Title:

Effect of housing system on reproductive behavior and on some endocrinological and seminal parameters of donkey stallions

Running title:

Housing influences on reproduction and endocrinology of male donkeys

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Summary

Reproductive management of male donkeys employed for artificial breeding has been poorly studied. The aim of this study was to evaluate the effect of housing system, with the animals grouped together in a paddock or kept in individual boxes, on sexual behavior, cortisol and testosterone concentration and seminal characteristics of adult male donkeys. The study included four Amiata donkey jacks (stallions) from which ejaculates, saliva and blood were collected during two distinct three weeks periods, one in the group and one in the box housing system. Overall, 27/36 and 28/36 ejaculates were collected in the paddock and in the box phases, respectively, and time needed for semen collection was shorter when donkeys were kept in paddocks compared to when they were kept in single boxes (14:57±07:27 and 20:52±09:31 min, $P<0.05$). Native semen

characteristics were not influenced by housing system, while cooled preservation in an Equitainer® showed that sperm motility parameters were significantly higher during the paddock period compared to the box period. Salivary cortisol was influenced by housing system, both before and 60 minutes after ejaculation, being statistically higher when donkeys were housed in paddocks. On the contrary, overall and basal testosterone concentrations were significantly higher when animals were kept in boxes. In conclusion, in the present study, good quality semen could be successfully collected from donkeys irrespective of the housing system despite some differences in hormone concentrations.

Keywords:

Donkey, Semen, Behavior, Cortisol, Testosterone

Introduction

Donkeys traditionally breed at pasture, with one male introduced in a group of females. In contrast, semen preservation for artificial insemination (AI) implies a different reproductive management of males, keeping them in single boxes to reduce the occurrence of injuries and for sanitary reasons (Burger et al 2012). Confinement stress has been studied in a variety of farm animals; however, less attention has been paid to its effects in equids (Harewood and McGowan 2005; Erber et al. 2013). In stallions, manipulation of socio-sexual conditions may result also in an extremely wide variation of testosterone concentrations, sexual behaviour, and aggressive behaviour (McDonnel and Murray. 1995; Aurich et al. 2015).Donkeys differ markedly from horses in their sexual behaviour both at pasture and at in-hand natural mating. Time needed to achieve erection and ejaculation is longer and successful semen collection rates are lower (Henry et al 1991; Henry et al 1998). To the best of our knowledge housing systems of donkey stallions kept for semen production has not been object of a controlled study to date.

Cortisol is commonly used for determination of the stress response in horses, however only a small number of studies were done on donkeys (Forhead et al. 1995; Veronesi et al. 2011; Fazio et al. 2013). Testosterone concentration in the periphery is a measure of testicular endocrine function. In horses, stress may affect testosterone concentration (Baker et al. 1982; Lange et al. 1997). Moreover, social interaction influences testosterone concentration, which is lower in "bachelors" (males living in groups) than in stallions living in contact with mares (McDonnel and Murray 1995) or other stallions (Aurich et al. 2015). In addition, changes in social environment may have an effect on seminal characteristics, together with modifications of testosterone concentration (Burger et al. 2015).

The aim of this study was to evaluate the effect of housing system, with the animals grouped together in a paddock or kept in individual boxes, on sexual behavior, cortisol and testosterone concentrations and seminal characteristics of adult male donkeys. We hypothesized that transfer of male donkeys from group to individual housing has pronounced effects on these end points.

Materials and methods

Animals and outline of the study: the present study was approved by the ethical committee (OBA) of the University of Pisa, according to D.lvo 26/2014. The study included four Amiata donkey jacks (stallions), aged 3 and half years (born between May and July 2012). The males were kept together in a paddock (10 x 15 mt.), without semen collections, for two months (September and October 2015). Thereafter, semen collections were attempted thrice weekly (Tuesday, Thursday, Saturday) during 3 weeks in the month of November. On the same days salivary and blood samples were collected for determination of cortisol and testosterone, respectively. In December the donkeys were moved to single boxes (3.5 x 3.5 mt) with a small outside paddock (3.5 x 6 m), where they still had visual but no physical contact to each other. They were not subjected to semen

collections for the subsequent two months (December 2015 and January 2016), while in February 2016 semen, saliva and blood collections were again attempted thrice weekly (Tuesday, Thursday, Saturday) for 3 weeks. During both housing periods, female donkeys (jennies) were kept on the same premises at about 30 to 50 meters in distance from the males. Jacks were maintained under natural light conditions, and fed meadow hay, bought as a single stock and used throughout the study, and a commercial feed for horses (humidity 12,2%, protein 16.3%, oils and lipids 1.7%; cellulose 6.8%; ashes 2.7%; sodium 75 mg/kg; Equifioc, Molitoria Val di Serchio, Lucca, Italy), in accordance with the NRC recommendations for energy (National Research Council, 2007). During the paddock-period, hay was given at libitum, while in boxes the amount of hay and feed was calculated in order to keep fairly constant weight and BCS. Weight was 280.3±30.4 kg when donkeys were moved to boxes and 283.5±27.0 kg at the end of the study, with a difference of -3, +1, +10 and + 5 kg in the single animals, while BCS was always evaluated between 5 and 6.

From three donkey stallions of the same Amiata breed and size (age: 3, 9, and 11 years), kept in another facility without changing housing conditions throughout the study, blood and saliva were sampled once monthly between November and February. The samples were evaluated for testosterone and cortisol and served as controls. These stallions were used neither for mating nor for semen collection during the period of study.

Semen collection and evaluation: Semen was collected using a Missouri artificial vagina and the jack jumping on an estrous jenny. For each donkey's semen collection attempt, time was limited to 45 minutes. If semen could not be collected within this time, the donkey was returned to his paddock or box. The time from the entrance to the collection area until ejaculation was measured.

Immediately after collection and determination of total volume, semen was filtered. Volume after filtration, gel volume and sperm concentration (using a Thoma counting chamber) were

92 determined.Motility was evaluated under a phase contrast microscope at 200x magnification after
93 dilution of raw semen 1:2 in INRA96® (IMV Technologies, Nouzilly, F). . Smears for assessment of
94 sperm morphology (Spermac stain, Minitube, DE) were prepared and evaluated once weekly. For
95 each sample, 200 spermatozoa were evaluated under a light microscope at 1000x. For each
96 donkey, aliquots of one ejaculate during both the second and the third week in each housing
97 system were diluted 1:4 in INRA96 and evaluated for motility after 24 and 48 hours of
98 preservation in an Equitainer®(Hamilton Research Inc., Ipswich, MA; 5 °C) using the computerized
99 semen analysis system CEROS 12.1 Analyzer (Hamilton Research Inc, South Hamilton, MA) as
100 previously described (Rota et al. 2010).

101 Sperm plasma membrane functional integrity was evaluated by hypo-osmotic swelling-test (HOS-
102 test, Rota et al. 2010). At least 100 spermatozoa were assessed and classified as HOS positive
103 (HOS+) when showing the typical swelling or bending of the tail.

104 Blood and salivary samples schedule: Blood samples were collected from the four donkey stallions
105 once weekly (Thursday) for determination of testosterone concentration, while salivary samples
106 were taken twice weekly (Tuesday, Saturday) for determination of cortisol.

107 The schedule for blood sampling was the following:

108 Sample 1: 8:30

109 Sample 2: at the exit from paddock/boxSample 3: immediately after ejaculation

110 Sample 4: 30 minutes after ejaculation

111 Sample 5: 60 minutes after ejaculation

112 The schedule for salivary sampling was the following:

113 Sample 1: 8:30

114 Sample 2: at the exit from paddock/box

Sample 3: immediately after ejaculation

Sample 4: 10 minutes after ejaculation

Sample 5: 30 minutes after ejaculation

Sample 6: 60 minutes after ejaculation

In both schedules, if an ejaculate was not obtained only Samples 1 and 2 were collected.

Hormonal analyses: blood samples were taken from the jugular vein, placed in serum sample tubes, and allowed to clot for 30 min. Serum was separated by centrifugation (1000 g for 10 min) and frozen at -20°C until determination of testosterone concentration by direct enzyme immunoassay (Testosterone ELISA, Demeditec Diagnostics, Kiel, Germany) without extraction (Schrammel et al. 2015). The assay was validated for donkey plasma in our own laboratory. Recovery of testosterone standard added to plasma was 103%, and increasing dilutions of plasma samples resulted in changes in optical density parallel to the standard curve. Intra and inter-assay CV% were 4.0 and 5.7%, respectively. The lower detection limit was 0.01 ng/ml. Saliva samples were collected using a cotton-based swab (Salivette®, Sarstedt, Numbrecht-Rommelsdorf, Germany) and returned into a polypropylene tube. Tubes were then centrifuged for 10 min at 1000 g and the obtained saliva was frozen at −20° C until analysis. A commercial enzyme immunoassay without extraction (Demeditec Diagnostics, Kiel-Wellsee, Germany) was used for saliva cortisol determination. The assay was validated for donkey saliva in our laboratory (Bonelli et al. 2017). Recovery of cortisol standard added to donkey saliva was 108%, and increasing dilutions of saliva samples resulted in changes in optical density parallel to the standard curve. Intra and inter-assay CV% were 8.0 and 10.7%, respectively. The lower detection limit was 0.01 ng/ml.

Statistical analysis: analyses were performed using the statistical package Minitab 17.2.1 (Minitab Inc., State College, USA). All data were assessed for normal distribution by the Anerson Darling Test. When data were not normally distributed the Box-Cox transformation was employed before analysis. The difference in proportion of successful semen collection between the two housing systems was evaluated by chi-square test. For the time needed to obtain an ejaculate, all semen parameters, basal (Sample 1) and final (Sample 6) salivary cortisol and basal serum testosterone concentration the General linear model (GLM) was employed, including the effect of housing system, donkey, and the interaction between the two.The effects of housing system, donkey and sample on salivary cortisol and serum testosterone concentration before semen collection (Samples 1 and 2), and on the whole set of collected samples were evaluated by GLM for repeated measures. When a sample effect was present, different sampling times were compared by paired t-test. Differences were considered significant when $P<0.05$. All values are given as mean ± standard deviation.

Results:

Semen collections

In two donkeys, semen collection was possible at all times, while for BE1 and BE2, semen collection failed in seven and ten attempts, respectively. Overall, 27/36 and 28/36 ejaculates were collected in the paddock and in the box phases, respectively (Chi-Square = 0.077, P-Value = 0.781). Table 1 shows mean time elapsed between the arrival in the semen collection area and ejaculation. Time needed for semen collection was shorter when donkeys were kept in paddocks compared to single boxes (14:57±07:27 and 20:52±09:31 min, $P<0.05$). Overall, time needed for semen collection was significantly longer for donkey BE2 compared to donkey BA ($P<0.05$).

161 Semen evaluation

162 Table 2 shows the seminal characteristics for each donkey in the two housing systems. One of
163 donkey BE2 ejaculates, while housed in paddock, was lost and thus not evaluated. The donkey,
164 but not the housing system significantly affected the semen characteristics sperm concentration,
165 total number of spermatozoa, estimated motility and sperm morphology ($P<0.05$). Concerning the
166 three considered semen volumes (total, gel fraction, and post-filtration) there was neither an
167 effect of donkey nor of housing system, but an interaction could be detected ($P<0.05$). Finally,
168 neither donkey nor housing system influenced the proportion of spermatozoa with intact plasma
169 membranes as evaluated by the HOS test.

170

171 Evaluation after cooled preservation in an Equitainer® showed that total motility (MTOT) and
172 average path velocity (VAP) at at 24 and 48 hours and progressive motility (MPRO) at 24 hours
173 were significantly higher during the paddock period compared to the box period ($P<0.05$, Table 3).

174

175 Salivary cortisol concentration

176 Before semen collection, salivary cortisol concentration was neither influenced by donkey nor by
177 the housing system, if evaluating only Sample 1, while evaluating Samples 1 and 2 combined
178 cortisol was higher in paddock (Table 4, $P<0.05$). Sixty minutes after ejaculation (Sample 6) salivary
179 cortisol was higher in donkeys kept in paddocks than in boxes (Table 4, $P<0.01$). No significant
180 effect of donkey was observed. Mean salivary cortisol concentration (Samples C1 and C2) of the
181 three control donkeys in November and February was 4.77±1.18 and 11.20±7.05, respectively.

Overall, salivary samples collected from the donkeys at the semen collections were 207/218 attempts (11 samples did not contain enough saliva for analysis or were lost). There was no significant effect of donkey or housing system on salivary cortisol concentration, but an effect of sampling time was seen ($P<0.05$, Figure 1). Cortisol concentration in Sample 1 was significantly lower than in Samples 3 and 5 (Paired t-test, $P=0.022$ and $P=0.008$, respectively), and in Sample 2 was lower than in Sample 5 ($P=0.040$).

Serum testosterone concentration

Both donkey and housing system influenced basal testosterone concentration (Sample 1) ($P<0.05$). Testosterone concentration was higher when the donkeys were housed in single boxes compared to paddocks (5.33±2.87 and 3.30±2.09, respectively; $P<0.05$, Table 5). However, this effect was lost when samples 1 and 2 were both included into the analysis ($P>0.05$, Table 5). Mean serum testosterone concentration in November and February in the three control donkeys was 7.31±4.26 and 8.25±4.55, respectively.

For the evaluation of the changes in testosterone concentration during successful semen collection, samples 1 to 5 were evaluated., Overall, testosterone concentration was higher when the donkeys were housed in single boxes compared to paddocks (6.77±5.04 and 5.23±3.70, respectively; $P<0.05$, Figure 2), and was higher in BA compared with the other three donkeys, and in BO than in BE1 ($P<0.05$). There was no significant effect of sampling time (sample).

Discussion

In the present study we could demonstrate that young male donkeys can be housed either together in paddocks or in single boxes while used for semen collections without significant effects on raw semen characteristics or behavior. The two periods of the study, however, differed significantly in some of the evaluated parameters, such as time needed for semen collection, salivary cortisol concentration 60 minutes post-ejaculation, serum testosterone concentration and sperm motility after cooled preservation in INRA96.

A semen sample was collected at all attempts from two donkeys, while success rate for BE1 and BE2 was low. Interestingly, both donkeys had a significantly lower overall testosterone concentration than BA, which had both the highest levels of testosterone and the overall shorter time needed for semen collection among the four donkeys. It may therefore be hypothesized that higher testosterone was associated with more pronounced sexual behavior in donkeys. In stallions, however, there is little correlation between androgen concentration and the sexual behavior characteristics (Pickett et al. 1981; Silva Rua et al. 2015). Low concentration of peripheral testosterone was accompanied with physiologic sexual behavior and administration of exogenous testosterone to horse stallions did not enhance libido (Berndtson et al. 1979; Waheed et al. 2015). Veronesi et al. (2010), however, found a correlation, albeit low, between testosterone concentration and sexual behavior. The relation between testosterone and stallion behavior is thus still not clear, and even less information is available for the donkey species.

The time needed for semen collection was shorter when donkeys were in paddock than when in single boxes, despite basal (sample 1) and overall testosterone concentration in boxes was higher. In both cases, the flock of jennies was not in sight but at a distance of 30-50 mt, males could therefore most probably hear and smell their presence and perceive the presence of pheromones

(Carluccio et al 2013a). During semen collection, moreover, donkey stallions had physical contact with an estrous jenny, they should thus not be considered isolated from females. In an early study, success rate of twice-weekly semen collections for 12 months ranged 33 to 90%, and a slight improvement was observed leaving the donkey and the jenny free in a paddock (Henry et al. 1998). It is known that changes of social environment (e.g. passage from bachelor to harem status) may modify serum testosterone concentration and behavior of stallions (McDonnel and Murray 1995). No such studies have been done in donkeys. Due to differences in male behavior between horses and donkey, the situation is not easily comparable. It was suggested that breeding jacks continuously confined in stalls may develop behavioral problems (Canisso et al. 2009) but this was not the case in the present investigation. In both housing conditions, semen collections were also successfully performed outside the breeding season under short days and long nights (10-28 November, 9-27 February, day length always lower than 11h). In a study by Carluccio et al (2013b) on Martina Franca donkey stallions, time from exposure to the female and effective erection (reaction time) was shorter in spring and summer (7.5 and 6.9 minutes) compared to autumn and winter (10.1 and 9.8 minutes). Thus, the shorter time needed in November (autumn-winter) in the present study is most probably not due to a seasonal influence.

Neither individual donkeys nor housing systems influenced basal (Sample 1) salivary cortisol concentration. Similarly, horse geldings housed in paddocks or in individual boxes had similar salivary cortisol levels, except in February, when concentration in group-housed animals was higher (Aurich et al. 2015). In all donkeys of the present study, however, basal salivary cortisol concentration was higher when housed in paddocks, and combining Samples 1 and 2, both taken prior to semen collection, the difference became statistically significant. Donkeys were kept in the respective housing systems for at least two months before sampling started, and were thus accustomed to their environment. This protocol made sure that acute stress due to the change of

environment was excluded (Erber et al. 2013). It might be possible that an effect of season was also present, as in horses salivary cortisol was higher in December compared to February (Aurich et al. 2015). Nevertheless, this trend was not observed in the three control donkeys included in the present study, for two of which mean salivary cortisol (C1 and C2) was numerically higher in February, compared to November.

Salivary cortisol levels 60 minutes after semen collection were significantly higher in donkeys kept in paddocks compared to individual boxes, suggesting that the conflicts and interactions when returning to their mates stimulated a stress response. Despite the fact that in this situation overall testosterone concentration was lower, semen characteristics were not negatively affected. In studies investigating variation in donkey semen quality over time, season affected only semen pH (Gastal et al. 1997), semen volume and sperm velocity (Carluccio et al. 2013b). Ejaculatory frequency affected total number, viability and morphologic head defects (Gastal et al. 1997). In stallions, change of environment from continuous contact only with males to continuous contact with a female increased the total sperm number in stallions (Burger et al. 2015). However, the model investigated in the present study did not change raw semen characteristics, and as to our knowledge, no other studies on semen quality of donkeys housed in different conditions are available.

Nevertheless, the change of the housing system had pronounced effects on sperm motility during cooled preservation. Total and progressive motility after 24 hours decreased by about 20% and a 10%, respectively, when donkeys were housed in box, and the difference exacerbated after 48 hours. Despite very similar raw semen motility parameters, significant differences after cooling were observed when the seasonal effect was investigated: in November-December semen had a higher rate of decline in motility than in May-June (Contri et al. 2010). It is thus possible that semen preservation disclosed hidden changes that occurred to sperm cells with the change of

housing system. However, as in stallions motility in cooled semen decreased between November and February, a seasonal effect can also not be ruled out (Schmid-Lausigk et al. 2014).

In conclusion, in the present study, good quality semen could be successfully collected from donkeys irrespective of the housing system despite some differences in hormon concentrations.

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Conflict of interest

None of the authors has any conflict of interest to declare.

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364 Table 1: Proportion of successful semen collections and time needed to obtain an ejaculate
365 (mean±SD) for the four donkeys in the two housing systems.

Donkey	Housing system	Successful semen collections	Time needed to obtain the ejaculate
BA	Paddock	100% (9/9)	12:05 (n=9)
	Box	100% (9/9)	15:34 (n=7)
BE1	Paddock	66.6% (6/9)	17:49 (n=6)
	Box	55.5% (5/9)	23:58 (n=5)
BE2	Paddock	33.3% (3/9)	15:07 (n=3)
	Box	55.5% (5/9)	32:30 (n=4)
BO	Paddock	100% (9/9)	16:06 (n=7)
	Box	100% (9/9)	17:19 (n=7)
All donkeys	Paddock	75.0% (27/36) A	14:57±07:27 (n=25) A
	Box	77.8% (28/36) A	20:52±09:31 (n=23) B

366 Within column, A≠B (P<0.05)

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369 Table 2: Mean values (mean±SD) for the four donkeys in the two housing systems of semen
370 volume before and after filtration, gel volume, concentration and total number of spermatozoa,
371 subjective total motility, plasma membrane intact spermatozoa, according to the water test and
372 morphologically normal spermatozoa.

Donkey	Housing	Total volume (ml)	Volume post filtration (ml)	Gel volume (ml)	Sperm conc. ($x10^6$/ml)	Total sperm count ($x10^6$)	Subjective motility (%)	HOS test + (%)	Normal morphology (%)
BA	Paddock	42.8	37.0	4.7	267.7	9411.1	68.3	61.3	41.0
	Box	41.0	38.0	0.0	266.6	9972.7	70.0	58.3	41.8
BE1	Paddock	28.3	27.2	0.0	119.4	3206.0	26.7	72.8	17.5
	Box	20.5	19.4	0.0	71.0	1647.2	47.5	75.2	21.2
BE2	Paddock	84.5	33.0	50.0	102.5	3194.5	68.3	68.3	62.3
	Box	36.8	21.4	15.0	168.2	4035.3	68.0	51.0	60.0
BO	Paddock	23.6	21.9	0.0	716.7	15735.4	89.4	69.2	91.3
	Box	42.7	39.7	0.0	467.3	18318.7	91.1	65.1	88.8
All	Paddock	36.0 ±22.9 A	28.3 ±13.7 A	5.5 ±19.9 A	362.5 ±278.7 A	9332.3 ±6662.4 A	66.1 ±26.2 A	67.2 ±8.9 A	52.4 ±28.5 A
	Box	36.6 ±15.4 A	31.8 ±13.4 A	2.6 ±9.7 A	274.0 ±162.0 A	9816.6 ±7564.0 A	71.6 ±18.7 A	62.6 ±12.7 A	53.6 ±27.7 A

373 Within column, A≠B (P<0.05)

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376 Table 3: Mean values (mean±SD) for the four donkeys in the two housing systems of total motility,
377 progressive motility and velocity on the average path (VAP) evaluated after 24 and 48 hours of
378 cooled preservation in INRA96®.

		Total motility 24 hours	Progressive motility 24 hours	VAP 24 hours	Total motility 48 hours	Progressive motility 48 hours	VAP 48 hours
Donkey	**Housing**	(%)	(%)	(μm/sec)	(%)	(%)	(μm/sec)
All	Paddock	67.1±23.8 A	36.1±19.9 A	114.4±23.5 A	52.1±25.7 A	17.0 ±10.8 A	44.4±25.7 A
	Box	45.7 ±27.2 B	24.7 ±21.0 B	85.5±23.7 B	25.7±20.6 B	9.7±10.7 A	19.3±17.8 B

379 Within column, A≠B (P<0.05)

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Table 4: Salivary cortisol concentration (mean±SD) before semen collection (Samples 1 and 2 combined) and 60 minutes after semen collection (Sample 6) of the four donkeys in the different housing systems.

Donkey	Housing	Cortisol (ng/ml) (Sample 1)	Cortisol (ng/ml) (Samples 1 and 2)	Cortisol (ng/ml) (Sample 6)
BA	Paddock	6.91±5.25	5.96±3.98	12.01±7.53
	Box	4.93±0.88	5.32±1.62	4.21±1.70
BE1	Paddock	6.17±3.84	6.02±5.83	13.82±6.15
	Box	5.15±2.58	4.97±2.16	4.77±5.18
BE2	Paddock	11.16±13.33	5.97±5.66	3.72±1.64
	Box	4.67±2.43	4.59±2.39	1.41±1.64
BO	Paddock	6.95±3.71	8.59±4.20	8.21±3.05
	Box	6.21±3.55	6.89±3.74	6.30±7.12
All donkeys	Paddock	7.32±5.30 A	7.40±5.21 A	10.04±5.96 A
	Box	5.34±2.42 A	5.71±2.74 B	4.54±4.70 B

Within column, A≠B ($P<0.05$)

Table 5: Serum testosterone concentration (mean±SD) as basal value (Sample 1) or all samples combined (Samples 1-5) of the four donkeys in the different housing systems.

Donkey	Housing	Testosterone (ng/ml) (Sample 1)	Testosterone (ng/ml) (Samples 1 and 2)	Testosterone (ng/ml) (Samples 1 to 5)
BA	Paddock	4.80±0.29	8.15±5.51	7.90±4.04
	Box	9.46±1.50	10.06±1.82	13.16±5.55
BE1	Paddock	1.08±0.29	1.33±0.40	1.63±0.76
	Box	2.36±1.04	2.34±0.73	3.14±1.65
BE2	Paddock	2.81±1.04	2.92±1.11	3.12±1.46
	Box	4.05±0.89	3.97±0.65	4.31±0.63
BO	Paddock	4.50±0.14	6.03±3.16	5.65±2.66
	Box	5.44±0.14	5.02±0.56	5.64±0.89
All donkeys	Paddock	3.30±2.09 A	4.61±4.05 A	5.23±3.70 A
	Box	5.33±2.87 B	5.35±3.11 A	6.77±5.04 B

Within column, A≠B ($P<0.05$)

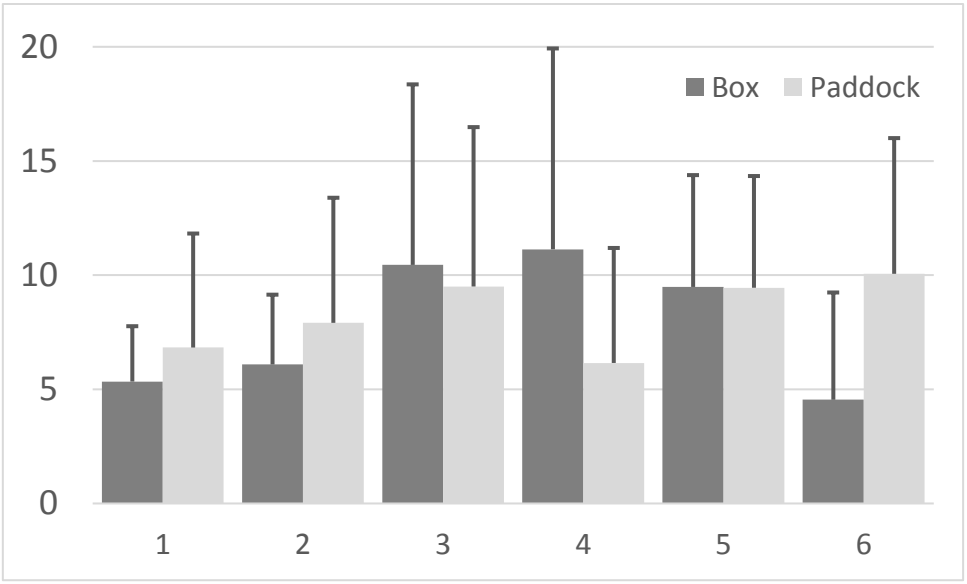
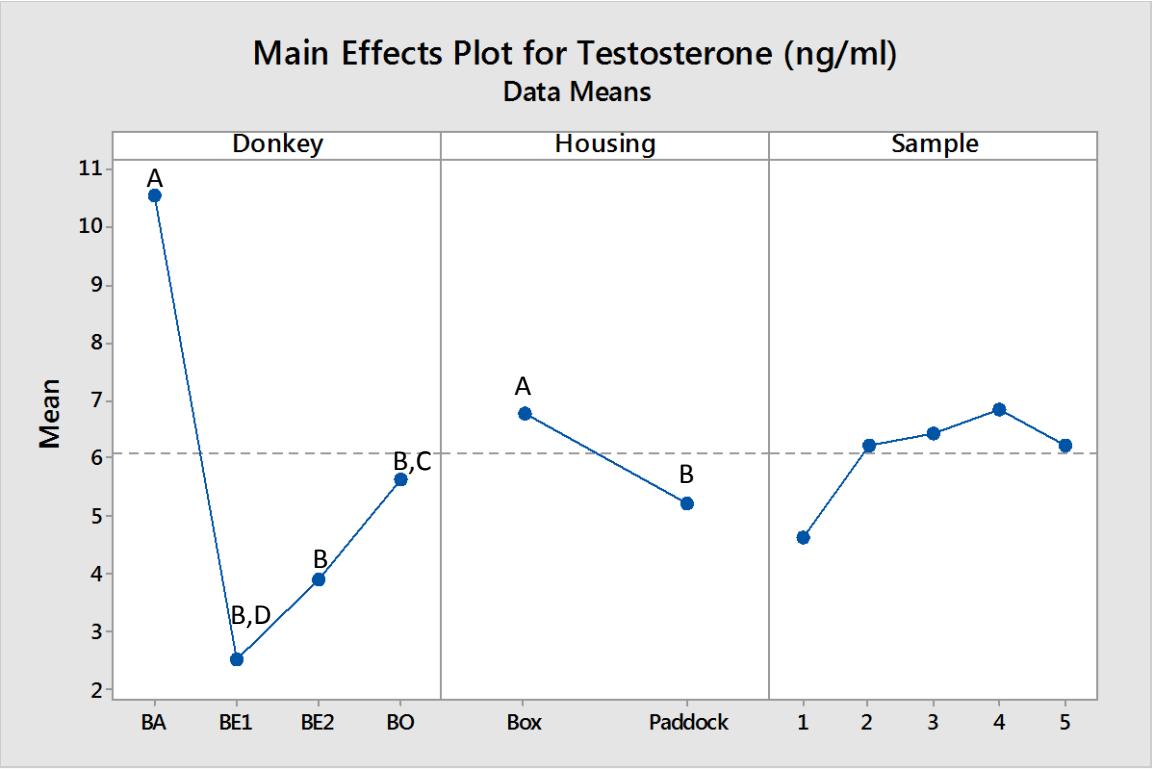


Figure 1: Salivary cortisol of the four donkeys in the two housing system at the six sampling times (mean and standard deviation). Sample 1: 8:30; Sample 2: at the exit from paddock/box, before going to semen collection area; Sample 3: immediately after ejaculation; Sample 4: 10 minutes after ejaculation; Sample 5: 30 minutes after ejaculation; Sample 6: 60 minutes after ejaculation.



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404 Figure 2: Main effects plot showing the mean serum testosterone observed in the 4 donkeys, in

405 the two housing system, and at the 5 sampling times. Within main effect: A≠B and C≠D by P<0.05.

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