

1 Title:

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

4 Running title:

5 **Housing influences on reproduction and endocrinology of male donkeys**

6 Authors:

7 Rota Alessandra¹, Sgorbini Micaela¹, Panzani Duccio¹, Bonelli Francesca¹, Baragli Paolo¹, Ille
8 Natascha², Gatta Domenico¹, Sighieri Claudio¹, Casini Lucia¹, Maggiorelli Maria Maddalena¹, Aurich
9 Christine², Camillo¹ Francesco.

10 ¹Department of Veterinary Sciences, University of Pisa, viale delle Piagge 2, 56122 Pisa, Italy

11 ²Artificial Insemination and Embryo Transfer, Department for Small Animals and Horses,
12 Vetmeduni Vienna, Veterinaerplatz 1, 1210 Vienna, Austria

13 **Summary**

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

23 characteristics were not influenced by housing system, while cooled preservation in an Equitainer®
24 showed that sperm motility parameters were significantly higher during the paddock period
25 compared to the box period. Salivary cortisol was influenced by housing system, both before and
26 60 minutes after ejaculation, being statistically higher when donkeys were housed in paddocks. On
27 the contrary, overall and basal testosterone concentrations were significantly higher when animals
28 were kept in boxes. In conclusion, in the present study, good quality semen could be successfully
29 collected from donkeys irrespective of the housing system despite some differences in hormone
30 concentrations.

31 **Keywords:**

32 Donkey, Semen, Behavior, Cortisol, Testosterone

33 **Introduction**

34 Donkeys traditionally breed at pasture, with one male introduced in a group of females. In
35 contrast, semen preservation for artificial insemination (AI) implies a different reproductive
36 management of males, keeping them in single boxes to reduce the occurrence of injuries and for
37 sanitary reasons (Burger et al 2012). Confinement stress has been studied in a variety of farm
38 animals; however, less attention has been paid to its effects in equids (Harewood and McGowan
39 2005; Erber et al. 2013). In stallions, manipulation of socio-sexual conditions may result also in an
40 extremely wide variation of testosterone concentrations, sexual behaviour, and aggressive
41 behaviour (McDonnel and Murray. 1995; Aurich et al. 2015). Donkeys differ markedly from horses
42 in their sexual behaviour both at pasture and at in-hand natural mating. Time needed to achieve
43 erection and ejaculation is longer and successful semen collection rates are lower (Henry et al
44 1991; Henry et al 1998). To the best of our knowledge housing systems of donkey stallions kept for
45 semen production has not been object of a controlled study to date.

46 Cortisol is commonly used for determination of the stress response in horses, however only a
47 small number of studies were done on donkeys (Forhead et al. 1995; Veronesi et al. 2011; Fazio et
48 al. 2013). Testosterone concentration in the periphery is a measure of testicular endocrine
49 function. In horses, stress may affect testosterone concentration (Baker et al. 1982; Lange et al.
50 1997). Moreover, social interaction influences testosterone concentration, which is lower in
51 “bachelors” (males living in groups) than in stallions living in contact with mares (McDonnel and
52 Murray 1995) or other stallions (Aurich et al. 2015). In addition, changes in social environment
53 may have an effect on seminal characteristics, together with modifications of testosterone
54 concentration (Burger et al. 2015).

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

59 **Materials and methods**

60 Animals and outline of the study: the present study was approved by the ethical committee (OBA)
61 of the University of Pisa, according to D.lvo 26/2014. The study included four Amiata donkey jacks
62 (stallions), aged 3 and half years (born between May and July 2012). The males were kept together
63 in a paddock (10 x 15 mt.), without semen collections, for two months (September and October
64 2015). Thereafter, semen collections were attempted thrice weekly (Tuesday, Thursday, Saturday)
65 during 3 weeks in the month of November. On the same days salivary and blood samples were
66 collected for determination of cortisol and testosterone, respectively. In December the donkeys
67 were moved to single boxes (3.5 x 3.5 mt) with a small outside paddock (3.5 x 6 m), where they
68 still had visual but no physical contact to each other. They were not subjected to semen

69 collections for the subsequent two months (December 2015 and January 2016), while in February
70 2016 semen, saliva and blood collections were again attempted thrice weekly (Tuesday, Thursday,
71 Saturday) for 3 weeks. During both housing periods, female donkeys (jennies) were kept on the
72 same premises at about 30 to 50 meters in distance from the males. Jacks were maintained under
73 natural light conditions, and fed meadow hay, bought as a single stock and used throughout the
74 study, and a commercial feed for horses (humidity 12,2%, protein 16.3%, oils and lipids 1.7%;
75 cellulose 6.8%; ashes 2.7%; sodium 75 mg/kg; Equifioc, Molitoria Val di Serchio, Lucca, Italy), in
76 accordance with the NRC recommendations for energy (National Research Council, 2007). During
77 the paddock-period, hay was given at libitum, while in boxes the amount of hay and feed was
78 calculated in order to keep fairly constant weight and BCS. Weight was 280.3 ± 30.4 kg when
79 donkeys were moved to boxes and 283.5 ± 27.0 kg at the end of the study, with a difference of -3,
80 +1, +10 and + 5 kg in the single animals, while BCS was always evaluated between 5 and 6.

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

86 Semen collection and evaluation: Semen was collected using a Missouri artificial vagina and the
87 jack jumping on an estrous jenny. For each donkey's semen collection attempt, time was limited to
88 45 minutes. If semen could not be collected within this time, the donkey was returned to his
89 paddock or box. The time from the entrance to the collection area until ejaculation was measured.
90 Immediately after collection and determination of total volume, semen was filtered. Volume after
91 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/box Sample 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

115 Sample 3: immediately after ejaculation

116 Sample 4: 10 minutes after ejaculation

117 Sample 5: 30 minutes after ejaculation

118 Sample 6: 60 minutes after ejaculation

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

120

121 Hormonal analyses: blood samples were taken from the jugular vein, placed in serum sample
122 tubes, and allowed to clot for 30 min. Serum was separated by centrifugation (1000 g for 10 min)
123 and frozen at -20°C until determination of testosterone concentration by direct enzyme
124 immunoassay (Testosterone ELISA, Demeditec Diagnostics, Kiel, Germany) without extraction
125 (Schrammel et al. 2015). The assay was validated for donkey plasma in our own laboratory.
126 Recovery of testosterone standard added to plasma was 103%, and increasing dilutions of plasma
127 samples resulted in changes in optical density parallel to the standard curve. Intra and inter-assay
128 CV% were 4.0 and 5.7%, respectively. The lower detection limit was 0.01 ng/ml. Saliva samples
129 were collected using a cotton-based swab (Salivette®, Sarstedt, Numbrecht-Rommelsdorf,
130 Germany) and returned into a polypropylene tube. Tubes were then centrifuged for 10 min at
131 1000 g and the obtained saliva was frozen at -20° C until analysis. A commercial enzyme
132 immunoassay without extraction (Demeditec Diagnostics, Kiel-Wellsee, Germany) was used for
133 saliva cortisol determination. The assay was validated for donkey saliva in our laboratory (Bonelli
134 et al. 2017). Recovery of cortisol standard added to donkey saliva was 108%, and increasing
135 dilutions of saliva samples resulted in changes in optical density parallel to the standard curve.
136 Intra and inter-assay CV% were 8.0 and 10.7%, respectively. The lower detection limit was 0.01
137 ng/ml.

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

151 **Results:**

152 Semen collections

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

160

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.

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

188

189 Serum testosterone concentration

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

196

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

202

203

204 **Discussion**

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

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

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

227 (Carluccio et al 2013a). During semen collection, moreover, donkey stallions had physical contact
228 with an estrous jenny, they should thus not be considered isolated from females. In an early study,
229 success rate of twice-weekly semen collections for 12 months ranged 33 to 90%, and a slight
230 improvement was observed leaving the donkey and the jenny free in a paddock (Henry et al.
231 1998). It is known that changes of social environment (e.g. passage from bachelor to harem status)
232 may modify serum testosterone concentration and behavior of stallions (McDonnel and Murray
233 1995). No such studies have been done in donkeys. Due to differences in male behavior between
234 horses and donkey, the situation is not easily comparable. It was suggested that breeding jacks
235 continuously confined in stalls may develop behavioral problems (Canisso et al. 2009) but this was
236 not the case in the present investigation. In both housing conditions, semen collections were also
237 successfully performed outside the breeding season under short days and long nights (10-28
238 November, 9-27 February, day length always lower than 11h). In a study by Carluccio et al (2013b)
239 on Martina Franca donkey stallions, time from exposure to the female and effective erection
240 (reaction time) was shorter in spring and summer (7.5 and 6.9 minutes) compared to autumn and
241 winter (10.1 and 9.8 minutes). Thus, the shorter time needed in November (autumn-winter) in the
242 present study is most probably not due to a seasonal influence.

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

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

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

268 Nevertheless, the change of the housing system had pronounced effects on sperm motility during
269 cooled preservation. Total and progressive motility after 24 hours decreased by about 20% and a
270 10%, respectively, when donkeys were housed in box, and the difference exacerbated after 48
271 hours. Despite very similar raw semen motility parameters, significant differences after cooling
272 were observed when the seasonal effect was investigated: in November-December semen had a
273 higher rate of decline in motility than in May-June (Contri et al. 2010). It is thus possible that
274 semen preservation disclosed hidden changes that occurred to sperm cells with the change of

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

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

279

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283 Conflict of interest

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

285

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361

362

363

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)

367

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	Volume			Total				
		Total volume (ml)	post filtration (ml)	Gel volume (ml)	Sperm conc. (x10 ⁶ /ml)	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	28.3	5.5	362.5	9332.3	66.1	67.2	52.4
		±22.9	±13.7	±19.9	±278.7	±6662.4	±26.2	±8.9	±28.5
	Box	36.6	31.8	2.6	274.0	9816.6	71.6	62.6	53.6
		±15.4	±13.4	±9.7	±162.0	±7564.0	±18.7	±12.7	±27.7
		A	A	A	A	A	A	A	A

373 Within column, A≠B (P<0.05)

375

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®.

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

379 Within column, A≠B (P<0.05)

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

Donkey	Housing	Cortisol	Cortisol	Cortisol
		(ng/ml)	(ng/ml)	(ng/ml)
		(Sample 1)	(Samples 1 and 2)	(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

385 Within column, A≠B (P<0.05)

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389 Table 5: Serum testosterone concentration (mean±SD) as basal value (Sample 1) or all samples

390 combined (Samples 1-5) of the four donkeys in the different housing systems.

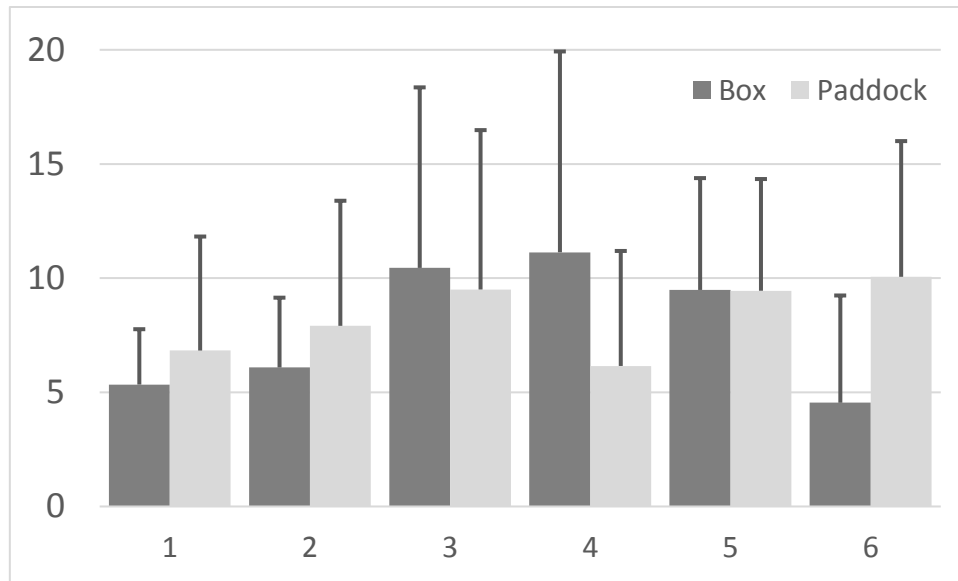
Donkey	Housing	Testosterone	Testosterone	Testosterone
		(ng/ml) (Sample 1)	(ng/ml) (Samples 1 and 2)	(ng/ml) (Samples 1 to 5)
BA	Paddock	4.80±0.29	8.15±5.51	7.90±4.04
	Box	9.46±1.50	10.06±1.82	13.16±5.55
BE1	Paddock	1.08±0.29	1.33±0.40	1.63±0.76
	Box	2.36±1.04	2.34±0.73	3.14±1.65
BE2	Paddock	2.81±1.04	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

391 Within column, A≠B (P<0.05)

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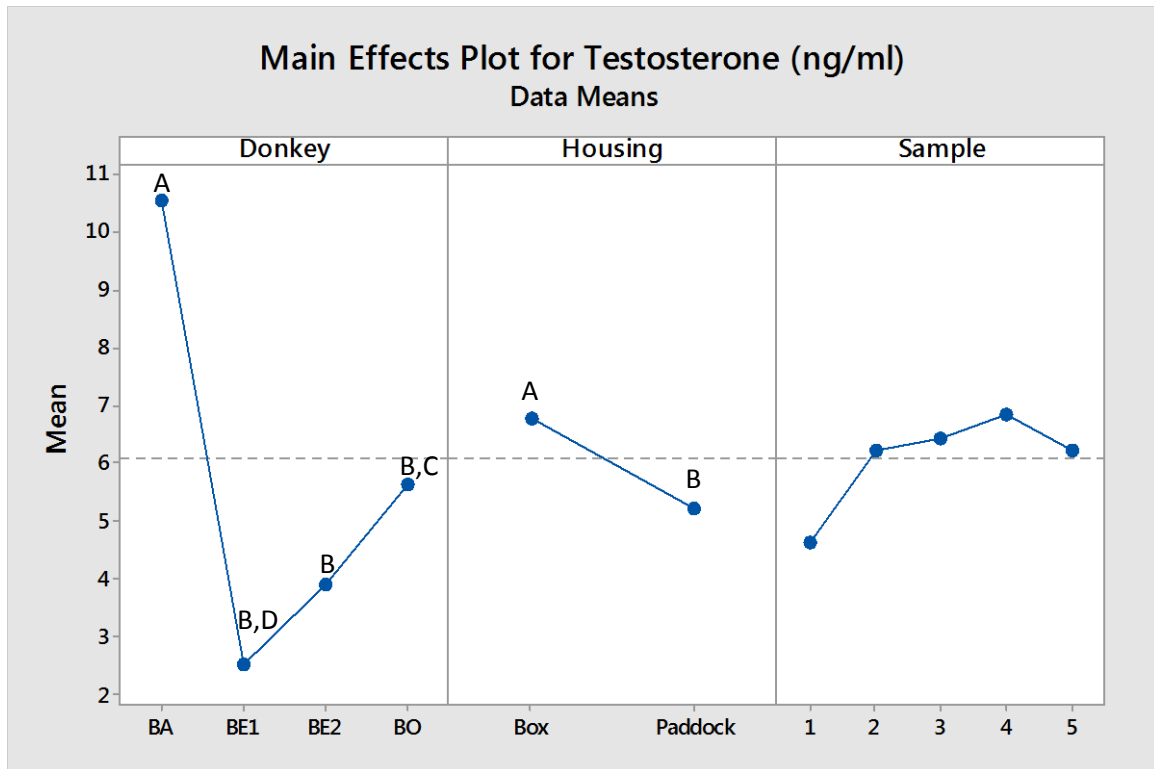
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397 Figure 1: Salivary cortisol of the four donkeys in the two housing system at the six sampling times
398 (mean and standard deviation). Sample 1: 8:30; Sample 2: at the exit from paddock/box, before
399 going to semen collection area; Sample 3: immediately after ejaculation; Sample 4: 10 minutes
400 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: $A \neq B$ and $C \neq D$ by $P < 0.05$.

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