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Original article

The stress response of Ragusano donkey (*Equus asinus*) to different semen collection techniques

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Abstract

The aim of the present study was to characterize the stress response of donkeys to different semen collection techniques, comparing the physiological patterns of hormonal (adrenocorticotrophic hormone: ACTH, cortisol) and biochemical variables (creatinine, total protein, urea, aspartate aminotransferase: AST, alanine aminotransferase: ALT), and routine seminal parameters and sexual behaviour. Twenty two healthy Ragusano donkeys were used and were randomly assigned to one of 2 groups based on different semen collection techniques. Group A was designated as the experimental group and included 16 donkeys submitted to semen collection by an artificial vagina (AV) “on the ground”; group B was designated as the control group, and included 6 donkeys submitted to semen collection by AV during the mount. The semen collection was performed in October, once a day for 10 consecutive days. Blood samples were collected in baseline conditions, before the onset of the treatments and within 30 min after, from the jugular vein of each subject. Two-way ANOVA showed a significant effect of semen collection technique and time points in group A for ACTH ($p=0.0084$), cortisol ($p=0.0004$) and creatinine ($p=0.0131$), with lower values after semen collection than before. A significant effect of semen collection technique and time points in group B for ACTH ($p<0.0001$) and cortisol ($p<0.0001$) was observed, with higher values after semen collection than before. The comparison between groups A and B values over different time points showed a significant effect after semen collection for ACTH ($p<0.0001$), cortisol ($p<0.0001$) and creatinine ($p<0.0001$), with the highest values in group B. This study provides the physiological evidence that semen collection on the ground in healthy donkeys could be used as an alternative strategy to induce a positive animal approach and economical advantages.

Key words: donkey, semen collection techniques, ACTH, cortisol, biochemical variables

Introduction

The qualitative and quantitative intra- and inter-stallion variations in sperm morphology and fertility, in different Equidae species, are widely known (Hoffmann and Landeck 1999, Love et al. 2000, Quartuccio et al. 2011a, Veronesi et al. 2011). A few studies reported conflicting results on the quality of stallion's semen obtained by different semen collection techniques (Lineberg et al. 1999, McDonnell 2005, Contri et al. 2010). Another point is that few data exist on appropriate sperm collection techniques for use in stallions with musculoskeletal and neurological deficits, to enable the propagation of genetic value (Cary et al. 2004, McDonnell 2005).

Neuroendocrine patterns and sexual behaviour are also involved in the response to physical or psychological strain to re-establish the body's homeostasis (McDonnell 1998). Significant increases in cortisol levels have been observed in response to excitement and stress in stallions and geldings (Colborn et al. 1991), with high levels at 10 min after mating, until 20 and 30 min after (Villani et al. 2006, Veronesi et al. 2010) and in donkeys 32 min after ejaculation (Veronesi et al. 2011). Although donkeys have shown many physiological, hormonal and reproductive similarities with horses, several specific differences have been observed also among different donkey breeds, according to genetic code, training time and under controlled or free range management systems (Gastal et al. 1996, Dugat et al. 2010, Fazio et al. 2013a).

It has also been recognised that semen collection in donkeys on the ground, without exposure to an oestrous jenny, can offer a simple, practical and efficient alternative method to the standard method (i.e. artificial vagina; AV) during normal semen collection technique or natural breeding.

In the available published data there is little information about the relationship between pituitary-adrenocortical response, some metabolic variables and reproductive parameters, probably co-responsible for a correct evaluation of good or poor reproductive performance in Equidae (*Equus asinus* and *Equus caballus*). On these bases, the purpose of the present study was to characterize the stress response of donkeys to different semen collection techniques, by AV in standing "on the ground" (group A) and during the mount (group B), comparing the hormonal patterns (adrenocorticotrophic hormone: ACTH, cortisol), biochemical variables (creatinine, total protein, urea, aspartate aminotransferase: AST, alanine aminotransferase: ALT), and routine seminal parameters (volume, sperm concentration, total spermatozoa number (TSN), progressive motility, viability, sperm morphology); sexual behaviour (mounts to successful collec-

tion, thrusts on ejaculatory mount, mount to ejaculation latency, collection time) was also considered.

Materials and Methods

All methods and procedures used in this study were in compliance with the guidelines of the Italian law (D.L. 04/3/2014 n. 26) and EU directive (2010/63/EU) on the protection of animals used for scientific purposes. The Animal Ethics Committee for the Care and Use of Animals of University of Messina concluded that the proposed study did not need ethical approval, as it did not qualified as an animal experiment under Italy law, but individual horse owner's consent was obtained for all horses to participate in the present study.

Animals

The study comprised twenty two healthy mature Ragusano donkeys, aged 9 ± 0.6 years, weighting 295 ± 30 kg, used as sires in the regular breeding program. The animals were divided in two groups on the basis of different treatment and were randomly assigned to one of 2 groups based on different semen collection techniques. Group A was designated as the experimental group and included 16 donkeys submitted to semen collection by AV on the "ground"; group B was designated as the control group, and included 6 donkeys submitted to semen collection by AV during the mount. Specifically, 16 donkeys (group A) ranged 8.98 ± 0.91 years and 6 donkeys (group B) ranged 8.33 ± 0.37 years.

Donkeys stabled on the Equine Breeding Reproduction Centre of Catania, Sicily, Italy ($37^{\circ} 30' 4''$ 68 N latitude; $15^{\circ} 4' 27''$ 12 E longitude) 380 m at sea level, in individual box stalls (4 x 4 m) on straw, under natural photoperiod and environmental temperature. The animals were individually fed with pelleted, complete supplement feed and vetch hay two times daily. Fresh water and mineral supplements were freely available to maintain good body condition. Individual live body weight (BW) was recorded in baseline conditions, using large animal scales. All donkeys had no history of medical problems in the preceding two weeks, had not received any pharmacological treatment for two weeks prior to the study and were healthy, based on physical examination.

Blood samples

Blood samples were taken from the jugular vein, twice a day over ten consecutive days, in baseline con-

ditions, immediately before sperm collection, in individual box stalls, and within 30 min after, at return of each donkey to respective box. The stress of the blood samples procedure was similar in both groups A and B. To reduce circadian variations on the hormonal measurements, all samples were collected in the morning, between 07:00 and 09:00 a.m., in quiet conditions by the same operator.

Analysis

All samples were collected into evacuated tubes (Venoject, Terumo®, Leuven, Belgium) and were immediately refrigerated at 4°C after collection, and subsequently (within 1 h) centrifuged at 3000 x g for 15 min at 20°C. Serum was harvested and stored in polystyrene tubes (Polyst test tube Sorvall CW1, Asti, Italy) at -20°C until assayed for cortisol and haematochemical parameters. Serum ACTH concentrations were analyzed in duplicate using a commercially available radioimmunoassay kit CIS-BioInternational, Gif-sur-Yvette, France). The hormone assay used has a range for the amount of ACTH detected of 0-440 pmol/L. The sensitivity of the assay ACTH was 0.44 pmol/L. The intra- and interassay CVs were 6.0% and 15.0%, respectively.

Total serum cortisol concentrations were analysed in duplicate using a competitive enzyme immunoassay (EIA, RADIM, Rome, Italy). During the first incubation, the cortisol sample competed with cortisol conjugated to horseradish peroxidase (HRPO) for the specific sites of the antiserum coated on the wells. Following incubation, all unbound material was removed by aspiration and washing. The enzyme activity bound to the solid phase is inversely proportional to cortisol concentration in calibrators and samples, and is made evident by incubating the wells with a chromogen solution (tetramethylbenzidine, TMB) in substrate-buffer. Colorimetric reading was carried out using a spectrophotometer at 450, 405 nm wave length (Sirio S, SEAC, Florence, Italy). Assay sensitivity was 5 ng/mL. The intra- and inter-assay CVs were 4.0% and 6.9%, respectively. Serum metabolic variables (creatinine, total protein, urea, AST, ALT) were analysed by a biochemistry autoanalyzer (SLIM, SEAC, Calenzano, Florence, Italy), using SEAC/Radim kits (Pomezia, Rome, Italy).

Semen collection

The semen collection was performed in October, once a day for 10 consecutive days, using a Colorado type artificial vagina (Animal Reproduction Systems,

Chino, CA), equipped with a collection bottle and a nylon filter to remove the gel fraction. The donkeys of group A were stimulated to achieve erection using a jenny that did not demonstrate any signs of oestrus (anovulatory period) placed 4-5 meters away; the donkeys of group B were stimulated to achieve erection using a jenny that demonstrated signs of oestrus. On reaching erection, the penis was carefully washed with warm water and dry with a cloth. During preparation of the male, the collector prepared the AV, adjusting the internal pressure and setting temperature at 50°C or so, taking care to fill the nozzle and the lumen of the AV with sterile non-spermicidal gel (K-Y, Johnson & Johnson, New Brunswick, NJ). In order to reduce collection time, the handler had the task of providing conditioned stimulus to the jack (pats on the chest, shoulders, approach and withdrawal of the jenny teaser), and during semen collection, kept the donkey's head down, thereby balancing the pressure of the body during copulation. Following successful erection, the penis was placed in the AV, and the jack responded to this stimulus by repeated pelvic thrusts, until obtaining ejaculation. Ejaculation was demonstrated by ragged tail of the jack (tail flagging), the display of rhythmic pulsations of the urethra on the ventral surface of the base of the penis.

Semen collection was continued for ten sessions over a three week period. At the end of this study, the jackasses were also bred naturally to evaluate willingness and ability. All semen collection sessions were videotaped for subsequent derivation of the following information: i) the number of semen collection sessions and total attempts to achieve the first of three consecutive successful collections; ii) the sexual behaviour: mounts to successful collection, thrusts on ejaculatory mount, mount to ejaculation latency, collection time.

Analysis of reproductive parameters

The extragonadal sperm reserve was minimized by the first three days of semen collection; hence, the ejaculates were only analysed from 4 to 10 sampling days. Immediately after semen collection, gel-free semen was evaluated for the routine seminal parameters, total volume of gel-free ejaculate was assessed using a graduated cylinder, sperm concentration was evaluated by photometer (Accucell – IMV Technologies, L'Aigle, France) calibrated for horses, but applicable also for donkeys by means of a previous validation with a Makler chamber (Sefi-Medical Instruments, Haifa, Israel); TSN was the product of the gel-free ejaculate volume (mL) and sperm concentration (10⁶/mL) (Makler 1978). Progressive motility was

Table 1. Mean values of routine semen parameters (mean \pm SD) in Ragusano donkey after semen collection on the “ground” (group A) and on the mount (group B).

| Parameters | Group A | Group B |
|--|--------------|--------------|
| Gel-free volume (mL) | 108 \pm 20 | 117 \pm 27 |
| Sperm concentration (x10 ⁶ /mL) | 134 \pm 24 | 130 \pm 18 |
| Total spermatozoa number (x10 ⁹) | 14 \pm 6 | 14 \pm 3 |
| Motility (%) | 80 \pm 10 | 83 \pm 10 |
| Viability (%) | 84 \pm 9 | 82 \pm 9 |
| Sperm morphology (%) | | |
| Head | 1 \pm 1 | 1 \pm 1 |
| Middle section | 8 \pm 3 | 7 \pm 3 |
| Tail | 3 \pm 2 | 2 \pm 1 |

Table 2. Sexual behaviours (mean \pm SD) in Ragusano donkey before and after semen collection on the “ground” (group A) and on the mount (group B).

| Parameters | Group A | Group B |
|--------------------------------------|------------|------------|
| Latency time T1 (min) | 9 \pm 4 | 7 \pm 3 |
| Ejaculation time T2 (sec) | 33 \pm 2 | 33 \pm 3 |
| Total semen collection time T3 (min) | 13 \pm 5 | 12 \pm 3 |
| Pelvic thrusts number PTN | 6 \pm 1 | 6 \pm 1 |

evaluated by placing a drop of undiluted semen in the Makler chamber at 37°C (Makler 1978); it was observed under a bright field microscope at 200 magnification, and estimated subjectively to the nearest 10%. The live and dead spermatozoa (viability) percentage and their morphology were evaluated on eosin-nigrosin-stained smears by counting 300 cells. The sperm morphology was expressed in percentages.

Statistics

Data are presented as mean \pm standard deviation (SD). Significant differences over different days were established using one-way repeated measures analysis of variance (one-way ANOVA). To compare baseline values and post-semen collection values over different time points, two-way repeated measures analysis of variance (2-way ANOVA) was applied. To compare group A and group B values over different time points, two-way repeated measures analysis of variance (2-way ANOVA) was applied. When the F statistic was significant, the differences between individual means over time were then assessed using a post hoc multiple comparison test (Bonferroni). The level of significance was set at $p < 0.05$. All calculations were performed using the PAST software (PALaeontological STatistics, ver. 2.00, Hammer & Harper). The correlation (r) between hormonal, haematochemical variables, seminal parameters and age was performed by using Spearman test.

Results

Hormonal and metabolic parameters

Significant changes in hormonal data are presented as mean (\pm SD) over a period of 10 days in Fig. 1. Two-way ANOVA showed a significant effect of semen collection technique and time points in group A for ACTH ($p = 0.0084$), cortisol ($p = 0.0004$) and creatinine ($p = 0.0131$); lower values after semen collection than before for ACTH concentrations from the 7th to the 9th ($p < 0.05$) day; for cortisol concentrations from the 6th to the 10th ($p < 0.05$) day and for creatinine concentrations at the 7th ($p < 0.05$), 8th ($p < 0.01$), 9th ($p < 0.05$) and 10th ($p < 0.01$) day, were observed.

A significant effect of semen collection technique and time points in group B for ACTH ($p < 0.0001$) and cortisol ($p < 0.0001$) values was shown; higher values after semen collection than before for ACTH concentrations from the 8th to the 10th ($p < 0.01$) day and for cortisol concentrations from the 1st to the 7th day ($p < 0.05$) and from to 8th to the 10th ($p < 0.01$) day, were observed.

The comparison between group A and group B values over different time points showed a significant effect after semen collection for ACTH ($p < 0.0001$), cortisol ($p < 0.0001$) and creatinine ($p < 0.0001$); the highest values in group B for ACTH concentrations from the 7th to the 9th ($p < 0.05$) day; for cortisol concentrations at the 6th ($p < 0.0001$),

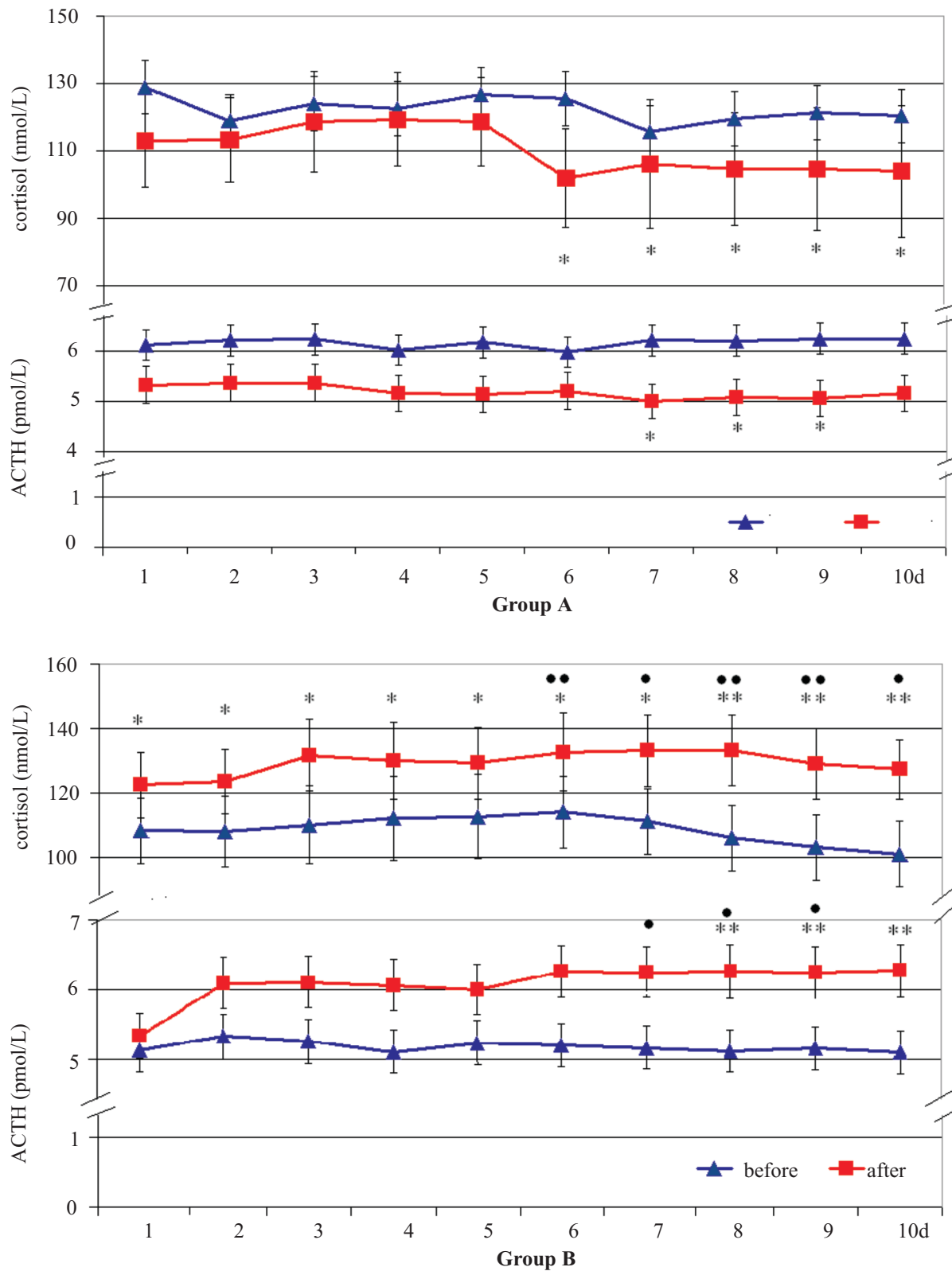


Fig. 1. Serum cortisol and ACTH profiles (M ± SD) in Ragusano donkey before and after semen collection on the “ground” (group A) and on the mount (group B).

Footnote

Asterisks indicate significant differences vs. before *p<0.05; **p<0.01; symbols indicate significant differences vs. group A ●p<0.05; ●●p<0.01

7th ($p < 0.05$), 8th, 9th ($p < 0.001$) and 10th ($p < 0.05$) day and for creatinine concentrations from the 1st to the 10th ($p < 0.0001$) day after semen collection, were observed.

Compared to baseline values, no significant differences for total protein, urea, AST and ALT pattern after semen collection were observed in both group A and group B, nor between groups A and B.

Reproductive parameters

The routine seminal parameters and sexual behaviours are presented as mean (\pm SD) over a period of 10 days in Tables 1 and 2, respectively.

Two-way ANOVA did not show a significant effect of semen collection technique and time points for all reproductive pathways. Positive and significant correlations between latency time and total semen collection time ($p = 0.0066$; $p = 0.0059$), ejaculation time and total spermatozoa number ($p = 0.05$), and spermatozoa's motility and viability ($p = 0.003$; $p = 0.002$) were observed in groups A and B, respectively.

Discussion

Hormonal, metabolic and reproductive pathways found in the present study are in agreement with data obtained in Ragusano (Gastal et al. 1996, Dugat et al. 2010, Fazio et al. 2011a, Fazio et al. 2011b, Quartuccio et al. 2011b) and Martina Franca donkeys (Veronesi et al. 2011). The data obtained showed that group A, after semen collection "on the ground", without the exposure to an oestrous jenny, showed an early but constantly lower ACTH and cortisol concentrations compared with the baseline values. This trend is not in line with cortisol increases in response to excitement, mating and stress described previously in stallions and geldings (*Equus caballus*) (Colborn et al. 1991, Villani et al. 2006, Fazio et al. 2013a).

It is thus reasonable to assume that the donkey's physical component of sexual stimulation may combine with the psychic one in order to enhance the hypophysis-adrenocortical response. There are a number of reasons why this may be.

Firstly, the present study was designed to evaluate the effects of different semen collection techniques by AV "on the ground" and on the mount in the donkey (*Equus asinus*) after exposure to anoestrous or oestrous females, respectively; previous experiments evaluated the effects of mating on plasma cortisol in stallions (*Equus caballus*) and donkeys (*Equus asinus*) after exposure to oestrous females.

Secondly, although oestrone sulphate can provide a sensitive assessment of testicular endocrine activity, modulating sex gland activity and maintenance of libido in stallions (Claus et al. 1992), the negative correlation between plasma cortisol and oestrogen levels described for Equine species (Asa et al. 1983) could explain the cortisol decrease obtained in donkeys after semen collection "on the ground". Nevertheless, the cortisol trend is probably also related to the different sexual behaviours of *Equus asinus* and *Equus caballus*. (McDonnell 1998, Noue et al. 2001). Another possibility is that the constant low cortisol concentrations observed after semen collection in group A could be related to the possible inhibition of adrenocortical activity reported when the presentation of stressful stimuli involved a consummatory event (Levine et al. 1989); in the present experimental design the specific consummatory event could be represented by the ejaculation response. Overall, the magnitude of cortisol decreases after the last day of semen collection what may suggest a stimulus-response relationship and a probable habituation. Some evidence also suggests that the physical component of sexual stimulation, represented by the oestrous jenny, may combine with the psychic one to enhance cortisol release. Taken together, these findings suggest that the relative contribution made by olfactory, visual and tactile stimuli, in the presence of oestrous jenny, or some combination of these factors, increased activation of the adrenocortical response. Moreover, the absence of a significant correlation between ACTH and cortisol concentrations in both groups confirms previous data obtained in horses and donkeys (Fazio et al. 2013a), indicating that there is probably a different response of these hormones under the same conditions, according to the different mental and physical reproductive stimuli; as a matter of fact, several studies in different animal species showed that there are many biological factors capable of modulating the adrenocortical and glucocorticoid release, independently of pituitary ACTH modulation during different stress conditions (Bornstein et al. 2008, Medica et al. 2011).

Concentration of serum creatinine, which is produced in skeletal muscle, shows a significant decrease after semen collection only in group A. This result was, probably, due to inconsistent muscle effort, related to the relative workload involved in the "on the ground" position.

On the other hand, the activity of serum AST, which is a leakage enzyme responsive to muscle injury and damage, and is a diagnostic but not prognostic marker (Thomassian et al. 2007), shows a tendency to increase after semen collection in both groups A and B, possibly due to short duration and/or low intensity

of performance. It can be concluded that Ragusano donkeys adapted better to the effort of reproductive performance, and the creatinine pathway, related to skeletal muscle metabolism, proved to be the most sensitive indicator together with moderate-intensity fitness.

Unmodified changes on total protein, urea and serum ALT activity therefore showed that reproductive performance, including semen collection “on the ground” and on the mount, for Ragusano donkeys did not cause any injury to myocardium, skeletal muscles or liver cells over 10 consecutive days.

The very low mean gel-free volume and the sperm concentration observed in groups A and B confirmed the high variability, reported in Amiata and Pega donkeys, and also in Ragusano donkeys, according to long and short day season (Contri et al. 2010, Quartuccio et al. 2011a). Total spermatozoa number (TSN) is comparable to that obtained by Gastal et al. (1997) in donkeys; unlike, the higher TSN values of $24\text{-}31 \times 10^9$ were described in the Martina Franca donkey (Contri et al. 2010). The present data for forward motility, viability and spermatic anomaly percentage in Ragusano donkey confirmed that the *Equus asinus* stallion represents the high and desirable spermatic productivity and quality (Gastal et al. 1997), as shown by high spermatozoa mobility and viability and low spermatic anomaly percentages, irrespective of semen collection techniques.

The latency time (T1) and the total semen collection time (T3) are very similar to those previously obtained in the donkey (*Equus asinus*) with different semen collection methods (Gastal et al. 1996, Fazio et al. 2011a). Moreover, the ejaculation time (T2) and the pelvic thrusts number (PTN) confirmed previous data described in domesticated donkey breeding under controlled or free range management systems (Gastal et al. 1996). Moreover, the present data confirmed that the coping capacity towards the alternative strategy for semen collection “on the ground” varies widely among behavioural pathways, as showed by a wide standard deviation described at T1, T2 and T3 along time. Furthermore, positive and significant correlations observed in both groups between latency time and total semen collection time, ejaculation time and total spermatozoa number, and spermatozoa motility and vitality showed that, probably, a stallion with the best T2 could potentially be the most desirable specimen of great reproductive value.

What is clear is that there are advantages and disadvantages in different semen collection techniques, with the apparent shift of energy metabolism in a catabolic or anabolic direction and with a wide range of endocrine changes.

In conclusion, the endocrine, metabolic and reproductive mechanisms involved during semen collection “on the ground” in Ragusano donkeys are probably different from those involved in the normal semen collection technique with AV or natural breeding, according to each individual, taking into account the breed, age, function, environmental and nutritional factors, etc. The organism is highly complex and in order to achieve a perfect functioning of the hypothalamic-pituitary-gonadal axis there is a need for perfect interactions between neuroendocrine basis and functional aspects.

The determination of reference values for donkey hormonal, metabolic and reproductive patterns during semen collection “on the ground” and on the mount, by artificial vagina, represents a basic requirement for future implementation of this alternative strategy for semen collection; this approach could be useful for considerable reduction of costs and represents a clinically useful collection method for some stallions (*Equus asinus*) that, due to physical injury or hind limb pain, experience difficulties in mounting a jenny, enabling the propagation of genetic value, even in absence of oestrous females. The utilization of semen collection “on the ground”, without the presence of oestrous jenny, could be extremely useful in some specific cases. Hence, to perfect the technique, a reproductive, clinical and technical knowledge is essential.

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