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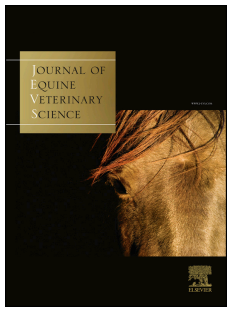

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Studies on the use of PGF2α and GnRH analogues for Timed Artificial Insemination in jennies

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Abstract

Donkey farming is expanding due to the rediscovery of nutritional properties of jennies' milk for human consumption. In livestock, Timed Artificial Insemination (TAI) allows to manage big herds without estrus detection, but there are very few studies on estrus synchronization in jennies. Aims of this study were to compare three different combinations of PGF2α and GnRH analogues (GnRH) for TAI in jennies and to compare the estrus/diestrus status diagnosed by ultrasonography (US) with serum progesterone concentration. Nine fertile jennies were submitted to three TAI protocols: PPG (PGF2α, PGF2α, GnRH), PGPG (PGF2α, GnRH, PGF2α, GnRH) and GPG (GnRH, PGF2α, GnRH). Ovarian activity was monitored until ovulation and blood samples were taken for progesterone determination. Artificial Insemination (AI) was done with a fresh-diluted semen. The comparison of the three TAI protocols showed a trend for difference in pregnancy rates per synchronized jennies (from 11% with PPG up to 56% with PGPG), even though not statistically significant. Follicle diameter or the presence/absence of a CL at the beginning of the treatment did not affect synchronization response or pregnancy rate. Dominant follicle diameter, at the time of the last GnRH treatment, significantly affected the ovulation response. US was confirmed to be highly accurate for the determination of the estrus/diestrus status. This study demonstrated the possibility to achieve reasonable synchronization and pregnancy rates in jennies using TAI protocols adapted from other species.

Keywords: Donkey; timed artificial insemination; pregnancy rate.

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1. INTRODUCTION

In the last years, the rediscovery of the nutritional properties of jennies' milk revived the interest in donkey breeding, especially in European Mediterranean countries. Jenny's milk is the most similar to the human one, representing the best choice for feeding children intolerant to cow's milk proteins [1–3].

Synchronization of estrus and ovulation is an excellent tool to improve the use of Timed Artificial Insemination (TAI) and consequently genetic selection in livestock [4–7].

In bovine reproduction, estrus synchronisation is possible by using many different protocols allowing the application of TAI to manage big herds of cows without estrus detection. The most used protocols are Ovsynch [6] which consists of serial injections of GnRH analogues (GnRH), PGF2α, GnRH followed by AI, and his variants Cosynch-72 [8] and Double-Ovsynch. This last is mostly used for managing the post-partum AI [9]. Cow's pregnancy rates after the application of frozen semen TAI are reported to range from 49% to 64% [10].

In small ruminants, progestagen-impregnated sponges and eCG are being used to synchronize estrus and ovulation during the breeding season or superficial anestrus. This allow to inseminate ewes with fresh transported semen at a fixed time, without the detection of estrus [5,11] and with pregnancy rates between 49% and 72% [12–14].

In horses, several drug combinations and associations such as Progesterone and 17-β-Estradiol, Allyl trembolone, PGF2α, hCG and GnRH have been used for the synchronization of follicular growth and ovulation [7,15–17]. On the other hand, this species is characterized by an individual approach to reproduction, thus estrus synchronisation and TAI are seldom used.

Blanchard published a study on estrus synchronisation in Mammoth jennies (*Equus asinus americanus)* [18], using a combination of Progesterone and 17-β-Estradiol or two injections of PGF2α 16 days apart [18].

Hystorically, hCG is the treatment of election to induce ovulation in mares and jennies [19–22]. However, repeated injections of hCG in mares induce the production of antibodies against this heterologous protein [23]. For this reason, GnRH was succesfully employed as an alternative to hCG for the induction of ovulation in mares and jennies [20–22,24–26].

Diagnostic ultrasound (US) is a non-invasive technique that allows to explore the characteristics of soft tissues. In equine reproduction, this is applied to evaluate the dynamic changes of the uterus and ovaries [27]. In mares, the presence of endometrial oedema during the follicular phase has been described and uterine oedema grade [28] is related to the estrus stage and ovulation time

[29]. In jennies, uterine oedema is not always observed during estrus [30], while corpus luteum appears similar to those reported in mares [30,31].

The aims of this study were i) to compare the outcome of three different combinations of PGF2α and GnRH analogues for TAI in jennies in terms of estrus synchronization, ovulation and pregnancy rates; ii) to evaluate ex post if the ultrasound examination itself agrees with the progesterone evaluations and thus it is accurate enough for the diagnosis of the estrus/diestrus status of jennies.

2. MATERIALS AND METHODS

2.1 Animals – This study was carried out at the Department of Veterinary Sciences, Pisa University (43° 41' 00" North, 10° 21' 00" East), from August to February.

The study was approved by the Ethical Committee of Pisa University (protocol number 0066075/2017).

Nine cyclic, non-lactating Amiata jennies, 6-12 years old, with a body condition score between 3 and 4 out of 5 [32], an average weight of 280 kg and known to be fertile, were kept in paddocks and fed with hay from mixed-grass meadows and water *ad libitum*. Semen was collected from a fertile donkey stallion, 4 years old, with a body condition score of 3 out of 5 [32], stabled in box and also fed with hay from mixed-grass meadows and water *ad libitum*.

2.2 Estrus synchronization protocols and ovarian activity monitoring – Jennies were submitted to three protocols for the synchronization of estrus and ovulation based on different combinations of analogues of PGF2α (Alphaprostol, Gabbrostim®, Vetem Spa, Monza-Brianza, Italy), 1.5 mL, im, and GnRH agonists (GnRH) (Buserelin acetate, Suprefact®, Sanofi Spa, Milano), 0.4 mL, sc.

The three protocols used a different combination of treatments and were named as follows:

PPG → PGF2α+PGF2α+GnRH;

PGPG → PGF2α+GnRH+PGF2α+GnRH;

GPG → GnRH+PGF2α+GnRH.

The time schedule of the three protocols are descripted in Table 1.

Jennies' ovarian activity was monitored weekly by ultrasounds (US) using a machine equipped with a linear probe of 5-7.5MHz (Mindray DP30, Shenzhen, China). At day 0, corresponding to seven days after the last PGF2α injection, jennies were evaluated for estrus: regardless to the

grade of the uterine oedema, jennies were judged to be in estrus when having a follicle of a diameter ≥28 mm and no corpus luteum. Jennies in estrus were judged as responding to the synchronization protocol, submitted to the treatment with GnRH analogue and AI and to daily US until the detection of ovulation.

Ovulation response was considered positive if ovulation occurred within two days after the treatment with GnRH.

Table 1: Time schedule of the three protocols used for estrus synchronization in jennies

Protocol	**Day -22**	**Day -15**	**day -7**	**day 0**
PPG	PGF2α	-	PGF2α	GnRH + AI
PGPG	PGF2α	GnRH	PGF2α	GnRH + AI
GPG	-	GnRH	PGF2α	GnRH + AI

2.3 Semen collection, AI and pregnancy diagnosis – Semen was collected by using a Colorado-model artificial vagina (ARS, Chino, CA), with the jackass jumping on an estrus jenny. Collection was made after one or two days of sexual rest. Immediately after collection and estimation of the total volume, semen was filtered through a sterile gauze to remove the gel fraction. Thereafter, volume and sperm concentration (using a Thoma counting chamber) were determined. Subjective motility was evaluated after dilution in the extender INRA96 (IMV Technologies, France).

Jennies were inseminated at the time of ovulation induction with 1 billion of spermatozoa diluted in INRA 96, at room temperature. Insemination was performed within one hour from semen collection (fresh semen).

Pregnancy diagnosis was made 14 days after ovulation by US and confirmed at 16 days, then jennies were treated with PGF2α to induce luteolysis and a new estrus cycle.

2.4 Blood samples and P4 determination – During PGPG and GPG protocols blood was collected weekly by jugular venipuncture the same day of the treatment and one week after the induction of ovulation. Blood was centrifuged at 3000 rpm for 10 minutes, serum fraction was separated and stored at −20°C until the P4 determination.

Progesterone was measured by an ELISA assay on 96-well plate (Immuno Nunc Maxisorp C96) coated with goat anti-mouse IgG (Uptima UP462140, Interchim) overnight at 4 °C. After washing with Tris-Tween20 the plates were incubated with the secondary antibody (mice monoclonal anti-progesterone, AbD, Serotec (10 µL in 16 mL tris-BSA) together with 10 µL of samples overnight at 4°C. The following day 50 µL/well of progesterone-alpha alkaline phosphatase conjugate (Immunometrics Ltd.) (10 µL P4-pal 6 mL tris-BSA) were added to each well and incubated in the dark for 1 hour. Plates were washed with tris-tween20 and then incubate with pNpp (Sigma-Aldrich) for about 2 hours at 37°C and absorbance recorded at 405 nm with a plate reader (TECAN). Intra-assay coefficient of variation averaged 8.5% and assay sensitivity was 0.25 $ng \cdot mL^{-1}$. Estrus was defined as [P4] < 1 ng/mL and diestrus was defined as [P4] ≥ 1 ng/mL;

2.5 Statistical analysis – Statistical analysis was performed using GraphPad Prism version 6.00 for Mac Os X (GraphPad Software, La Jolla, CA, www.graphpad.com).

Due to the reduced number of jennies, Fisher Exact test with Bonferroni correction was performed to evaluate the effect on:

- Synchronization response, ovulation response and pregnancy rates among the 3 protocols;
- Synchronization response according to the presence or the absence of a follicle of diameter ≥ 28 mm, the day of the beginning of the protocol;
- Synchronization response according to the presence or the absence of CL, the day of the beginning of the protocol;
- Ovulation response according the presence of a follicle between 28 and 35 mm or ≥36 mm of diameter, at Day 0;
- Pregnancy rates according the response or not to the induction of ovulation.

Sensitivity, specificity, positive and negative predictive value [33] have been calculated considering 'true estrus' jennies with at least one follicle ≥ 28 mm and absence of a CL at US and [P4] < 1 ng/mL, and 'true diestrus' jennies with the presence of a CL at US and [P4] ≥ 1 ng/mL.

- Sensitivity (denoted in %) was defined as the number of jennies correctly identified, by US, as being in estrus or diestrus divided by the total number of jennies with P4 < 1 ng/mL (for estrus) or with P4 ≥ 1 ng/mL (for diestrus), respectively.

- Specificity was the number of jennies identified, by US, as not being in estrus or diestrus, divided by the total number of jennies with P4 < 1 ng/mL (for estrus) or with P4 ≥ 1 ng/mL (for diestrus), respectively.
- Positive predictive value was defined as the probability to have P4 < 1 ng/mL after diagnosis of estrus, at US, (number of jennies in estrus/number of jennies diagnosed in estrus) or to have P4 ≥ 1 ng/mL after diagnosis of diestrus, at US, (number of jennies in diestrus/number of jennies diagnosed in diestrus).
- Negative predictive value was defined as the probability to have P4 <1 ng/mL after diagnosis of non-estrus at the ultrasound examination (number of jennies not in estrus/number of jennies diagnosed not in estrus after ultrasound examination), or after diagnosis of non-diestrus at the ultrasound examination (number of jennies not in diestrus/number of jennies diagnosed not in diestrus) or to have P4 ≥ 1 ng/mL.

3. RESULTS

Synchronization and ovulation responses and pregnancy rates of the 3 protocols were not significantly different (Table 2).

The presence of a follicle of < 28 mm or ≥ 28 mm in diameter at the beginning of the protocol resulted in 11/18 and 8/9 positive responses to the estrus synchronization, respectively (P>0.05).

Presence or absence of a CL at the beginning of the protocol resulted in 10/14 and 9/13 positive responses to estrus synchronization, respectively (P>0.05).

Follicular diameter between 28 and 35 mm or ≥36 mm at Day 0 resulted in 5/12 and 7/7 ovulations within 48 hours, respectively (P<0.05).

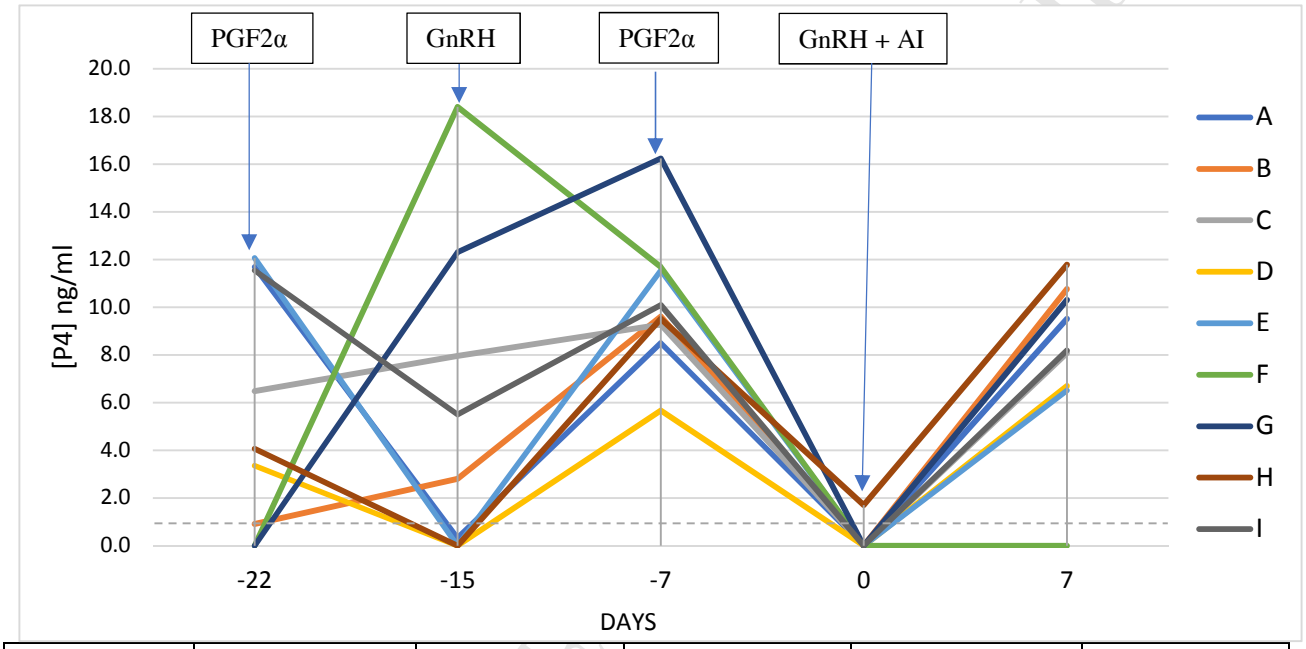
Pregnancy rates according to a response or not to the induction of ovulation were 7/12 and 2/7, respectively (P>0.05).

Table 2: Jennies responding to the synchronization protocol, to the induction of ovulation and pregnant (per inseminated jennies and per the total of the treated jennies) according to three different protocols (P>0.05).

Protocol	**Synchronization response (%)**	**Ovulation response (%)**	**Pregnancy rate/AI (%)**	**Pregnancy rate/Treated (%)**
PPG	6/9 (67%)	3/6 (50%)	1/6 (17%)	1/9 (11%)
PGPG	8/9 (89%)	5/8 (63%)	5/8 (63%)	5/9 (56%)

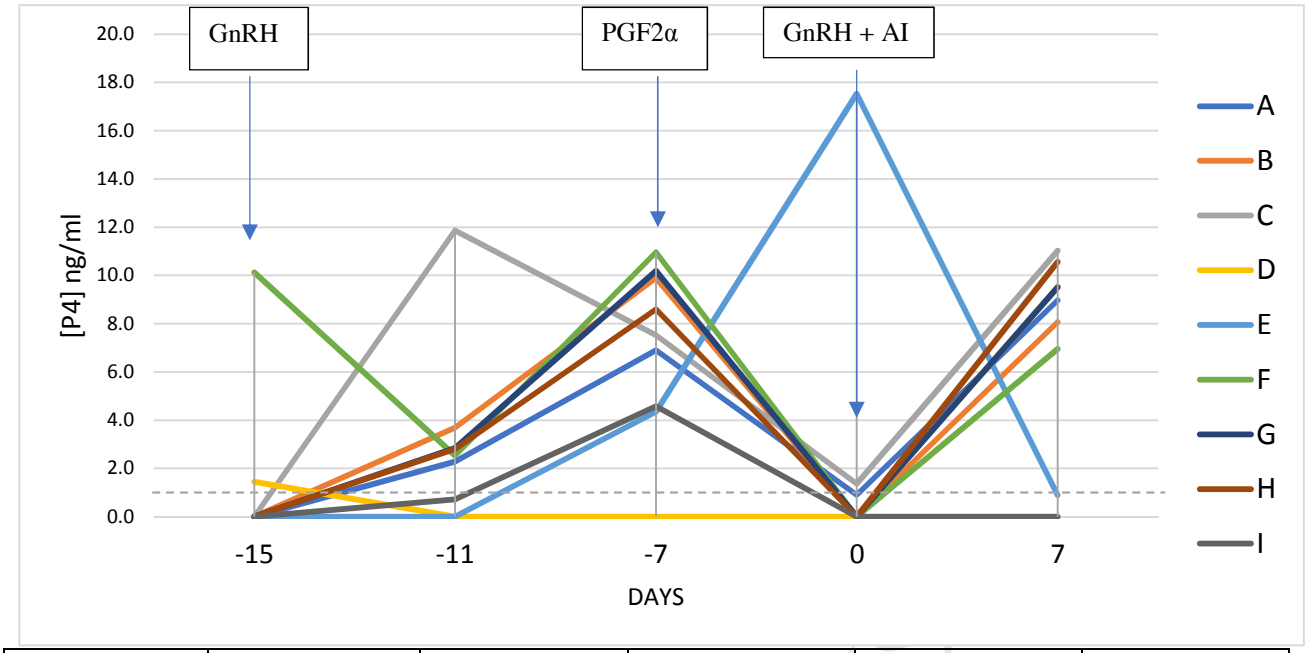
GPG	5/9 (55%)	4/5 (80%)	3/5 (60%)	3/9 (33%)

Figure 1 and 2 describe the serum progesterone profile, according to the different steps of the PGPG and GPG protocols, respectively.



	Day -22 (*PGF2α*)	Day -15 (GnRH)	Day -7 (*PGF2α*)	Day 0 (GnRH + AI)	Day +7
[P4]<1 ng/ml	3/9	4/9	0/9	8/9	1/9
[P4]>1 ng/ml	6/9	5/9	9/9	1/9	8/9

Figure 1: Serum P4 levels of 9 jennies (A to I) submitted to the PGPG protocol.
Day -22 and -7 = injection of PGF2α.
Day -15 = injection of GnRH analogue.
Day 0 = injection of GnRH analogue and AI in 8 jennies but not in jenny H judged not in heat according to the presence of a CL at the US examination.



	Day -15 (GnRH)	Day -11	Day -7 (*PGF2α*)	Day 0 (GnRH + AI)	Day +7
[P4]<1 ng/ml	7/9	3/9	1/9	7/9	1/9
[P4]>1 ng/ml	2/9	6/9	8/9	2/9	8/9

Figure 2: Serum P4 levels of 9 jennies (A to I) submitted to the GPG protocol.
Day -15 = injection of GnRH analogue.
Day -7 = injection of PGF2α.
Day 0 injection of GnRH analogue and AI in 5 jennies but not in jennies A, C, E and I judged not in heat according to the presence of a CL and/or absence of a follicle ≥ 28 mm at the US examination.

Sensitivity of US-based estrus diagnosis was 97.1% and specificity was 54.3%, while the positive and negative predictive values were 92.6% and 75.9 %, respectively.

Sensitivity of US-based diestrus diagnosis was 92.6% and specificity was 82.6%, while the positive and negative predictive values were 88.7% and 88.4%, respectively.

DISCUSSION

In the last years, fostered by an increasing consciousness of the importance of biodiversity in animal farming and through the rediscovery of the nutraceutical properties of jennies' milk [1–3], donkey breeding is gaining more and more interest. This is mostly true in European Mediterranean

222 countries [34], whereas in China donkey breeding has a great economic value for the production of
223 gelatine for traditional Chinese Medicine [35].

224 Artificial Insemination (AI) is universally accepted as the most powerful method to improve animal
225 breeding and genetic selection [36] and TAI protocols are widely used, especially in cows [6,8,9].

226 Few studies, however, investigated estrus synchronization in jennies [18,22] and research on AI in
227 this species is mainly performed by using frozen semen in experimental conditions [37–40].
228 Cryopreserved donkey semen has good post-thaw viability and motility [39,41] but gives still low
229 pregnancy rates ranging from 0% to 28% [40]. Today the best results were achieved by post
230 thawing re-extension of donkey frozen semen with seminal plasma [39] and by submitting jennies
231 to uterine lavage 10 hours post AI [40].

232 Working on Mammoth Asses (*Equus asinus americanus*) Blanchard et al. [18] reported that 76% of
233 jennies treated once with PGF2α, regardless to the stage of estrus cycle, responded showing estrus
234 signs, with an interval to estrus of 4.4 ± 1.6 days [18]. Moreover, 10/10 (100%) and 8/11 (73%)
235 jennies had estrus behaviour after a 10 days treatment with progesterone and estradiol 17-β
236 followed by PGF2α, or after two administrations of PGF2α 16 days apart, respectively [18]. In
237 Blanchard's studies jennies were not bred.

238 Recently, Zeng and colleagues [22] reported synchronisation rates from 17% up to 46% using
239 protocols based on different PGF2α analogues and/or oral progesterone in intensive farming of
240 Dezhou donkey in China. In this study, jennies were submitted to induction of ovulation with a
241 GnRH analogue and to repeated AIs every 48 hours until the detection of the ovulation, using 1
242 billion of fresh semen and achieving a pregnancy rate of 31% [22]. Oliveira and colleagues [40]
243 inseminated jennies with 1×10^9 or 500×10^6 viable fresh spermatozoa, every 48 hours after the
244 detection of a follicle of 33 up to 35 mm in diameter until ovulation, with a conception rate of 73%
245 (11/15) and 40% (6/15), respectively.

246 Physiologically, luteolysis in donkey species occurs between 15 and 17 days from ovulation [42];
247 PGF2α administration is commonly used to induce luteolysis and to shorten estrus cycle [43]. A
248 recent manuscript reported that PGF2α is able to induce a complete luteolysis from day 6 after
249 ovulation [44] and not from day 3 [45]: the presence of a functional CL susceptible to luteolysis
250 was confirmed as to be a prerequisite for estrus synchronization with PGF2α. The results of the
251 present manuscript, although based on a small number of animals and on a restrictive definition of

252 positive response to the treatment, confirm the efficacy of PGF2α and GnRH analogue for estrus
253 synchronization in jennies. On the other hand, even though there were no statistically significant
254 differences between the three protocols employed, the PGPG protocol alone showed a pregnancy
255 rate (close to 60%) comparable with the best results reported in literature for donkeys.
256 Noteworthy, this pregnancy rate was obtained with one single TAI and not after repeated AIs as in
257 the case of the cited papers [22,40].

258 In the present study, the cumulative response to the induction of ovulation by GnRH, 63%, was
259 lower, compared with a previous study done in the same animals few years ago [46]. This result is
260 probably due to the different size of follicles at the time of GnRH injection: ≥ 28 in the present
261 study and 33±2 in the previous one. Indeed, the results of the present study indicate that ovulation
262 rate was significantly lower in follicles between 28 and 35 mm of diameter, compared to larger
263 follicles. This agrees with previous observations in jennies reporting a negative correlation
264 between follicle size and the interval between treatment and ovulation [21]. The effect of follicle
265 size on ovulation success was previously evaluated in Dezhou jennies: when the follicle size was
266 25-30 mm, 31-35 mm or 36-40 mm ovulation rate within 48 hours was 0%, 0% and 50% with hCG
267 and 72%, 96% and 100% with a slow releasing GnRH analogue [22].

268 Comparing the three protocols of the present study, synchronization response and ovulation
269 response ranged 55-89% and 50-80%, respectively, and pregnancy rates per AI and per treated
270 jenny ranged 17-63% and 11-56%, respectively. In spite of the absence of statistically significant
271 differences, probably due to the small number of animals employed, the PGPG protocol resulted in
272 pregnancy rates per treated jenny two- and five-times higher compared with those obtained with
273 GPG and PPG treatments, respectively.

274

275 The presence of uterine folds is a good indicator of estrus status in mares [29,47], but not in
276 jennies where uterine folds are seldom evident even during the estrus phase [30]. Estrus
277 behaviour of jennies is evident and well known [48,49] but for them to show it a jackass stallion is
278 needed, and this is not always available especially in small farms. This reason urged us to evaluate
279 if the US examination itself was accurate enough for the diagnosis of the estrus/diestrus status of
280 jennies' reproductive tract. The use of the serum progesterone levels as an ex post control of the
281 results of US examination showed that US-based diagnosis was accurate enough for the
282 determination of the estrus phase and highly accurate for the diestrus one. The lower specificity of

283 estrus compared to the diestrus diagnosis, reflects the fact that [P4] is not <1 ng/ml in estrus only,
284 but also in the early estrus and immediately after ovulation. This observation could be helpful for
285 practitioners asked to manage reproduction of a small number of animals as well as big herds of
286 jennies without the use of a teaser donkey stallion.
287 In conclusion, in spite of the small number of animals employed, this study showed that protocols
288 for TAI can be applied in the donkey species and that the combination of double PGF2α, each one
289 followed by a GnRH analogue, give reasonable results in terms of estrus synchronization and
290 pregnancy rates. In addition, the simple transrectal US examination seemed to be able to diagnose
291 the status of estrus or diestrus in jennies. The possibility of managing jennies' reproduction
292 without estrus detection should be better studied in a large number of animals.

293

294 Conflicts of interest

295 The authors declare no conflicts of interest.

296

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Highlights

- Acceptable synchronization and pregnancy rates were obtained with a double injection of alfaprostol, each one followed by buserelin acetate, in jennies.
- The follicle diameter or the presence of a corpus luteum at the beginning of the protocol had no effect on synchronization rate.
- The follicle diameter at the day of ovulation induction affected the treatment-ovulation interval.
- Transrectal US examination was high accurate to diagnose the estrus/diestrus status in jennies.