Manuscript Details

Manuscript number	JEVS_2019_9_R1
Title	Determination of salivary cortisol in donkey stallions
Article type	Short communication

Abstract

Salivary cortisol provides information about free plasma cortisol concentration and salivary sampling is a non-invasive well-tolerated procedure. The aim of this study was to validate a commercial enzyme immunoassay for the determination of salivary cortisol in donkeys. Saliva samples were collected in 4 donkey stallions on thirteen non-consecutive days at 8:30 AM to avoid circadian variation. Animals were already accustomed to be handled. Saliva was collected by using a swab inserted at the angle of the lips, placed onto the tongue for 1 min and returned into a polypropylene tube. Tubes were centrifuged and at least 1 ml of saliva was aspirated from each sample and frozen at −20° C until analysis. A commercial enzyme immunoassay kit without extraction was used for determination of cortisol in saliva. Median cortisol concentrations with minimum and maximum value were calculated. Recovery of cortisol standard in donkey saliva was between 97.3% and 99.7% and serial dilution of donkey saliva samples with assay buffer resulted in changes in optical density parallel to the standard curve. Cross-reactivity of the antiserum was 10.4% with 11-deoxycortisol, 5.2% with corticosterone, 0.4% with 11-deoxycorticosterone, 0.2% with cortisone and <0.1% with testosterone, progesterone and estradiol. The intra-assay coefficient of variation was 10.7%, the inter-assay variation was 8.0% and the minimal detectable concentration was 0.01 ng/ml. The results of the present study demonstrate the validity of a commercial kit to determine the concentration of cortisol in donkey saliva, as already reported in other species.

Keywords	donkey; salivary cortisol; validation; stress; enzyme immunoassay.

Corresponding Author	Micaela Sgorbini
Corresponding Author's Institution	Department of Veterinary Sciences, Veterinary teaching Hospital
Order of Authors	FRANCESCA BONELLI, Alessandra Rota, Christine Aurich, Ille Natascha, Francesco Camillo, Duccio Panzani, Micaela Sgorbini
Suggested reviewers	Fabrizio Rueca, Fulvio Laus

Submission Files Included in this PDF

File Name [File Type]

Cover letter Bonelli et al. cortisol.docx [Cover Letter]

Cortisol in donkeys - Answer to reviewers.docx [Response to Reviewers]

Highlights SALIVARY CORTISOL pos revision.docx [Highlights]

DETERMINATION OF SALIVARY CORTISOL IN DONKEY_post revision.docx [Manuscript File]

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Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

Dear Editor,

here is our revised paper titled “Determination of salivary cortisol in donkey stallions”, authored by Bonelli et al. The Ethical Committee, University of Pisa, approved this study. This study was supported totally by funds from the University of Pisa (100%) (Progetti di Ricerca di Ateneo: PRA_2016_53). No conflict of interest exits.

Yours sincerely,

Prof.ssa Micaela Sgorbini

Reviewer 1 (answers and comments in yellow).

The paper describe the validation of use for a commercial enzyme immunoassay for the determination of salivary cortisol in donkeys. The paper is well written and in my opinion provide useful information to the scientific community. The authors validate a method for evaluation of salivary cortisol in donkey, paving the way for further studies that will investigate the diagnostic use in clinic or in the assessment of well-being.

Minor revision:
Line 6: highlights should convey the main findings and not aims of the paper.
Answer: the highlights have been corrected and the main findings have been better reported.

Line 13: Change "The Kit" with "A kit" or revise the sentence.
Answer: The sentence has been revised.

Line 83: Citation number 30 should be changed and "Mat and Met" section consequently revised. (For example see: Pearson and Ouassat, 2000 also cited in Quaresma M. et al, The veterinary Journal, 2013).
Answer: the citation has been changed.

Line 93: it is 2017?
Answer: sorry for this mistake, the years were 2015 and 2016, as written in line 92.

Line 95: delete "were".
Answer: done.

Line 97: what the authors mean with "*special* restraint"?
Answer: the sentence has been better formulated.

Line 181: change: "...despite in the majority of the samples, the quantity of saliva was good enough for..." with "despite in the majority of the samples the quantity of saliva was good enough for..."
Answer: correctio done.

Reviewer 2 (answers anc comments in green)

Determination of salivary cortisol in donkey stallions

Immunoassays have to be evaluated before being used in a given species. The manuscript describes a validation of a commercial enzyme immunoassay for measuring the cortisol concentration in donkey. Data of cortisol concentrations in saliva of 4 donkey stallions measured with this method are included. A denser sampling regime (more frequently than once a day) would have given more information (circadian rhythm and episodic spikes).

Answer: thank you for this comment. The salivary samplings schedule used in this paper was mandatory because the samples have been performed at the same time for two different protocols (please see Rota et al., 2018. Effect of housing system on reproductive behaviour and on some endocrinological and seminal parameters of donkey stallions. Reprod Dom Anim, 53(1): 40-47.

The manuscript may benefit if the scopes of applications of the different matrices (blood, saliva, urine and faeces) are discussed, as not in all cases measuring cortisol in saliva is the best way to monitor cortisol production. In the discussion it would be desirable to compare cortisol concentrations in the saliva of donkyes and horses.

Answer: the comparison between saliva cortisol concentrations of donkeys and horses has been added to the discussion section (please see lines 178-184, in grey).

Material and methods: how much saliva did you use per well?

Answer: the amount of saliva collected was at least 1 ml (please see line 100), because this the minimum amount needed for all the laboratory procedures.

Reviewer 3 (answer and comments in blue)

This is a nice manuscript that needs a few improvements in the english language. See comments below.

In general, the biggest question is why no comparison to blood cortisol levels was done?

Answer: Thank you for this comment. The salivary cortisol evaluation has been performed to verify the effect of housing or semen collection in donkey stallion (please see Rota et al., 2018. Effect of housing system on reproductive behaviour and on some endocrinological and seminal parameters of donkey stallions. Reprod Dom Anim, 53(1): 40-47). Our study was performed as the validation of the kit used in the previous study and the sampling scheduling (saliva and blood) was mandatory in the way it has been performed.

How do you explain the high values of cortisol in your study compared to horses? Any ideas why basal levels in donkeys could be that much higher? How do you explain the big individual differences for the samples?

Answer: An explanation about the reason why in donkeys we found higher values of saliva cortisol has been added to the discussion section (please see 178-184, grey).

Highlights: Line 15 – donkeys.

Answer: correction done.

Abstract: Line 18 - well-tolerated

Answer: correction done.

Introduction:

Line 39/40 – belated physiological responses to a stressor

Answer: correction done.

Line 56-58 – repitition of lines 50/51 , rephrase or emphasize why putting more than once

Answer: the repetition has been delated.

Line 61 – other equids??? Why do I need to mention them?

Line 61 - … the donkey achieved a …

Answer: correction done.

Line 63 – donkeys have become

Answer: correction done.

Line 78 – cleaned daily

Answer: correction done.

Line 80 - according to NRC

Answer: correction done.

Line 81 to 83 – move to results section. How was body weight determined? Neccessary fort he paper?

Lines 82-85 have been delated.

Line 87 – all 4 jacks were subjected to semen collection

Answer: correction done.

Line 97 – special restraint? Please xplain in more detail what type of action was taken.

Answer: the sentence has been rewerded.

Line 100 – 700g? 1800g? not clear how centrifuged.

Answer: sorry for this mistake. The saliva samples have been centrifuged at 1000g for 10 min, as in the paper by Rota et al 2018, Reprod Dom Anim, 53(1): 40-47.

Table 1 – explain individual differences in discussion. Values are pretty high in genereal compared to basal lines in horses for example.

Line 149 – always stress?

Answer: “always” has been changed with “often” (Peeters et al. 2011, Equine Vet J, 43(4): 487-93.

Line 152 – delete „well“

Answer: correction done.

Line 160 - change none to no effect

Answer: correction done.

Line 161-163 – reference 39 is about mice, not horses. Please check and use correct reference.

Answer: a new reference has been added.

Conclusions

Line 180-182 – please rephrase

Answer: the sentence has been rephrased.

References:

Line 285 - correct references, spelling mistakes

Answer: correction done.

Highlights

- The sampling procedure was easy to perform and stress-free for the animals.
- The commercial kit used to determine the concentration of cortisol in donkey saliva was valid.
- Cortisol evaluation in saliva could be considered highly suitable in donkeys

Determination of salivary cortisol in donkey stallions

Short title: Salivary cortisol in donkeys

Francesca Bonelli[a], Alessandra Rota[a], Christine Aurich[b], Natascha Ille[b], Francesco Camillo[a], Duccio Panzani[a], Micaela Sgorbini[a]

[a]Department of Veterinary Sciences, via Livornese snc, 56122 San Piero a Grado (PI), Italy

[b]Centre for Artificial Insemination and Embryo Transfer, University of Veterinary Sciences, Vienna, Austria.

Corresponding Author:

Micaela Sgorbini

Department of Veterinary Sciences

via Livornese snc

56122 San Piero a Grado (PI), Italy

micaela.sgorbini@unipi.it

Abstract

Salivary cortisol provides information about free plasma cortisol concentration and salivary sampling is a non-invasive well-tolerated procedure. The aim of this study was to validate a commercial enzyme immunoassay for the determination of salivary cortisol in donkeys.

Saliva samples were collected in 4 donkey stallions on thirteen non-consecutive days at 8:30 AM to avoid circadian variation. Animals were already accustomed to be handled. Saliva was collected by using a swab inserted at the angle of the lips, placed onto the tongue for 1 min and returned into a polypropylene tube. Tubes were centrifuged and at least 1 ml of saliva was aspirated from each sample and frozen at −20° C until analysis. A commercial enzyme immunoassay kit without extraction was used for determination of cortisol in saliva. Median cortisol concentrations with minimum and maximum value were calculated.

Recovery of cortisol standard in donkey saliva was between 97.3% and 99.7% and serial dilution of donkey saliva samples with assay buffer resulted in changes in optical density parallel to the standard curve. Cross-reactivity of the antiserum was 10.4% with 11-deoxycortisol, 5.2% with corticosterone, 0.4% with 11-deoxycorticosterone, 0.2% with cortisone and <0.1% with testosterone, progesterone and estradiol. The intra-assay coefficient of variation was 10.7%, the inter-assay variation was 8.0% and the minimal detectable concentration was 0.01 ng/ml.

The results of the present study demonstrate the validity of a commercial kit to determine the concentration of cortisol in donkey saliva, as already reported in other species.

Key words: donkey; salivary cortisol; validation; stress; enzyme immunoassay.

Introduction

Domestic animals react to stress through physiological responses, which are the results of individual emotional reactivity [1]. The hypothalamic-pituitary-adrenocortical (HPA) system is activated in response to a stressor. While early response is under catecholaminergic control, late physiological response to stressor is commonly assessed by determination of glucocorticoid concentrations, such as cortisol.

An increase in hypothalamic-pituitary-adrenocortical activity indicates a physiological response to different stressors, and measurement of plasma corticosteroids is frequently used to study middle and longtime stress response [2].

Cortisol was primarily obtained from blood, plasma or urine. Blood sampling itself (by syringe or by catheterization) can produce stress in the animal causing a rise in cortisol levels [3,4]. Urine and fecal samples can be easily and non-invasively collected without submitting animals to stress [5,6], but the volume/unit weight varies between animals and species [5] and time periods between application of the stressor and collection of the urine or feces cannot be easily controlled. On the other hand the animals are hardly affected by saliva sample collection [7,8], thus saliva can be considered a non-invasive monitoring of HPA functioning in animals.

Cortisol is passively diffused into the salivary glands, not bound to proteins and its concentration is not affected by salivary flow rate. Therefore, the salivary cortisol provides direct information about free plasma cortisol concentration, which is the only biologically active fraction in the organism, owing to it being able to bind to cell receptors [9,10].

~~Furthermore, the collection of saliva is a non-invasive procedure, which animals usually tolerate better than blood sampling.~~ For all of these reasons, the interest in measurement of salivary cortisol concentration in animals has increased during the past few years.

Salivary cortisol concentration has been evaluated in human [8,11,12], dogs [13-16], cattle [3,17-

19], pigs [20,21] and horses [9,10,22-24].

In the last decades ~~other equids,~~ the donkey~~,~~ achieved a relevant position in the human society thanks to their employment in animal-assisted therapy [25] and milk production [26-28]. Therefore, the welfare and stress responses of donkeys have become an interesting field of research related to its behavior. To the authors' knowledge there are no validated commercial kits for the determination of salivary cortisol levels in donkeys. We followed the hypothesis that determination of cortisol concentration in donkey saliva is possible. It was the aim of this study to validate a commercial enzyme immunoassay for the determination of salivary cortisol concentrations in donkeys.

Materials and methods

This study was conducted between September 2015 and February 2016 and was approved by the Ethical Committee (OPBA) of the University of Pisa, according to D.lvo 26/2014. Four Amiata donkey stallions (jacks), aged 3 years (born between May and July 2012) were included in this study.

The jacks were kept together in a paddock (10 x 20 mt) for three months (September-November 2015). Then donkeys were housed for three months (December 2015-January 2016) in single boxes (3.5 x 3.5 mt) with a small outside paddock (3.5 x 6 mt), where they could see each other, but had no physical contact. Boxes were cleaned daily and bedded with straw. All the donkeys had free access to water from a water bowl and were fed meadow hay *ad libitum* and commercial concentrate (Equifioc®, Molitoria Val di Serchio, Lucca, Italy), according to NRC recommendations for adult donkeys [29]. ~~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 Body Condition Score (BCS) was always evaluated between 5 and 6/9~~

~~[30].~~

Saliva samples were collected on thirteen non-consecutive days at 8:30 AM to avoid circadian variation [10] using a cotton-based swab (Salivette®, Sarstedt, Numbrecht-Rommelsdorf, Germany) as described in horses [22,23]. Sampling time for cortisol evaluation was scheduled in line with another project running simultaneously [31]. Briefly, all the 4 jacks were subjected to semen collections thrice weekly (Tuesday, Thursday and Saturday) during 3 weeks in the month of November 2015 and February 2016. One saliva sampling took place the week before the beginning of semen collection (November 2015), while the other 12 samplings were carried out every Tuesday and Saturday when semen collection was scheduled (6 samplings in November 2015 and 6 samplings in February 2016). Thus, 7 saliva sampling were done in November 2015 and 6 in February 2016.

Samplings were always made before feeding and cleaning procedures of the boxes and before semen collection, when scheduled, with animals ~~were~~ at rest. Special care was used to minimize stress during the sampling procedure, in particular, the same operator handled the animals throughout the study and only manual restrain during saliva samplings was applied. The swab was grasped with a surgical clamp, inserted at the angle of the lips into the mouth of the donkey and placed gently onto the tongue for 1 min and afterwards returned into a polypropylene tube. Tubes were then centrifuged for 10 min at 1000 g, at least 1 ml of saliva was aspirated from each sample and the obtained saliva was frozen at −20° C until analysis.

A commercial enzyme immunoassay kit without extraction (Demeditec Diagnostics, Kiel-Wellsee, Germany) was used for determination of cortisol in saliva. The assay was validated for asinine saliva in the laboratory of the unit for Obstetrics, Gynecology and Andrology, Department for Small Animals and Horses, Vetmeduni (Vienna, Austria). Cross-reactivity of the antiserum was determined at 50% binding with 11-deoxycortisol, corticosterone, 11-

deoxycorticosterone, cortisone, testosterone, progesterone and estradiol-17β. Recovery of cortisol standards added to donkey saliva was tested. Parallelism between serial dilutions of saliva samples with assay buffer and respective changes in optical density with respect to the standard curve were determined. The intra-assay and inter-assay coefficients of variation were determined from duplicates of control saliva with low and high cortisol concentrations run in each assay (n=5). The minimal detectable concentration was defined as 2 standard deviations from zero binding.

The Graph Pad Prism 6 (USA) software was used for statistical analysis. Data were analyzed for normal distribution by Kolmogorov-Smirnov test. Because not all data were normally distributed, results were expressed as median values of cortisol concentration and range calculated for each day of sampling. Values are given as ng/ml.

Results

Saliva samples could be easily collected in all the donkeys enrolled in this study. The sampling procedure was well accepted by all the animals. In one donkey (n. 4 table 1), the amount of saliva collected was occasionally too small for analysis (samples 2nd, 6th and 9th).

Recovery of cortisol standard in donkey saliva was between 97.3% and 99.7% and serial dilution of donkey saliva samples with assay buffer resulted in changes in optical density parallel to the standard curve. Cross-reactivity of the antiserum at 50% binding was 10.4% with 11-deoxycortisol, 5.2% with corticosterone, 0.4% with 11-deoxycorticosterone, 0.2% with cortisone and <0.1% with testosterone, progesterone and estradiol. The intra-assay coefficient of variation was 10.7%, the inter-assay variation was 8.0% and the minimal detectable concentration was 0.01 ng/ml.

Results of cortisol concentrations determined in saliva collected from donkeys on different days

are presented in Table 1.

	Salivary cortisol concentration (ng/ml)												
Donkeys	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
1	3.9	3.6	10.8	3.6	6.2	1.5	4.6	3.4	5.8	5.4	4.6	5.6	4.8
2	0.5	7.25	8.7	8.11	7.5	1.73	1.7	4.7	5.9	2.9	8.1	1.9	1.9
3	9.4	5.36	20.6	7.72	1.0	6.6	2.8	3.7	9.2	3.0	5.0	6.9	2.1
4	3.1	*	4.7	10.38	9.5	*	9.7	5.6	*	3.7	11.8	4.2	2.7
Median	3	5	9	7.5	6.6	1	3	3.5	5	3	6.5	4.5	2
Range	0-9	3-7	4-20	3-10	1-9	1-6	1-9	3-5	5-9	2-5	4-11	1-6	1-4

Tab 1. Results of salivary cortisol concentration obtained in 4 individual donkey stallions on 13 non-consecutive days. Legend: *Amount of saliva not sufficient for cortisol analysis.

Discussion

The production of cortisol is regulated by the corticotrophic axis and can be altered in different circumstances. Cortisol concentrations can rise in stressful situations, thus it is considered to be an indicator of animal welfare [4]. Although traditionally cortisol concentrations have been determined in plasma or serum, their determination in samples easily collectable under field conditions (saliva, milk, urine, feces and hair) can be of interest for animal welfare studies in different species [8,32-35]. Thus, in our study, a commercial kit assay system, originally used in humans for measuring concentration of cortisol, was adapted and validated for donkey saliva samples.

The evaluation of salivary cortisol has some advantages in large animals. Cortisol concentration in saliva corresponds to the free fraction of cortisol in plasma, thus salivary cortisol represents a better indicator of the possible effects of the corticotrophic axis on the animal organism than plasma cortisol [17]. Moreover, blood collection often produces stress in the animal that can

cause cortisol levels to rise [3,8]. On the other hand, saliva sampling, especially in already handled animals, can be an easy and stress-free procedure as confirmed by our results.

All donkeys included in the study ~~well~~ tolerated the procedures and the amount of saliva collected was good enough in the majority of the samples. The results of the present study demonstrate the validity of a commercial kit to determine the concentration of cortisol in donkey saliva, as already reported in dogs [13-15] and horses [9,10,22,23,37].

In the specificity tests, all the cross reactions observed were in line with the manufactory performance characteristic declared. A very low cross-reaction has been found with 11-deoxycortisol (10.4%) and corticosterone (5.2%). The 11-deoxycortisol is a direct precursor of cortisol and is found in plasma at lower concentrations than cortisol. Literature showed that the 11-deoxycortisol have no glucocorticoid effect and almost no mineral corticoid effect [38]. Corticosterone is the predominant glucocorticoid produced in some species (i.e. mice, rats and birds) [39]. Even if corticosterone is also synthesized in cortisol-dominant species, like equids, its glucocorticoid activity in these species is still under assessment [40]. Cross-reactivity with 11-deoxycorticosterone, cortisone and with testosterone, progesterone and estradiol were negligible.

Intra- and inter-assay coefficients of variation are used to control the potential analytic error in biological assay systems [41]. Our results showed a slightly increased intra- and inter-assay CV % compared with the manufactory performance characteristic declaration (intra-assay CV of 3.8-5.8% and inter-assay CV of 6.4-6.2%). However, the validation results indicated that the method was adequate for salivary cortisol measurements given that it is generally accepted that the CV must be lower than 20% for analytical determinations [41]. The minimal detectable concentration found in our study was slightly below those indicated by the manufactory guidelines (0.01 ng/ml *vs* 0.024 ng/ml, respectively) and published in cows [17,42], but similar to results in pigs [20] and horses [10].

The cortisol concentration in the saliva obtained in donkey stallions was higher respect to equine mares or geldings [9,22,23], but similar to equine foals during the immediate postnatal period [37].

In a recent study [10], saliva cortisol concentration has been assessed in equine stallion during the winter-time (November-February). Saliva cortisol concentration obtained in jacks was higher than in stallion [10]. Donkeys enrolled in this study were similar to horses included in the previous study concerning sex and age. Moreover the saliva cortisol concentration was assessed during the same season and using the same method. Thus, the higher saliva cortisol concentration obtained in donkeys might be related to species.

Conclusions

In conclusion, given the advantages of the stress-free collection and processing of saliva and the good performance of the assay, the use of cortisol detection in saliva could be considered highly suitable in donkey stress research. This study has some limitation. Salivary and blood cortisol concentrations have not been compared. However, when this was done in horses, the relationship was very good [9,10]. Also, despite in the majority of the samples, the quantity of saliva was good enough for running the assay, an improvement in saliva collection procedure need to be addressed.

Acknowledgements

The study was funded by University of Pisa (Progetti di Ricerca di Ateneo: PRA_2016_53). We are grateful to Ente Terre Regionali Toscane for allowing us to employ the animals for this study.

Fundings

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

1. Koolhaas JM, De Boer SF, Coppens CM, Buwalda B. Neuroendocrinology of coping styles: towards understanding the biology of individual variation. Front. Neuroendocrinol 2010;31:307-21.
2. Pacak K, Palkovits M. Stressor specificity of central neuroendocrine responses: Implications for stress-related disorders. Endocr Rev 2001;22:502–48.
3. Fell LR, Shutt DA. Adrenocortical response of calves to transport stress as measured by salivary cortisol. Can J Anim Sci 1986;66: 637-41.
4. Cook NJ, Schaefer AL, Lepage P, Morgan Jones S. Salivary vs. serum cortisol for the assessment of adrenal activity in swine. Can J Anim Sci 1996;76:329-35.
5. Palme R, Fischer P, Schildorfer H, Ismail MN. Excretion of infused C-steroid hormones via faeces and urine in domestic livestock. Anim Reprod Sci 1996;43:43–63.
6. Mostl E, Palme R. Hormones as indicators of stress. Dom Anim Endocrinol 2002;23:67-74.
7. Fell LR, Shutt DA, Bentley CJ. Development of a salivary cortisol method for detecting changes in plasma “free” cortisol arising from acute stress in sheep. Aust Vet J 1985;12:403-6.
8. Cooper TR, Trunkfield HR, Zanella AJ, Booth WD. An enzyme-linked immunosorbent assay for cortisol in the saliva of man and domestic farm animals. J Endocrinol 1989;123:R13–6.
9. Peeters M, Sulon J, Beckers JF, Ledoux D, Vandenheede M. Comparison between blood serum and salivary cortisol concentrations in horses using an adrenocoticotropic hormone challenge. Equine Vet J 2011;43:487-93.

10. Aurich J, Wulf M, Ille N, Erber R, von Lewinski M, Palme R, et al. Effects of season, age, sex, and housing on salivary cortisol concentrations in horses. Domest Anim Endocrinol 2015;52: 11-6.
11. Aardal E, Holm AC. Cortisol in Saliva. Reference ranges and relation to cortisol in serum. Eur J Clin Chem Clin Biochem 2005;33:927-32.
12. Turpeinen U, Hamalainen, E. Determination of cortisol in serum, saliva and urine. Best Pract Res Clin Endocrinol Metab 2013;27(6):795-801.
13. Koyama T, Omata Y, Saito A. Changes in salivary cortisol concentrations during a 24-hour period in dogs. Horm Metab Res 2003;35:355-7.
14. Wenger-Riggenbach, B, Boretti, FS, Quante, S, Schellenberg S, Reusch CE, Sieber-Ruckstuhl NS. Salivary cortisol concentrations in healthy dogs and dogs with hypercortisolism. J Vet Intern Med 2010;24:551–6.
15. Bennet A, Hayssen V. Measuring cortisol in hair and saliva from dogs: coat color and pigment differences. Domest Anim Endocrinol 2010;39(3):171-80.
16. Di Nardo F, Anfossi L, Ozella L, Saccani A, Giovannoli C, Spano G., et al. Validation of a qualitative immunochromatographic test for the noninvasive assessment of stress in dogs. J Chromatogr B Analyt Technol Biomed Life Sci 2016;1028:192-8.
17. Chacón Pérez G, García-Belenguer Laita S, Illera del Portal SJC, Palacio Liesa J. Validation of an EIA technique for the determination of salivary cortisol in cattle. Span J Agric Res 2004;2(1):45-51.
18. Negrao JA, Porcionato MA, de Passillè AM, Rushen J. Cortisol in Saliva and Plasma of Cattle After ACTH Administration and Milking. J Dairy Sci 2004;87:1713-8.

19. Hernandez CE, Thierfelder T, Svennersten-Sjaunja K, Berg C, Orihuela A, Lidfors L. Time lag between peak concentrations of plasma and salivary cortisol following a stressful procedure in dairy cattle. Acta Vet Scand 2014;56:61.
20. Escribano D, Fuentes-Rubio M, Cerón JJ. Validation of an automated chemiluminescent immunoassay for salivary cortisol measurements in pigs. J Vet Diagn Invest 2012;24(5):918-23.
21. Escribano D, Gutiérrez AM, Tecles F, Cerón JJ. Changes in saliva biomarkers of stress and immunity in domestic pigs exposed to a psychosocial stressor. Res Vet Sci 2015;102:38-44.
22. Schmidt A, Biau S, Möstl E, Becker-Birck M, Morillon B, et al. Changes in cortisol release and heart rate variability in sport horses during long-distance road transport. Domest Anim Endocrinol 2010a;38:179-89.
23. Schmidt A, Möstl E, Wehnert C, Aurich J, Müller J Aurich C. Cortisol release and heart rate variability in horses during road transport. Horm Behav 2010b;57:209-15.
24. Bohak ZS, Szabo F, Beckers JF, Melo de Sousa N, Kutasi O, Nagy K, et al. Monitoring the circadian rhythm of the serum and salivary cortisol concentrations in the horse. Domest Anim Endocrinol 2013;45:38-42.
25. Borioni M, Marinaro P, Celestini S, Del Sole F, Magro R, Zoppi D, et al. Effect of equestrian therapy and onotherapy in physical and psycho-social performances of adults with intellectual disability: a preliminary study of evaluation tools based on the ICF classification. Disabil Rehabil 2012;34(4):279-97.
26. Carroccio A, Cavataio F, Montalto G. Intolerance to hydrolysed cow's milk proteins in infants: clinical characteristic and dietary treatment. Clin Exp Allergy 2000;30:1597-1603.

27. Muraro MA, Giampietro PG, Galli E. Soy formulas and non bovine milk. Ann Allergy Asthma Immunol 2002;89(S):97-101.
28. Camillo F, Rota A, Biagini L, Tesi M, Fanelli D, Panzani D. The Current Situation and Trend of Donkey Industry in Europe. J Equine Vet Sci 2018;65:44-9.
29. National Research Council. Nutrient requirements of horses: sixth revised edition. Washington, DC: The National Academies Press; 2007.
30. Pearson RA, Ouassat M. A Guide to Body Condition Scoring and Live Weight Estimation of Donkeys. Centre for Tropical Veterinary Medicine, University of Edinburgh; 2000.
31. Rota A, Sgorbini M, Panzani D, Bonelli F, Baragli P, Ille, N et al. Effect of housing system on reproductive behavior and on some endocrinological and seminal parameters of donkey stallions. Reprod Dom Anim 2018;53(1):40-7.
32. Verkerk GA, Phipps AM, Carragher JF, Matthews LR, Stelwagen K. Characterization of milk cortisol concentration as a measure of short term stress response in lactating dairy cows. Anim Welfare 1998;7:77-86.
33. Cirimele V, Kintz P, Dumestre V, Goullé JP, Ludes B. Identification of ten corticosteroids in human hair by liquid chromatography-ionspray mass spectrometry. Forensic Sci Internat 1999;107(1-3):381-8.
34. Fritsche S, Schmidt G, Steinhart H. Gas chromatographic-mass spectrometric determination of natural profiles of androgens, progestogens and glucocorticoids in muscle tissue of males cattle. Eur Food Res Technol 1999;209:393-9.
35. Morrow CJ, Kolver ES, Verkerk GA, Matthews LR. Urinary corticosteroids: an indicator of stress in dairy cattle. Anim Prod Sci. 2000;60:218-21.

36. Palme R, Robia C, Baumgartner W, Möstl E. Transport stress in cattle as reflected by an increase in faecal cortisol metabolite concentration. Vet Rec 2000;146:108-9.

37. Nagel CC, Erber R, Ille N, Wulf M, Aurich J, Möstl E, Aurich C. Heart rate and salivary cortisol concentrations in foals at birth. Vet J 2015;203:250-2.

38. Lòpez-Calderòn A. Glandulas suprarrenales. In: Fisiologia humana, 2nd edition. Madrid, Spain: Mc Graw-Hill Interamericana; 1999.

39. Gong S, Miao Y-L, Jiao G-Z, Sun M-J, Li H, Lin J, et al. Dynamics and Correlation of Serum Cortisol and Corticosterone under Different Physiological or Stressful Conditions in Mice. PLoS ONE 2015;10(2):e0117503.

40. Koren L, Whiteside D, Fahlman A, Ruckstuhl K, Kutz S, Checkley S et al. Cortisol and corticosterone independence in cortisol-dominant wildlife. General and Comparative Endocrinol 2012;177:113-119.

41. Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), and Center for Veterinary Medicine (CVM). Guidance for industry: Bioanalytical method validation. Washington, DC: U.S. Department of Health and Human Services. 2001; Retrieved from http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf

42. Schrama JW, Heetkamp MJW, Verstegen MWA, Schouten WGP, Van Der Veen F, Helmond FA. Responses of young calves on two levels of feeding to transportation. J Anim Sci 1996;63:79-89.