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Faecal cortisol metabolite excretion and stress in Standardbred Trotters under field conditions and during treadmill training

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Summary

Faecal cortisol metabolite concentrations are useful indicators of stress in domestic and game animals. Their excretion by trained or raced horses was used as a suitable indicator of stress in the present study. The reference range of concentrations of faecal 11,17-dioxoandrostanes (11,17-DOA), a group of cortisol metabolites, for racing standardbreds was calculated from data of 18 healthy regularly trained and raced trotters. The 95 % confidence interval was 14-31 nmol/kg faeces. Some unfit horses showed values out of this reference range, e.g. 2 of 4 horses with recurrent exertional rhabdomyolysis both had 11,17-DOA concentrations of 50 nmol/kg.

In a further trial, the relation between faecal 11,17-DOA levels of 5 treadmill-trained trotters and exercise was investigated. Median 11,17-DOA levels gradually increased from 39 nmol/kg at the start of the training program to a maximum of 146 nmol/kg prior to the 3rd training session. The 11,17-DOA levels of one rather nervous horse even reached 1,478 nmol/kg, prior to its 3rd training session. The day after the 1st,2nd ,3rd and 4th training session, median 11,17-DOA levels were 33, 55, 65 and 115 nmol/kg, respectively. The median 11,17-DOA levels prior to 4 standard exercise test (SET) were 26, 43, 216 and 43 nmol/kg, respectively. The median post SET 11,17-DOA levels were 63, 281, 197 and 81 nmol/kg.

Plasma cortisol levels increased after exercise, but at 18 hrs it appeared that the circadian pattern was restored. The type of exercise and the time in relation to the exercise had significant effects on plasma cortisol concentrations. Plasma cortisol concentrations prior to SETs were higher than those prior to training.

Increased baseline 11,17-DOA levels prior to exercise indicated increased hypothalamic-pituitary-adrenal axis activity during the entire trial. Hence, all training of horses and performing SETs indicates stress. However, when horses became accustomed to the exercise they excreted lower levels of faecal cortisol metabolites, suggesting successful adaptation to the stress of intensive exercise.

Abbreviations: AST = aspartate aminotransferase; CK = creatine kinase; CI = confidence interval; 11,17-DOA = 11,17-dioxoandrostanes; EDTA = ethylenediaminetetraacetic acid; EIA = enzyme immunoassay; RER = recurrent exertional rhabdomyolysis; SET: standard exercise test; IU/L = international units per litre

Schlüsselwörter Pferd**,** Stress, Nebennierenaktivität, Standard Exercise Test (standardisierter Leistungstest), Kreuzschlag.

Zusammenfassung

Ausscheidung von fäkalen Kortisolmetaboliten und Stress bei Trabern unter Feldbedingungen und bei Laufbandtraining

Konzentrationen von fäkalen Kortisolmetaboliten können als Indikatoren für Stress bei Haus- und Wildtieren verwendet werden. Deren Ausscheidung durch trainierte und beim Rennen eingesetzte Pferde wurde in dieser Studie als Stressindikator herangezogen. Die Referenzwerte für 11,17-Dioxoandrostane (11,17-DOA), eine Gruppe von Kortisolmetaboliten im Kot, wurden aus Daten von 18 Tieren berechnet, die regelmäßig trainiert und in Rennen eingesetzt wurden.

Das 95 %-Konfidenzinterval der 11,17-DOA-Konzentrationen von 18 gesunden Pferden betrug 14-31 nmol/kg. Einige nicht ausreichend trainierte Pferde zeigten Werte außerhalb des Referenzbereichs, so hatten z.B. 2 von 4 Pferden mit Symptomen eines Kreuzschlags eine Konzentration von 50 nmol/kg.

Weiters wurde der Zusammenhang zwischen der Ausscheidung der 11,17-DOA mit der gefragten Leistung von 5 auf einem Laufband trainierten Trabern untersucht. Die mediane 11,17-DOA-Konzentration stieg allmählich von 39 nmol/kg, beim Anfang des Trainingsprogramms, bis zu einem Maximum von 146 nmol/kg, vor der dritten Trainingseinheit, an. Besonders ausgeprägt war dies bei einem sehr ängstlichen Pferd, das vor der 3.Trainingseinheit 1.478 nmol 11,17-DOA/kg Kot hatte.

Die medianen 11,17-DOA-Konzentrationen am Tag nach dem 1., 2., 3. und 4. Training lagen bei 33, 55, 65 and 115 nmol/kg. Die Medianwerte vor 4 Standardleistungstests (SLT) waren jeweils 26, 43, 216 und 43 nmol/kg. Die medianen 11,17-DOA Spiegel nach den SLTs waren 63, 281, 197 and 81 nmol/kg.

Nach der Arbeit waren die Plasmakortisolspiegel erhöht, nach 18 Stunden war der zirkadiane Rhythmus wieder ausgeprägt. Die Art der Belastung und die Zeit in Relation zur Belastung hatten keine signifikanten Effekte auf die Plasmakortisolkonzentrationen. Diese war vor den standardisierten Leistungstests signifikant höher als vor den Trainingseinheiten.

Der angestiegene basale fäkale Kortisolmetaboliten-Spiegel vor der Belastung weist auf eine erhöhte Aktivität der Hypothalamus-Hypophysen-Nebennierenrinde-Achse

während des ganzen Versuches hin. Das heißt, dass das Trainieren von Pferden und das Ausführen eines SLTs Stress indizieren. Im weiteren Verlauf des Versuches hatten sich die Pferde jedoch an diese Prozedur gewöhnt und zeigten nur noch eine geringgradig erhöhte 11,17-DOA Ausscheidung.

Introduction

Welfare and stress are terms associated with animal protection. Although animal protectionists mainly involve themselves in food production animals, the welfare of equines, especially those used in horse sports, is sometimes questioned by these persons. In order to keep discussions with protectionists objective, there is a need to develop unbiased parameters of welfare and stress. Plasma cortisol levels in this respect are not very helpful, since sound interpretation is hindered by effects difficult to control. First, there is a circadian pattern in horses (IRVINE and ALEXANDER, 1994). In addition, frequent blood sampling itself may result in increased cortisol levels.

Alternatively, the quantification of the faecal concentration of 11,17-dioxoandrostanes (11,17-DOA), a group of cortisol metabolites, may be used as a parameter of adrenocortical activity (PALME and MÖSTL, 1997). The method has been proven to be useful in measuring effects of stressors on livestock (PALME et al., 1999, 2000; PESENHOFER et al., 2006; LEXEN et al., 2008), game animals and horses (MÖSTL et al., 1999; MERL et al., 2000; DEHNHARD et al., 2001; GORGASSER et al., 2007).

Since faecal 11,17-DOA output is not continuous and since mixing of gut contents takes place, a certain summation and averaging effect may be expected. This may smooth the effects of the circadian cortisol production rhythms.

The aim of this study was to find out if treadmill training and racing cause stress that is detected by monitoring the faecal excretion of 11,17-DOA. Data was convenience data gathered during the conduct of a main study on amino acid kinetics (VAN DEN HOVEN et al., 2009). The intent was not to produce definitive results, but to gain insight into the patterns of response. For reference values of faecal 11,17-DOA excretion in working standardbreds, the 95 % confidence interval (95 % CI) was calculated from samples of 18 normal standardbreds in training at a nearby race track.

Material and methods

Calculation of confidence intervals

Horses

Faecal samples were collected from 22 standardbred trotters that were regularly exercised at a race track (Krieau, Vienna, Austria). On the day of collecting the faecal samples, blood samples from all horses were taken too, to detect presence of training induced muscle damage, since standardbreds are prone to develop recurrent exertional rhabdomyolysis (RER). The blood samples were analysed for CK and AST activities. Based on a CK activity of 800 IU/L the population (HARRIS et al., 1998) was divided in 18 healthy horses and 4 cases of RER. Data from the latter were excluded for calculation of the reference range.

Faecal 11,17-dioxoandrostanes levels during treadmill training and racingstandard exercise tests

Horses

5 trotters, 1 mare and 4 males (Tab. 2) in full training status were purchased from a trainer. When not at work and when weather conditions were good, the horses were kept out on 2 paddocks. At night they were individually housed in boxes on straw and with free access to water. All horses were submitted to muscle biopsy sampling prior to and after the standard exercise tests (SETs) for another study.

Diets and feeding regime

The horses were fed ad libitum with grass hay and with oats (between 1 and 4 kg /day) in the morning (7 a.m.) and evening (4 p.m.), in amounts meeting the individual needs of the horse to maintain body mass, or at least keep fluctuations within a 10 kg range. Horses were supplemented with 90 g mineral supplement/day ("Sport-Mineral", Lexa Tierernährung Xaver Scheule GmbH, Kirchhelm, Germany). Estimated daily intake of digestible energy was between 230-294 kJ/kg body mass. Estimated digestible crude protein intake was between 1.0 and 1.5 grams/kg.

Experimental design

The experimental part of the study was approved by the Ethical Committee for Animal Experiments (protocol no. 68.205/142-BrGT/2004). To avoid training induced effects, horses in full training had been purchased from a trainer.

Training

The horses were exercised from Monday to Friday. On Monday, Wednesday and Friday horses were exercised on a treadmill (Mustang 2200, Kagra AG, Furthwangen, Switzerland) and on the other days they were either walked for 1 h or ridden for 30 minutes. Treadmill training consisted of slow training (warm-up: 500 m at 2 m/s, slow trot for 7,000 m at 5 m/s, cool down: 500 m at 2 m/s) twice weekly and speed training once per week (warm-up: 500 m at 2 m/s and 2,000 m 5 m/s; 2,000 m at 8-9 m/s, followed by 500 m at 2 m/s, then 2,000 m at 10-11.5 m/s, cooling-down over 1,000 m at 5 m/s and finally 5 min of walking).

Standard exercise tests

After a 2-week period of training on the treadmill, the horses were submitted to a SET twice within 1 week (Monday and Friday). This was repeated after 3 weeks. The SET shared the warm-up protocol of the training, but the 2 bouts of speed were replaced by one incremental exercise test, which started with 500 m trot at 5 m/s on a 3 % slope, followed by 3 min trotting at 7 m/s; thereafter speed increased every minute with 1 m/s, until the horses had reached a pulse rates > 200 beats per minute. This speed was maintained until the horse showed signs of exhaustion. At this point the treadmill was stopped within 30 s and

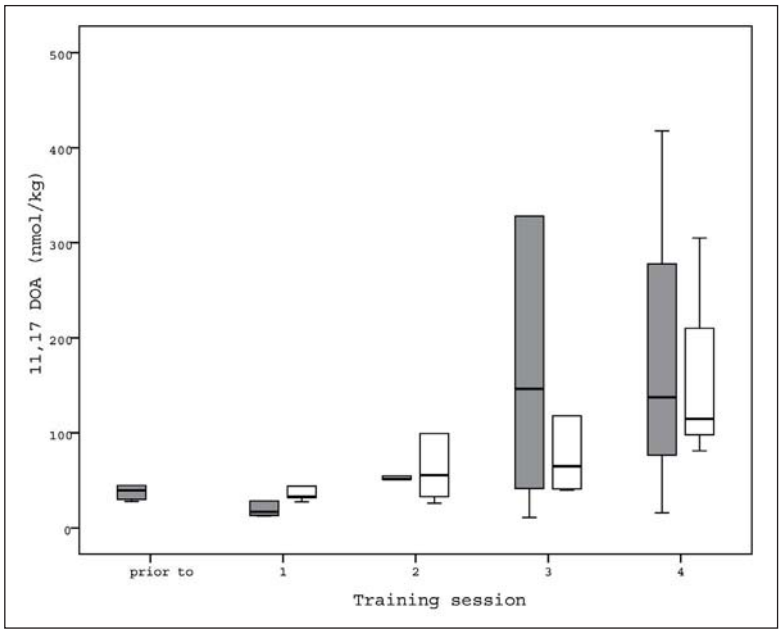

Fig.1: Box plots of faecal 11,17 DOA concentrations of 5 standardbreds at the start of the training program (prior to), and immediately before (black grey box) and after (white box) each of 4 training sessions

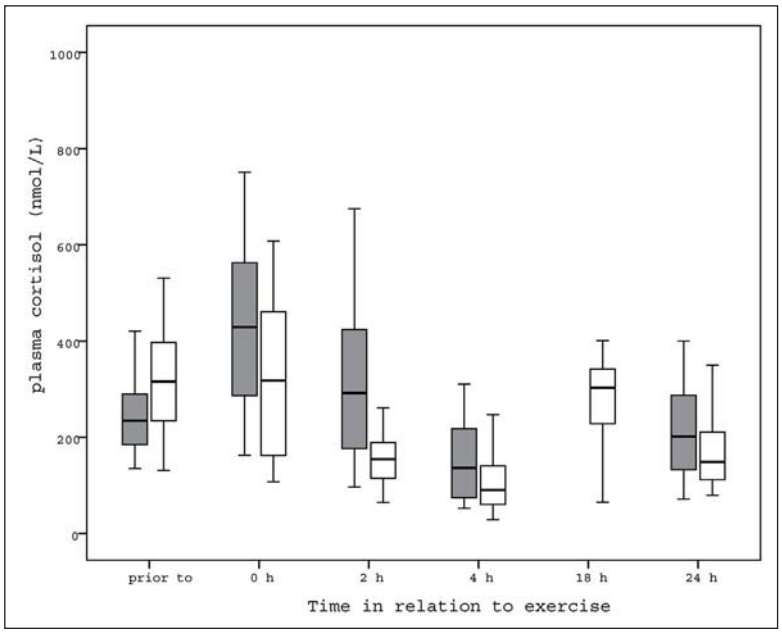

Fig.2: Box plots of plasma cortisol levels before and after training (black grey box) and SET sessions (white box); at 18 h after training no samples could be collected.

muscle biopsies were taken immediately. Afterwards horses were walked until pulse and respiration were normal and the sweat had dried up. Pulse rates were monitored with Polar® Equine heart rate monitors (Polar Electro, Oy, Finnland).

Blood samples

9 ml of blood were collected from the jugular vein into EDTA tubes. Blood was sampled immediately prior to exercise (-1), immediately after the end of exercise (0) and 1.5, 2, 4, 18 and 24 hours after exercise. Blood was centrifuged within 45 minutes and plasma was separated. About 2-3 mL of plasma was frozen at -20 °C until cortisol analysis.

Faecal samples

The morning of the day on which the horses were exercised on the treadmill (both during training and SET) and 18-24 hours afterwards, fresh faecal samples were collected rectally. Samples were stored at -20 °C till analysis.

Steroid analyses

Blood cortisol and faecal 11,17-DOA were determined according to the methods described by MÖSTL et al. (1999) and MERL et al. (2000). Quantification of 11,17-DOA was performed with an 11-oxoaetiocholanolone EIA as described by PALME and MÖSTL (1997).

Statistics

The 95 % CIs were calculated with descriptive statistic options. Plasma cortisol data needed to be normalised by log transformation and was subsequently analysed with a mixed linear model (SPSS 14.5, SPSS Inc., Chicago, Illinois), taking each sample as repeated within-subject factor, exercise type and time in relation to exercise were taken as fixed factors and the individual was taken as random factor. Faecal 11,17-DOA data were also analysed with the mixed linear model. Furthermore, the first order autoregressive option was used in the analysis of effects. All lev-

Tab. 1: Post exercise CK and AST activities in IU/L

	Median	Minimum	Maximum
CK healthy	74	42	113
CK RER	4,535	851	7,421
AST healthy	176	129	428
AST RER	890	433	3,400

Tab. 2: Age, body mass, gender and character of experimental horses

Horse no.	Age (year)	Body mass (kg)	Gender	Character
1	5	429	mare	cooperative, but easy to excite
2	6	407	gelding	cooperative, but very nervous
3	5	427	gelding	cooperative, mildly nervous
4	2	405	stallion	calm and cooperative
5	2	392	stallion	calm and cooperative

Tab. 3: Median, minimal and maximal concentrations of 11,17-dioxoandrostanes (11,17-DOA; nmol/kg) in faeces of 5 standardbred trotters collected prior to and 1 day after each of 4 standard exercise test (SET)

SET no.	11,17-DOA before (min-max)	11,17-DOA after (min-max)
1	26 (10-58)	63 (26-90)
2	43 (37-532)	281 (65-4,085)
3	216 (13-1,602)	197 (20-1,462)
4	43 (0-519)	81 (55-866)

els of significance were set at $p \leq 0.05$. Differences were tested with Bonferroni test.

Results

Muscle enzyme activity

The post exercise CK and AST activities are given in Tab. 1. Based on the a priori defined activities of CK (HARRIS et al., 1998), it appeared that 4 horses at the race track showed evidence of RER. Their data was excluded for the calculation of the reference range.

Confidence interval of faecal 11,17-DOA

The 95 % CI of 11,17-DOA levels for the 18 healthy horses on the race track was 14-31 nmol/kg. 4 horses suffered RER, but only 2 had a faecal 11,17-DOA concentration of 50 nmol/kg. CK activity of these 2 horses was 5,900 and 3,171 IU/L. The 11,17-DOA levels of the other 2 RER cases were below 31 nmol/kg. CK activities of these horses were 850 and 7,400 IU/L. Remarkably, faecal 11,17-DOA levels of 2 healthy horses were clearly over the upper level value (51 and 73 nmol/kg, respectively).

Treadmill study (training and SET)

Faecal 11,17-DOA

Box plots of faecal 11,17-DOA concentrations prior to and 24 h after training are shown in Fig 1. Median base line concentrations were 39 nmol/kg (min-max: 28-71 nmol/kg) at the start of the experiment and 17 nmol/kg (min-max: 12-71 nmol/kg) prior to the first training. Median levels increased to 51 nmol/kg (min-max: 29-332 nmol/kg) prior to the 2nd training session and further to 146 nmol/kg (min-max: 11-1478 nmol/kg) prior to the 3rd training session, indicating prolonged or increased adrenal stimulation. This was especially pronounced in one nervous horse (no. 2), showing a faecal 11,17-DOA concentration of 1,478 nmol/kg at that moment. However, the animal eventually got used to all procedures and handling and showed lower pre-exercise 11,17-DOA levels on later occasions. The median 11,17-DOA of the horses prior to the 4th training was 137 nmol/kg (min-max: 16-418 nmol/kg). The day after training, median 11,17-DOA levels were 32 nmol/kg (min-max: 27-44 nmol/kg), 55 nmol/kg (min-max: 25-752 nmol/kg), 64 nmol/kg (min-max: 40-724 nmol/kg), and 115 nmol/kg (min-max: 81-305 nmol/kg) for the 1st,2nd,3rd and 4th training, respectively.

All median 11,17-DOA levels prior to and after the SETs are given in Tab. 3. Prior to the 3rd SET, median 11,17-DOA were higher than those before the 4th training session. Median faecal 11,17-faecal DOA levels were increased on the day after a SET, especially after the second SET. However, the variation was very large. Horse no. 2 excreted the highest amounts of 11,17-DOA (4,085 nmol/kg).

Performing a SET significantly (p=0.001) increased faecal 11,17-DOA excretion over the following 24-hour period. Furthermore, it appeared that in the period in which SETs 2 and 3 were performed, significantly (p=0.003; p=0.018) more 11,17-DOA were excreted by the horses than in the period before and the day after SET 1.

Plasma cortisol

Plasma cortisol levels were not normally distributed. Prior to both forms of exercise the levels were less than 36 nmol/L in 75 % of the horses, and less than 54 nmol/L in all, except horse no. 5. This animal showed a level of 86 nmol/L before being submitted for its 3rd SET and 77 nmol/L before it performed its 4th SET. Box plots of plasma cortisol levels before and after training and SETs are given in Fig. 2. Plasma cortisol levels increased after exercise, but, at least for the SETs, after 18 hrs it appeared that baseline levels were restored. At 18 hrs after training, no blood samples were collected, since this sampling point was at night and it was preferred not to disturb the horses. The time in relation to exercise had significant effects on the log-normalised plasma cortisol concentrations (p= 0.001). Highest mean levels were seen immediately after end of training and 1.5 h after end of SET.

The highest post exercise plasma concentration and the concentration of 11,17-DOA in a faecal sample collected 24 hours after the SET or training were not significantly correlated (r = 0.156, p=0.182).

Discussion

The welfare of an individual can be defined as the degree of how successfully it can cope with its environment (BROOM, 1986). A failure to cope often implies decreased fitness and hence stress. Stress is often associated with a delirious effect on an individual (BROOM and JOHNSSON, 1993), but this oversimplifies the complex phenomenon of stress and obscures its physiological role in survival and exercise. It is mainly the duration of a stressful event that determines the outcome. In short-term stress, glucocorticoids improve fitness by energy mobilisation (RAYNAERT et al., 1976) and may change behaviour (KORTE et al. 1993). In contrast, severe chronic stress decreases individual fitness by immunosuppression and atrophy of tissues (MUNCK et al., 1984). Exercise and training can be expected to represent a short-term stress for the horse. Regarding horse training, it should be realized that adverse behaviour might even be related to stress (McBRIDE and CUDDELFORD, 2001). Only a few studies have specifically addressed the issue of endocrine responses caused by training in horses (HYYPPÄ, 2005). One aspect of exercise is that the hypothalamic-pituitary-adrenal axis is strongly stimulated, which increases the circulating levels of cortisol (HYYPPÄ, 2005). The maximum plasma cortisol concentration can be observed between 5 and 30 min after finishing of short high-intensity exercise (JIMENEZ et al., 1998; NAGATA et al., 1999). These physiological exercise-induced changes hinder the monitoring of stress. Thus, alternative stress indicators must be used to detect and (semi)quantify stress caused by exercise.

In previous studies with castrated horses, colic cases and trained young quarter horses, faecal 11,17-DOA appeared to be a good marker of disturbance (MERL et al., 2000; GORGASSER et al., 2007). In a disorder like RER, anxiety or pain or even a forced resting day could trigger those factors that induce increased adrenal activity, and hence increased faecal 11,17-DOA concentrations. However, we observed inconsistent and lower than expected results in those cases. There was no relation between 11,17-DOA levels and CK activity. Nevertheless, we could not exclude that stress may have existed when RER developed in some cases.

For healthy standardbreds trained at a race track, faecal 11,17-DOA levels based on our results are between 14-31 nmol/kg in the morning of the day after exercise. It should be realised however, that baseline 11,17-DOA levels are affected by the horse's training status.

GORGASSER et al. (2007) found a median 11,17-DOA level of 22 nmol/kg in quarter horses in their first month of training, but individual levels varied from 4 to 66 nmol/kg faeces. MERL et al. (2000) reported median 11,17-DOA resting concentration of about 11 nmol/kg in healthy army horses. The same horses had median 11,17-DOA levels of 50 nmol/kg at the second day after they had been castrated. In horses suffering from colic, 11,17-DOA levels were even in excess of 33 nmol/kg.

These findings support our choice of an upper level of 30 nmol/kg for 11,17-DOA. Any faecal 11,17-DOA concentration above 30 nmol/kg is considered an indication of increased hypothalamus pituitary adrenal axis activity. Applied to the 11,17-DOA concentrations found in our 5 standardbreds, it follows that on an average horses were stressed by SET numbers 2, 3 and 4. Yet, the large variation suggests that different stress levels are experienced by horses. Some, however, did not appear to be stressed at all by the training or SET procedures.

Due to a half-life of about 2 hours (SLONE et al., 1983) and by the feed back mechanisms on the pituitary gland, post exercise cortisol levels can be expected to have returned to baseline values within a few hours after the end of exercise. Plasma cortisol after exercise was elevated on most occasions. However, plasma levels prior to exercise were increased already in some horses on some days, which may be interpreted as an indication of pre-exercise stress. This pre-exercise elevated cortisol may have affected post exercise levels too.

The plasma cortisol concentrations prior to and after exercise found by us appeared lower than those published by other authors. Compared to relatively newly published data, however, they were just slightly over the resting levels that could be reconstructed from data published by STULL and RODIEK (2000). A different cortisol assay is likely the cause for this (PALME et al., 1999).

Based on our findings we concluded that all training and handling of the horses, as well as subjecting them to SET resulted in frequent episodes of disturbance that could be interpreted as stress. As a physiological mechanism, stress per se is not inherently bad (MOBERG, 2000). However, if the episodes are too long, severe chronic stress may occur, which decrease individual fitness (MUNCK et al., 1984). As the trial advanced, horses became accustomed to all procedures and less faecal cortisol metabolites were excreted. Judged by the height of excretion of cortisol metabolites, more intense but short lasting stress was present when SETs were performed. Whether this falls within a normal biological response, or must be seen as

abnormal and potentially undermining the horse's welfare, has to be studied further.

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