male (STD 268, age 9 years.).

Fecal samples were collected early in the morning each day (5 day/week) over a period of 5.5 months between December 2005 and May 2006. Individuals were identified by their ear tags. Approximately 5 g of freshly deposited feces was collected in individual plastic bags and stored at -20ºC until analyzed.

2.2. Fecal sample processing

Steroid hormone metabolites (progestagens and estrogens) in all fecal samples were determined following standardized procedures. For extraction, fecal material was mixed thoroughly and a subsample of 0.18-0.2 g was extracted using ethanol (100%) (4.5 ml) and distilled water (0.5 ml); after 30 min of shaking (Multi-pulse vortexer, Glas-Col®, USA), samples were centrifuged at 2500 rpm for 20 min and the supernatant transferred to a glass tube. The fecal material was combined with an additional 4.5 ml of ethanol and 0.5 mL of distilled water, vortexed (1 min) and recentrifuged; the second supernatant was added to the initial one and evaporated with dry air. One mL of methanol was added to the dry extract and placed in a ultrasonic glass cleaner (Branson® 8510) for 20 min. The extracts were diluted in a dilution buffer and stored frozen until analysis. The mean (± sem) extraction efficiency was 80% for estrogens and 81.6 ± 5.8 for progesterone as determined by recovery of ^{3}H-estradiol and ^{14}C-progesterone.

2.3. Determination of fecal steroid metabolites with enzyme immunoassay (EIA)

Fecal steroid hormone determination followed the procedures described by Munro et al [31] for enzyme immuno assay (EIA). Antibodies for progesterone (monoclonal Pregnane CL425, 1:10.000 dilution) and estrogen (polyclonal E_2-R4972, 1:10.000 dilution) metabolites were provided by Coralie Munro (University of California, Davis, CA, USA). The CL425 cross reacts with various progesterone metabolites, including 4-pregnen-3,20-dione (100%), 4-pregnen-3a-ol-20-one (188%), 4-pregnen-3b-ol-20-one (172%), 4-pregnen- 11a-ol-3,20-dione (147%), 5a-pregnan-3b-ol-20-one (94%), 5a-pregnan-3b,20-dione (64%), 5a-pregnan- 3,20-dione (55%), 5b-pregnan-3b-ol-20-one (12.5%), 5-pregnan-3,20-dione (8.0%), 4-pregnen-11b-ol-3,20- dione (2.7%), and 5b-pregnan-3a-ol-20-one (2.5%) [32]. The R4972 cross reacts with estradiol 17B (100%) and estrone (3,3%). Before analysis, fecal extracts were diluted in phosphate-buffered saline 1:10 to 1:20 v/v for estrogens and 1:50 to 1:600 v/v for progesterone. Serial dilutions of pooled fecal extracts produced displacement curves parallel to those of the appropriate standard. The correlation coefficients of parallelism test were R^2= 0.985, R^2= 0.983, for progestagens, estrogens and testosterone, respectively. Inter-assay CVs were (mean±sem) 7.98±2.04, 8.25±1.1 for progestagens and estrogens respectively; intra-assay CVs less than 10%. Assay sensitivities were 1.17 pg/well (estrogens), 1.09 pg/well (progestagens). Absorbance was measured at 405 nm with an automatic microtiter plate spectrophotometer (Tecan®, sunrise, Austria) and the data were transferred to an interfaced computer (Magellan®, Austria). Hormone concentrations are expressed as ng/g wet feces.

2.4. Data analysis

The two phases of the estrous cycle –interluteal and luteal phases- were defined following Pickard et al [33]. The duration of the estrous cycle was calculated as the time between the onsets of two consecutive luteal phases. For each female, the average duration of each luteal and inter-luteal phase of each estrous cycle was calculated separately. Linear regression was used to investigate the variation in the duration of these phases. ANOVA test was used to investigate between individual and temporal/seasonal differences. One-sample *t*-test, with the mode as value of reference, was used to investigate intra-individual differences.

Plotting hormone concentration (progestagen and estrogen concentrations) with time showed a clear regular recurrence in progestagens but not in estrogens; however, the estrogen:progestagen ratio (E:P ratio) showed this recurrence. The E:P ratio has proven its ability for detecting the time of ovulation in humans [34] as well in other ungulates [33].

For each female, the average fecal concentration of progestagens, estrogens and E:P ratio were calculated separately for each luteal and inter-luteal phase of each estrous cycle. Linear regression was used to investigate the variation of each hormone concentration as well as the E:P ratio on the estrous cycles. ANOVA test was used to investigate between individual differences. One-sample t-test, with the mean value for progestogen and estrogen concentrations and E:P ratio of each luteal and interluteal phases as values of reference, was used to investigate intra-individual differences. All statistical analyses were performed using STATISTICA for Windows (Statsoft UK, Letchworth). Statistical differences were considered significant at $P< 0.05$, unless stated otherwise.

3. Results

Fecal progestagen excretion fluctuated regularly with a mean (±sem) frequency of 23.0±0.52 days. A range of 16 to 32 days, mode of 21 days, was recorded for n= 38 estrous cycles. The duration of the luteal phase varied between 12 to 27 days (mean±sem, 16.6±0.52, mode= 14) and the duration of the interluteal phase varied between 3 to 14 days (mean±sem, 6.5±0.49, mode= 4). The duration of the inter-luteal and luteal phases was inversely related (F=6.31, df=1, p=0.017, r=-0.4). The duration of the estrous cycles did not differ significantly between females; however, those differences were significant depending on the month (F=3.23, df=4 p= 0.024), with shorter estrous cycles in January (20.7±sem). Intra-individual variation in the duration of the estrous cycle was significant for one female (STD number 293, t=3.8, df=4, p= 0.02). Figure 1 shows a representative estrous cycle for the aoudad.

The average hormone concentration values, the E:P ratio, as well as their minimum and maximum values for each interluteal and luteal phases of the estrous cycle, are shown in Table 1. The E:P ratios were inversely related both in luteal and interluteal phases (interluteal phase: F=66.7, df =1,r^2=0.63, p<0.0001; luteal phase: F=39.9, df=1, r^2=0,47, p<0,0001).

Inter-individual significant differences (p<0.05) were found for hormone concentration and E:P ratio both in the interluteal and luteal phases of the estrous cycle. Age (the eldest female (14 years of age) *vs* females ≤ 5 years of age.) explained the highest significant difference (F=6.1, df=1, p= 0.02) found for the values of progestogen in the luteal phase (818.2±118.3 *vs* 497.5±47.1 respectively). No intra-individual significant differences were found in the hormone concentration values and for the E:P ratio in the two phases of each estrous cycles.

At the beginning of the study only two females were in estrous but, ten days after the male was introduced in the herd, the rest of females (n = 7) started to cycle at the same time. Globally, females maintained synchronicity showing some differences depending on the duration of each individual luteal phase. Figure 2 shows the ovarian cycles of three of the synchronized females (STD 294, 300 and 301).

All females, except the eldest one (STD 211), showed an interruption to the regularly pattern of progestagen secretion with the consequent start of an anoestrus period. In two cases, the anoestrus period started during the third week of February, in one case in the second week of March, in 4 cases (50%) in the first week of April and in one case in the first week of May. All except one (STD 297) of the females remained in anoestrus until the end of the study; this animal restarted cycling after 48 days (from February 17th to April 4th). There was a significant correlation (r^2= 0.497) between age and the onset of anoestrus, with younger females starting the anoestrus period before the older ones.

During the anoestrus period, the concentration of progestogen decreased, reaching concentration values equivalent to the interluteal phase of the estrous cycle. However, the secretion of estrogens remained unchanged, as during the estrous and, consequently, the E:P ratio during the anoestrus showed similar values to the interluteal phase (see average values in Table 1).

4. Discussion

This study shows the average duration of the estrous cycle in female aoudad with two well differentiated phases based on fecal progesterone concentrations: a luteal phase, when progesterone reaches its maximum values (539.3±46.2 ng g^{-1}), followed by an interluteal phase, characterized by minimum values of progesterone (128.4±10.3 ng g^{-1}). As expected, both phases were negatively correlated both with duration and progesterone concentration. Although there were no inter and intra-individual significant differences (except for one female, STD 293), the interval between successive peaks of progesterone was variable, ranging from 16 to 32 days (mode = 21 days). The duration of the period of sexual receptivity (interluteal phase) ranged form 3 to 14 days (mode = 4 days).

A comparison of several reproductive traits for some bovid species is shown in Table 2. The Aoudad estrous cycle and gestation are more similar to *Capra* than to the *Ovis* genera (see Table 2).

In spite of some dissimilarities between *Ammotragus* and *Ovis* for some reproductive traits, they show common endocrinological features, i.e., to maintain late pregnancies, in both genera the corpus luteum regresses before term of pregnancy, with the placenta being the major source of progesterone [16,44]; in contrast, the goat placenta produces little progesterone [45,46]

Aoudad captive populations show births over the whole year, although they exhibit a peak of births between March and May; however, births in summer and early autumn decrease significantly [12,22,40] which suggests a period of anestrus for this species in captivity, as shown in this study. The anestrus affected 8 out of 9 of the study females (88.8%). The period of anoestrus started by the third week of February and finished by the first week of May; this time interval explains the significant decrease in births found by Cassinello and Alados [22] in the same captive population.

Most wild goat and sheep species inhabiting northern latitudes are seasonal breeders. The species located further north show shorter breeding seasons than those living in southern locations; therefore, for the northern species, rut seasons take place in the autumn-early winter, with peaks of births in late spring-early summer; however, the species living further south have longer breeding seasons as their rut season starts early around the end of summer-early autumn and the birth season starts at the beginning of spring. In the case of the aoudad, the species naturally inhabits mountainous areas in a range of latitudes between 14º N (Kordofan, Sudan) and 35 ºN (northern of Morocco, Algeria and Tunisia).Photoperiod, climatic conditions and food availability are key factors explaining either the anoestrus periods or the breeding season [47] and, of course, the presence of males. In the case of the study captive population of aoudad, food availability and access to mates were not limiting factors, so that reproductive seasonality may be related to photoperiod as the captive breeding centre in Almería is located at 36º north latitude.

Our results showed a positive correlation between the age of the female and the time of anestrus onset. An mentioned earlier, onset of anoestrus by younger females could be related to the “female effect” occurring in populations living in captivity, as has been demonstrated in the Iberian ibex [48]. According to Santiago-Moreno et al [48], in captivity, the number of social interactions significantly increases and older (dominant) females may inhibit the ovulatory activity of younger (subordinate), females through the increase of cortisol levels derived from the stress associated with a limited food access [48] or by the secretion of inhibiting pheromones, as has been reported in other mammal species [49,50]. In the European mouflon, the onset of anestrus also takes place earlier in younger females (2 years old) than in older ones (> 3 years old) [51]. On the other hand, the so-called "male effect" would explain the synchronization of the study females just after the introduction of the vasectomized male (see Methods).

In sum, this study reveals that *Ammotragus* reproductive biology is more similar to that of *Capra* than *Ovis*, except for some endocrinological features. As stated in other studies mentioned here , the aoudad shares morphological, physiological and behavioural traits with either genera, or it is situated in an intermediate position, which is related to its taxonomic and phylogenetic relationship with *Ovis* and *Capra*, an issue yet to be clearly defined.

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Cover Letter

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Estación Experimental de Zonas Áridas (CSIC)

Almería, September 26th, 2011

Editor

Theriogenology

Dear Editor,

I will appreciate if you consider the manuscript titled **"Characterization of the estrous cycle and reproductive traits of the aoudad (*Ammotragus lervia*) in captivity"** for publication in THERIOGENOLOGY.

Sincerely

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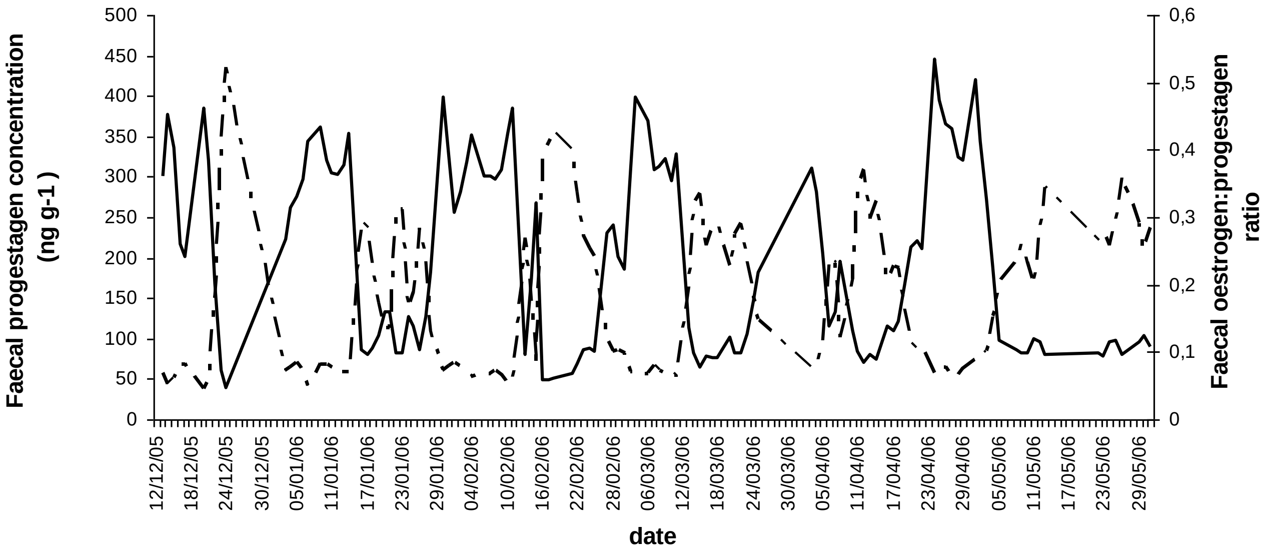
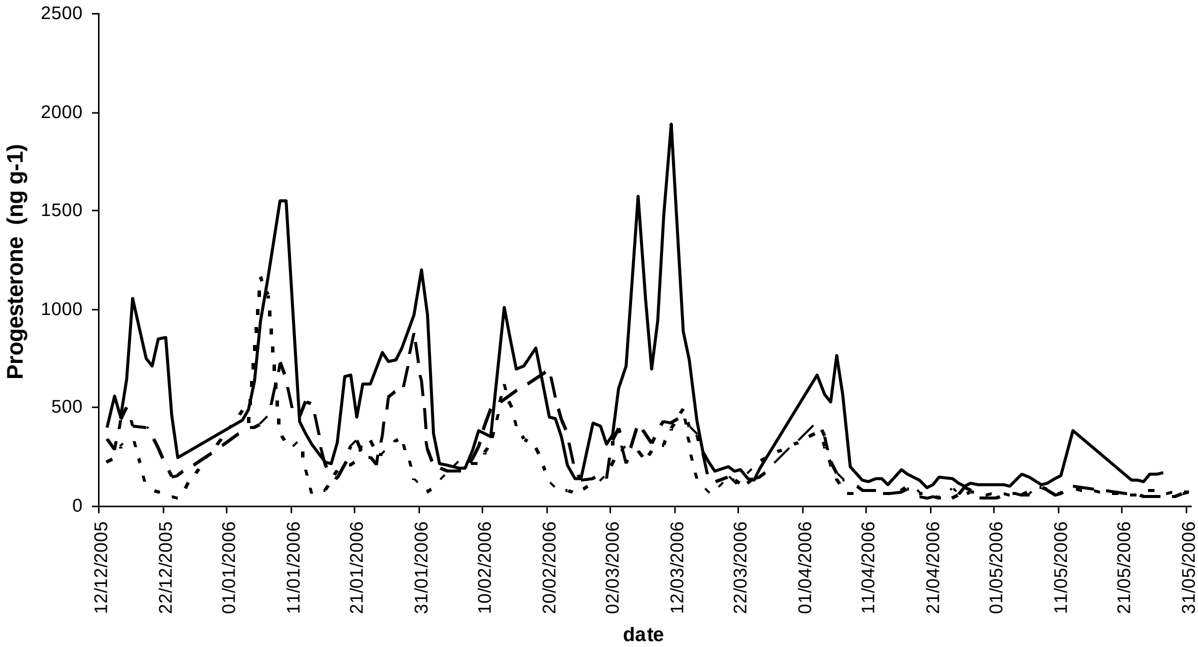
Figure

Figure 1



Figure

Figure 2



Tables

Table 1

Mean (±sem) and range values of fecal steroid concentrations during the different phases of the aoudad´s estrous cycles.

	Progestagens (ng g^{-1})	Estrogens (ng g^{-1})	E:P ratio
Interluteal phase	128.4±10.3	24.3±0.95	0.243±0.017
N=41	(54.8-284.8)	(16.3-41.7)	0.095 0.496)
Luteal phase	539.3±46.2	24.4±0.85	0.066±0.017
N=46	(182.6-1718.2)	(16.7-37.2)	(0.025-0.159)
Anoestrus	105.9±20.5	24.6±1.24	0.332±0.065
N=8	(42.5-208.2)	(20.4-30.4)	(0.095-0.7)

Tables

Table 2

Some comparative reproductive traits for different bovid species.

Species	Estrous cycle length (range) (in days)	Gestation length (range) (in days)	Presence/percentage of twins	References
Capra pyrenaica	19 (17-23) [1]	158 (157-160) [2]		[1]Santiago-Moreno et al. 2003[2] Granados et al. 2002
Capra ibex	20	167	yes	Stüve & Grodinsky 1987
Capra nubiana		147-180	yes	Shargal et al. 2008
Pseudois nayaur	24.9 (21-35)	168	yes	Kusuda et al. 2006
Domestic goats	21			Leyva-Ocariz et al. 1993; Simoes et al 2006
Ammotragus lervia	**23 (16-32) [1]**	154-161 [2]	23 [3] 54.8 [4] **triplets observed in the 3 cases in the study population**	**[1] present study** [2] Lobanov & Treus 1971 [3]Cassinello & Alados 1996 [4] Matschei 2006
Ovis orientalis	17 (16-18)		no [1]	[1] Santiago-Moreno et al. 2001
musimon			20.7 [2]	[2] If crossed with domestic sheep (Garel et al. 2005)
Ovis Dalli	18.2		no	Groodrowe et al. 1996
Domestic sheep	16			Leyva-Ocariz et al. 1993; Simoes et al 2006

Tables

Figure 1. Concentration of progesterone (continuous line) and E:P ratio (dash-dotted line) for individual ALS 293.

Figure 2. Ovarian cycle plot of three female aoudads showing estrus synchronization: females STD 294 (dotted line ……),.STD 300 (dashed line -------) and STD 301 (continuous line ———).

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