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Alterations of serum vitamin E and vitamin A concentrations of ponies and horses during experimentally induced obesity

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Abstract

Vitamin A, vitamin E and retinol-binding protein 4 (RBP4) are a focus of current obesity research in humans. The impact of body weight (BW) gain on fat-soluble vitamins and its associated parameters in equines has not been previously reported. Ten Shetland ponies and 9 Warmblood horses, all adult geldings, non-obese and healthy, were fed an excessive energy diet for 20 months to induce BW gain. Serum α-tocopherol (vitamin E), retinol (vitamin A), retinol-binding protein 4 (RBP4) and retinol/RBP4 ratio were analysed before BW gain induction and at six timepoints during the BW gaining period. The mean (±*SD*) % BW gain achieved during two years of excess energy intake was 29.9 ± 19.4% for ponies and 17 ± 6.74% for horses. Serum α-tocopherol increased significantly in ponies and horses during excess energy intake and circulating α-tocopherol levels correlated positively with α-tocopherol intake (r = .6; p < .001). Serum retinol concentrations showed variations during the study but without relation to intake. Serum RBP4 decreased at the end of the study. The retinol/RBP4 ratio increased with BW gain without differences between ponies and horses. In comparison with human research, the increase in the retinol/RBP4 ratio was unexpected and needs further elucidation.

KEYWORDS

body weight gain, equine, laminitis, retinol-binding protein 4, α-tocopherol

1 | INTRODUCTION

Obesity is an expanding health issue worldwide in humans and companion animals such as equines. It is known that obesity is associated with chronic inflammation and increased oxidative stress in humans (Cartier et al., 2008; D'Archivio et al., 2012). Oxidative stress is suspected to be partly responsible for some of the detrimental health consequences associated with human obesity such as atherosclerosis (Tibaut & Petrovič, 2016). Imbalances between the production of reactive oxygen species and antioxidant defences define oxidative stress (Pisoschi & Pop, 2015). Humans have several mechanisms to combat oxidative stress, namely production of endogenous antioxidants (e.g. uric acid, superoxide dismutase) and consumption of exogenous antioxidants (e.g. vitamin E, vitamin C; Pisoschi & Pop, 2015). Previous studies of oxidative stress in equine obesity and its consequences have revealed equivocal results. Holbrook, Tipton, and McFarlane (2012) found no changes in oxidative stress in obese horses exhibiting hyperinsulinaemia relative to non-obese horses with physiological plasma insulin concentrations. However, other studies reported reduced

antioxidative capacity in obese horses (Pleasant, Suagee, Thatcher, Elvinger, & Geor, 2013). Different sizes of the cohorts and different investigated markers may have caused these discrepancies. Vitamin E is a lipid-soluble compound that has different natural occurring isomers (α-, β-, γ- and δ-tocopherol) which protect cell membranes from damage caused by lipid peroxidation due to oxidative stress (Azzi et al., 2000).

Vitamin A (retinol) is the principal component of the endogenous synthesis of biologically active retinoids with essential functions that include cell differentiation, glucose and fatty acid metabolism and immune functions (Mody, 2017). Retinol is transported in the blood by retinol-binding protein 4 (RBP4), which is primarily synthesized in the liver, but also is described as an adipokine, which are cell-signalling proteins secreted by adipose tissue (Tamori, Sakaue, & Kasuga, 2006). Equivocal results were reported regarding the relationship between obesity and circulating RBP4 levels in humans and horses (Lewis, Shand, Frampton, & Elder, 2007; Liu, Wang, Li, Sun, & Xia, 2014; Selim et al., 2015; Ungru et al., 2012). It has been recommended in humans that the blood retinol/RBP4 ratio should be used for more physiologically relevant results, as RBP4 release is strongly influenced by retinol status (Mills, Furr, & Tanumihardjo, 2008). In human obesity, the retinol/RBP4 ratio was reported to be decreased in obese humans relative to non-obese age- and sex-matched controls (Mills et al., 2008). To the best of our knowledge, this ratio has not been determined in equines in previous studies of obesity.

We hypothesized that serum α-tocopherol levels would decrease with increasing BW due to higher demands for antioxidants in equine animals. We also expected the retinol/RB4 ratio to decrease with increasing BW in both horses and ponies.

2 | MATERIALS AND METHODS

2.1 | Animals

Ten Shetland ponies (geldings; mean (±*SD*) age 6 ± 3 years) and 9 Warmblood horses (geldings; mean (±*SD*) age 10 ± 3 years) owned by the Institute of Animal Nutrition, Nutrition Diseases and Dietetics of the Leipzig University were included in the study. The animals were bedded on straw in individual box stalls and had access to a dry lot for approximately 5 hr a day. The adaptation period of the animals lasted for at least 2 weeks. The Ethics Committee for Animal Rights Protection of the Leipzig District Government (No. TVV 32/15) approved the project in accordance with German legislation for animal rights and welfare. Animals were cared for according to the guidelines for the accommodation and care of animals used for experimental and other scientific purposes (2007/526/EC).

2.2 | Study design

Before the study was initiated, the ponies and horses had been fed meadow hay ad libitum for at least three weeks. Water and sodium chloride (in the form of salt blocks) were provided ad libitum throughout the study. The animals were fed individually, and the daily time budget for feed intake was approximately 19 hr. Basal blood samples were obtained in October 2015 (t0). Details about blood collection procedure are described below. The experimental ration, designed to induce obesity (see below), was then initiated. Blood samples were collected in April 2016 (t1), July 2016 (t2), October 2016 (t3), April 2017 (t4), July 2017 (t5) and December 2017 (t6).

TABLE 1 Calculated energy content and crude nutrients in the concentrates (as labelled by the manufacturer) and in hay (mean ± *SD* out of 20 analysed hay samples)

Variables	Concentrate 1	Concentrate 2	Hay
Dry matter	90	91	89.7 ± 1.62
Crude ash (% of DM)	7.9	6.7	5.37 ± 1.4
Crude fat (% of DM)	14.4	16.6	1.63 ± 0.48
Crude protein (% of DM)	13.3	14	8.34 ± 1.69
Crude fibre (% of DM)	10.4	7.5	34 ± 3.19
Calculated energy (MJ ME/kg DM)	14.7	16.3	6.94 ± 0.62

Abbreviations: DM, dry matter; ME, metabolizable energy; MJ, mega joule.

Following t0, the animals were gradually adapted to a ration which provided >150% of their ME maintenance requirements (ponies: MJ ME = 0.4 × $BW^{0.75}$; horses: MJ ME = 0.52 × $BW^{0.75}$) according to Flachowsky et al. (2014). The designated ration was attained after three months by gradually increasing the energy supply above maintenance requirement by increasing the respective concentrate. Finally, 60% of the energy intake was provided by meadow hay, and 40% was covered by a commercial grain-based concentrate. Due to problems according to the fat binding of the pelleted concentrate 1 (composition: wheat, flaxseed, barley, soya bean hulls, oat hulls bran, soya bean oil, molasses, soya bean meal and a vitamin–mineral mixture), there was an exchange to a muesli at t2 (concentrate 2: linseed, wheat flakes, malt, puffed corn, linseed cake, alfalfa, wheat bran, soya bean oil, corn flakes, cane molasses, sunflower seed extracted, wheat, chicory pulp, barley puffed, corn germ oil and a vitamin–mineral mixture) with moderate differences in nutrient composition (Table 1). Estimated energy content of the hay was calculated based on monthly nutrient analyses, by using the Weende system (Naumann & Bassler, 1999), according to the following equation: ME (MJ/kg dry matter) = −3.54 + (0.0129 crude protein + 0.0420 crude fat − 0.0019 crude fibre + 0.0185 nitrogen-free extractives; Flachowsky et al., 2014). As fat content in the concentrates exceeded > 8%, the energy content of the concentrate was estimated using the following equation: ME (MJ/kg dry matter) = (0.01192 crude protein + 0.04228 crude fat + 0.00793 crude fibre + 0.01676

nitrogen-free extractives) × estimated digestibility. Estimation of digestibility was performed according to the crude fibre content in % of dry matter (digestibility of organic matter [%] = 97.0 – 1.26 *x*, where *x* represents the crude fibre content in % of dry matter; Kamphues et al., 2014). Labelled nutrients of the concentrates were used for the estimations of the energy contents. See Table 1 for mean nutrient composition of the hay and the labelled nutrients in concentrate 1 and 2. Concentration of α-tocopherol, but not retinol of the two concentrates was different. Table 2 provides the nutrient composition of the ration and intake of α-tocopherol and retinol over the course of the study. BW, body condition score (BCS) and cresty neck score (CNS) were obtained weekly. Energy intake from the hay and concentrates was adapted monthly, based on the current BW (Table 3). Animals showed no feed refusals regarding the concentrate. Ponies showed furthermore no significant refusals of hay. In contrast, horses had mean hay refusals of 1.51 ± 0.43 kg during the experimental period.

TABLE 2 Estimated nutrient intake of ponies and horses based on calculated ration composition during two years of excess energy feeding

Variables	Ponies	Horses
Feed intake (kg DM/100 kg BW)		
Meadow hay	1.95 ± 0.16	1.53 ± 0.13
Concentrate	0.54 ± 0.08	0.48 ± 0.07
Nutrient content (% of DM)		
Crude fat	4.42 ± 0.42	4.70 ± 0.41
Crude protein	9.07 ± 1.85	9.20 ± 1.80
Crude fibre	29.1 ± 2.62	28.5 ± 2.50
Starch	7.45 ± 0.11	8.20 ± 0.12
Sugar	9.91 ± 1.07	9.45 ± 0.77
All-rac alpha tocopheryl acetate (IU/kg FM)		
Concentrate 1	280	
Concentrate 2	475	
Vitamin A (IU/kg FM)		
Concentrate 1	12,500	
Concentrate 2	12,500	
Intake of α-tocopherol (x-fold maintenance requirements)		
Concentrate 1	1.1 ± 0.2 (0.57 ± 0.1)	1.5 ± 0.3 (0.73 ± 0.13)
Concentrate 2	2.1 ± 0.04 (1.03 ± 0.02)	2.7 ± 0.1 (1.34 ± 0.04)
Intake of retinol (x-fold maintenance requirements)		
Concentrate 1 and 2	1.7 ± 0.2	2.3 ± 0.3

Abbreviations:: BW, body weight; DM, dry matter; ME, metabolizable energy; maintenance requirement of α-tocopherol: 5 IU/kg $BW^{0.75}$; maintenance requirement of retinol: 150 IU/kg $BW^{0.75}$ (Flachowsky et al., 2014), in brackets: Maintenance requirement of 10 IU/kg $BW^{0.75}$ for α-tocopherol for diets high in fat. The α-tocopherol and retinol concentrations in energy concentrates 1 and 2 are based on the manufacturer labelling. Values are presented as means (±*SD*).

2.3 | Blood sampling

A catheter (Braunuele MT® Luer Lock, B. Braun) was aseptically placed into the jugular vein after 8 hr of fasting. Blood samples were collected in tubes containing coagulation activator (Monovette, Sarstedt AG). After 30 min of clotting at room temperature, the samples were centrifuged at 865 *g* for 10 min and serum was removed for assessments of serum insulin, triglycerides, α-tocopherol, retinol and RBP4. Serum samples were gradually frozen from −20 to −80°C and stored until further analysis.

2.4 | Determination of BW, BCS and CNS

BW was obtained weekly using an electronic scale system for large animals (scale system: Iconix FX 1, Texas Trading, scale precision: 0.5 kg). BCS (Carroll & Huntington, 1988) and CNS (Carter, Geor, Staniar, Cubitt, & Harris, 2009) were assessed weekly by two evaluators (CS and DB) on a scale of 0–5 points, with 0 = very poor and 5 = very fat body condition. The mean of the evaluators' scores was calculated and used for the statistical analyses.

2.5 | Analysis of blood samples

Serum insulin levels were analysed using an immunoradiometric assay (IRMA, 125I, Demeditec Diagnostics GmbH). Triglycerides were analysed using an automated chemistry analyser (Roche Cobas C311, Roche Diagnostic GmbH).

Serum vitamin A (retinol) and vitamin E (α-tocopherol) were analysed using a gradient reverse-phase HPLC-system (Waters) as previously described (Kuhl et al., 2012). Briefly, serum samples were extracted twice with n-hexane, and the n-hexane layer was then evaporated under nitrogen (37°C) and reconstituted in mobile phase (isopropanol). Separation was performed using a C30 column (5 µm, 250 × 3.0 mm; YMC) and a photodiode array detector (Model 996; Waters). Retinol and α-tocopherol were quantified by comparison of retention time as well as peak areas with external standards by measuring the absorption at 325 nm for retinol and 290 nm for α-tocopherol. Accuracy and precision of the analyses were verified using standard reference material (SMR 968 a fat-soluble vitamins in human serum; National Institute of Standards and Technology, Gaithersburg, USA). The detection limit for α-tocopherol was 0.1 µg/ml and for retinol 0.004 µg/L. The coefficient of variability was <4% for all compounds.

Serum samples for RBP4 analysis were separated by 12% SDS-PAGE, using the buffer system of Laemmli (1970). After SDS-PAGE separation, the proteins were transferred from the gel onto a polyvinylidene difluoride membrane (Merck KGaA), blocked with 5% milk powder in buffer solution and incubated with cross-reacting rabbit anti-human RBP4 (Dako GmbH). After washing in buffer solution, the membranes were incubated with a secondary peroxidase-labelled polymer conjugated to goat anti-rabbit IgG (EnVision K4003;

TABLE 3 BW, intake of concentrate and hay in ponies and horses during two years of excess energy feeding

	Body weight (kg)		Intake of concentrate[a] (kg)		Intake of hay[b] (kg)	
Timepoint	Ponies	Horses	Ponies	Horses	Ponies	Horses
t0	118 ± 27	602 ± 42			ad libitum	ad libitum
t1	120 ± 27	615 ± 33	0.6 ± 0.1	2.64 ± 0.11	3.14 ± 0.5	12.4 ± 0.59
t2	132 ± 29	659 ± 35	0.96 ± 0.15	4.19 ± 0.19	2.71 ± 0.43	10.3 ± 0.55
t3	145 ± 30	700 ± 41	0.9 ± 0.14	3.83 ± 0.17	3.24 ± 0.49	12.4 ± 0.61
t4	148 ± 32	696 ± 45	0.92 ± 0.14	3.85 ± 0.17	3.13 ± 0.49	11.6 ± 0.58
t5	150 ± 32	701 ± 41	0.92 ± 0.14	3.86 ± 0.18	3.68 ± 0.55	13.7 ± 0.71
t6	151 ± 29	702 ± 42	0.93 ± 0.13	3.85 ± 0.16	2.85 ± 1.03	11.8 ± 0.61

Note: Values are presented as means (±*SD*).

[a]No feed refusals were monitored.

[b]Data considered feed refusals

Dako GmbH) for 1 hr. Antibody binding was visualized using the Chemiluminescence Blotting Substrate (Roche Diagnostics GmbH) according to the manufacturer's instructions. Band intensity of RBP was read with an imager (Bio-Rad) and analysed with the Bio-Rad Discovery software 1.1.

2.6 | Statistics

The data were analysed using a statistical software program (STATISTICA, version 12, StatSoft GmbH). The data were analysed for normal distributions using Shapiro–Wilk's test. ANOVA with repeated measurements was performed to analyse BW gain, serum insulin, α-tocopherol, retinol and RBP4 concentrations. Fisher's least significant difference test was applied as post hoc test. The retinol/RBP4 ratio was calculated and analysed by ANOVA with repeated measurements as well. Friedman's ANOVA was used to analyse the time effect on BCS and CNS. When significant differences were observed, the Wilcoxon signed rank test with Bonferroni's correction was performed as a post hoc test. The effects of the breed on non-parametric data were analysed using the Mann–Whitney *U* test. Correlations among variables were evaluated by calculating Spearman's correlation coefficients.

3 | RESULTS

3.1 | Effects on BW, adiposity scores and serum insulin concentrations

The mean (±*SD*) % BW gain recorded during two years of excess energy intake was 29.9 ± 19.4% for ponies ($p < .001$) and 17 ± 6.74% for horses ($p < .001$). Median BCS (25th/75th percentiles) increased ($p = .02$) from t0 to t6 significantly in both ponies (t0: 2.3 (1.2/3.4); t6: 3.9 (3.7/4.2)) and horses (t0: 2.7 (2.1/3.2); t6: 3.8 (3.7/3.9)). Ponies and horses showed an increase ($p = .02$) of median CNS (25th/75th percentiles) from t0 (ponies: 2.5 (0.8/3); horses: 2 (1.8/2.3)) to t6 (ponies: 3.5 (3.3/4.0); horses: 3.5 (3.5/4.0)). No significant differences in per cent BW gain, BCS and CNS were observed between ponies and horses. Mean (±*SD*) fasting serum insulin concentrations increased in ponies ($p = .009$) and horses ($p = .02$) from t0 (ponies: 4.26 ± 1.36 μU/ml; horses: 6.32 ± 2.35 μU/ml) to t6 (ponies: 13.9 ± 14.9 μU/ml; horses: 15.1 ± 10.3 μU/ml). One pony and one horse developed laminitis between t5 and t6. Both equines required medication for pain relief. Samples from the two affected equines at t6 were collected when they no longer had signs of lameness and at least 7 days after the last pain medication.

3.2 | Serum α-tocopherol concentrations

Figure 1a illustrates the significant increase in serum α-tocopherol concentrations during the BW gaining period in both ponies and horses. At t6, ponies had higher ($p = .004$) serum α-tocopherol concentrations than horses. Serum α-tocopherol concentrations were correlated with BCS and CNS in both breeds (BCS: ponies: $r = .6$; $p < .001$; horses: $r = .7$; $p < .001$; CNS: ponies: $r = .6$; $p < .001$; horses: $r = .6$; $p < .001$). No correlations were found between serum α-tocopherol and triglycerides (ponies: $r = -.1$; $p = .7$; horses: $r = .1$; $p = .8$). Serum α-tocopherol concentrations were correlated with the intake of α-tocopherol (ponies: $r = .6$; $p < .001$; horses: $r = .6$; $p < .001$).

3.3 | Serum retinol and RBP4 concentrations

Serum retinol concentrations increased from t3 to t6 in ponies ($p = .004$) and horses ($p < .001$). Horses had higher serum retinol concentrations at t0 ($p = .01$) and t6 ($p = .04$) compared to ponies (Figure 1b). The intake of vitamin A was not correlated ($p > .05$) with serum retinol concentrations in ponies or horses. No significant correlations between serum retinol and BCS or CNS were found in either ponies or horses (data not shown).

Serum RBP4 concentrations were not different between ponies and horses throughout the study. Between t0 and t2, serum RBP4

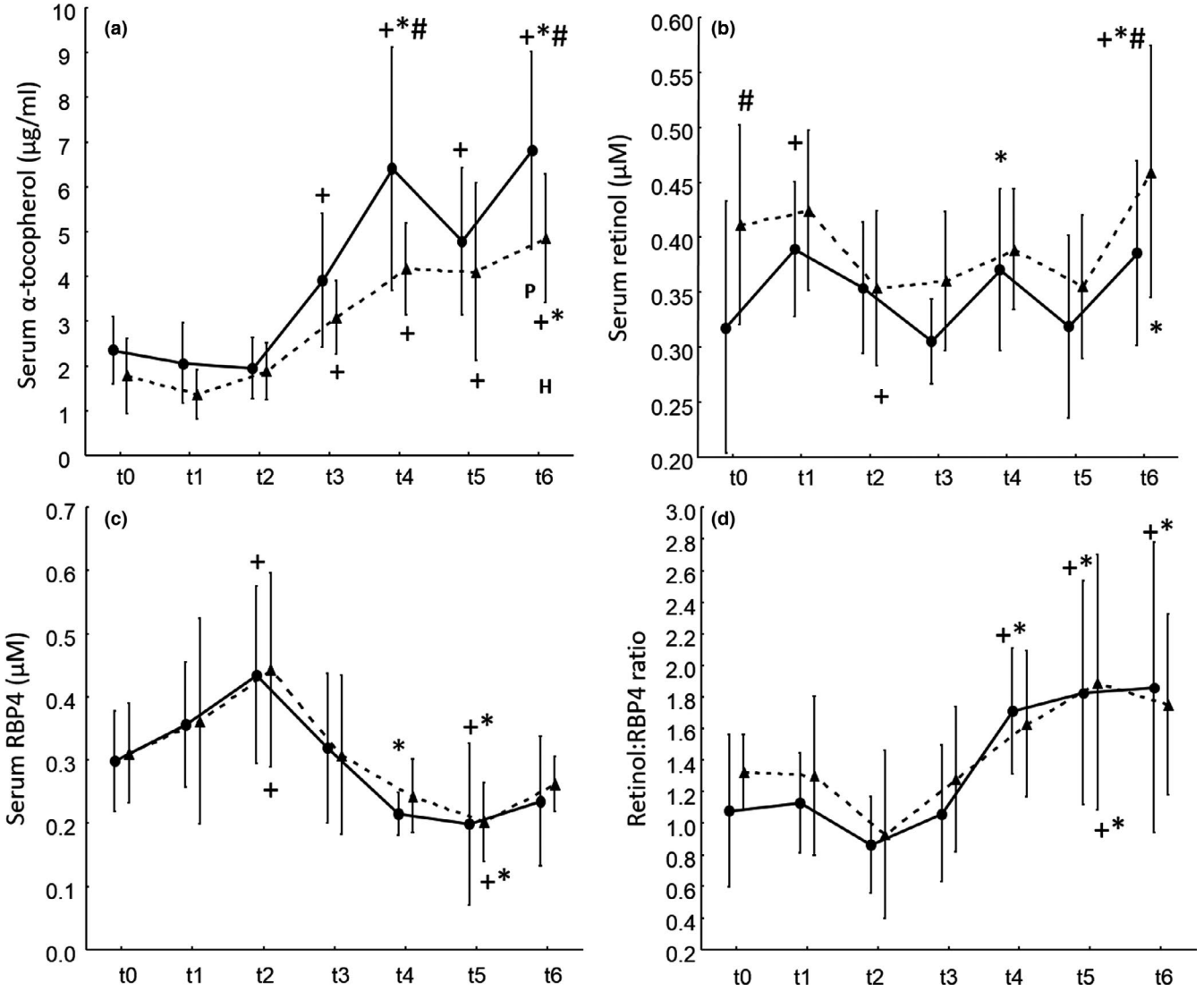

FIGURE 1 Effect of two years of 200% excess energy intake on serum α-tocopherol (a); breed: $p = .17$, time: $p < .001$, time × breed: $p = .09$; retinol (b); breed: $p = .81$, time: $p < .001$, time × breed: $p = .27$; RBP4 (c); breed: $p = .52$, time: $p < .001$, time × breed: $p = .9$; concentrations and retinol/RBP4 ratio (d); breed: $p = .46$, time: $p < .001$, time × breed: $p = .92$; in ponies (circles) and horses (triangles) at t0, t1, t2, t3, t4, t5 and t6 (reported as mean (circles or triangles) and standard deviation (whiskers), a: two laminitic equines indicated at t6 by P (pony) and H (horse); $^{+}$significantly different from t0; *significantly different from t3; + and * marked above the graphs are assigned to ponies; + and * marked below the graphs are assigned to horses; $^{\#}$significantly different between ponies and horses at the certain time point

concentrations increased in ponies and horses (ponies: $p = .006$; horses: $p = .01$). After t2, serum RBP4 concentrations decreased at t5 compared to t0 (ponies: $p = .04$; horses: $p = .04$) and t3 (ponies: $p = .01$; horses: $p = .04$; Figure 1c). Serum RBP4 concentrations had an inverse relationship with serum insulin concentrations in ponies ($r = -.4$; $p = .03$) but not in horses. Further negative correlations were detected between serum RBP4 concentrations and BCS and CNS in ponies (BCS: $r = -.4$; $p < .001$; CNS: $r = -.5$; $p < .001$) and horses (BCS: $r = -.4$; $p < .001$; CNS: $r = -0.3$; $p = .009$). No correlations between serum retinol and RBP4 concentrations were detected in either ponies or horses (data not shown).

The retinol/RBP4 ratio over time is presented in Figure 1d. No differences ($p > .05$) between ponies and horses were found. Ponies had an increase ($p < .01$) in the retinol/RBP4 ratio at t4, t5 and t6 compared to t0 and t3. In horses, the retinol/RBP4 ratio increased ($p = .02$) at t5 compared to t0 and t3. There also were positive correlations between the retinol/RBP4 ratio and BCS (ponies: $r = .6$; $p < .001$; horses: $r = .4$; $p = .002$), CNS (ponies: $r = .5$; $p < .001$; horses: $r = .3$; $p = .01$) and serum insulin concentrations (ponies: $r = .4$; $p = .01$; horses: $r = .1$; $p = .5$).

4 | DISCUSSION

As expected, the hypercaloric intake over two years induced significant increases in BW, BCS, CNS and fasting serum insulin in ponies and horses. However, the achieved BW gain was less than expected in the context of previous research by Carter, McCutcheon, et al. (2009) and Siegers, de Ruijter-Villani, van Doorn, Stout, and Roelfsema (2018) in

which the excess energy intake was provided by approximately 60% concentrate and 40% roughage. In contrast, the energy intake in the current study was provided by 40% concentrate and 60% roughage. Our feeding regimen was selected out of equine welfare considerations and it´s closer relation to practical horse feeding. The discrepancies in BW gain between the different studies might be related to the use of different energy evaluation systems and other influencing factors such as environmental temperature or voluntary exercise. A control group receiving an isocaloric diet would have been a powerful verification of our results. However, in most previous equine studies the comparison of lean (control) and obese animals was restricted to animals kept under different feeding and management conditions. To compensate the missing control group, we had a close follow-up of the equines under an identical feeding protocol. This allowed conclusions on changes in individual animals over time, as each animal served as its own control. We used t0 as a starting point at which all animals were non-obese and metabolically healthy.

Before the start of this study, the ponies and horses received no feed containing α-tocopherol other than conserved meadow hay, in which the α-tocopherol concentration was negligible according to literature (Hidiroglou, Batra, & Roy, 1994). The ponies and horses had no alterations of serum α-tocopherol concentrations from t0 to t2 while being fed energy concentrate 1. After t2, the concentrate feed was changed to energy concentrate 2, which contained roughly 1.7-fold higher concentrations of α-tocopherol than concentrate 1. Contrary to our initial hypothesis, serum α-tocopherol concentrations increased significantly in both breeds between t2 and t6. This suggests a dose–response relationship for α-tocopherol intake, which was further supported by the strong positive correlation between α-tocopherol intake and serum α-tocopherol concentrations. These results suggest that the increase in serum α-tocopherol after t2 was induced by feeding a range of 250–600 IU/day per pony (equals 2.6–3.2 mg α-tocopherol/kg BW) and 1,100–2,100 IU/day per horse (equals 2.3–2.9 mg α-tocopherol/kg BW), which was twofold to threefold the amount recommended (5 IU/kg $BW^{0.75}$; Flachowsky et al., 2014). The synthetic form of α-tocopherol (3a700 according to the regulation (EC) No 183/2003 on additives for use in animal nutrition), which was used in the present study, has been linked in humans to lower bioavailability than the natural RRR-α-tocopherol (Burton et al., 1998). Nonetheless, synthetic α-tocopherol is a well-studied feed additive in animal species, such as cattle (Horn et al., 2010). Our results are in accordance with former studies in equines. For example, Ronéus, Hakkarainen, Lindholm, and Työppönen (1986) found an almost linear dose–response relationship by supplementing different dosages of synthetic α-tocopherol over 112 days in clinically healthy Standardbreds. Kienzle, Kaden, Hoppe, and Opitz (2003) reported higher serum α-tocopherol concentrations during a high vitamin E supplementation regimen (4 mg/kg BW) compared to a moderate vitamin E intake (0.5 mg/kg BW) in adult equines. However, Pagan, Kane, and Nash (2005) did not detect any significant time effects of serum α-tocopherol concentrations after 56 days feeding of 5,000 IU synthetic α-tocopherol per day in unexercised Thoroughbreds.

The majority of previous research in humans on α-tocopherol, oxidative stress and obesity has reported negative associations between serum α-tocopherol levels and obesity (Botella-Carretero et al., 2010). However, some contradictory studies did report positive correlations of serum α-tocopherol and obesity in humans (Waniek et al., 2017). Our results also showed positive associations of serum α-tocopherol levels and obesity measurements in the ponies and horses used in this study. However, due to the strong link between serum α-tocopherol concentrations and feed intake of α-tocopherol found in the present study, the increase in serum α-tocopherol concentrations was most likely related to the increased vitamin E intake after t2 rather than obesity-associated alterations. However, A control group with lean animals but similar α-tocopherol intakes might have been a powerful verification of our results.

It is known that serum lipids strongly influence the serum concentrations of α-tocopherol in humans (Horwitt, Harvey, Dahm, & Searcy, 1972). Therefore, it is thought to be essential to evaluate serum lipids or fractions of the serum lipids to obtain an accurate measure of the α-tocopherol status. However, we did not find a correlation between serum α-tocopherol and serum triglycerides. Therefore, we assume that the increase in serum α-tocopherol in both breeds was not associated with hyperlipaemia.

As mentioned above, one pony and one horse developed laminitis at the final third of the study. Interestingly, the laminitic pony had 1.8 times lower serum concentrations of α-tocopherol compared to the mean of the pony cohort at t6. The serum concentration of α-tocopherol of the laminitic horse was 3.2 times lower compared to the mean of the horses at t6. Equine digital laminae lack superoxide dismutase, an important antioxidant, and these structures are therefore highly susceptible to oxidative damage (Loftus, Belknap, & Black, 2006). It is speculated that the decrease in serum α-tocopherol in both of our laminitic equines might have been induced by higher demands of antioxidants in the laminar tissue due to laminitis. This is an interesting finding which needs to be verified in further studies with more laminitic equines. As a limitation of the study, we did not determine markers of oxidative damage such as thiobarbituric acid-reactive substance which may have provided a more detailed insight into the very complex oxidant/antioxidant equilibrium in laminitis.

Conflicting data exist about the impact of human obesity on serum retinol concentrations. Some studies found decreased serum retinol concentrations in obese Caucasian individuals (Botella-Carretero et al., 2010), whereas others found increasing amounts of serum retinol with increasing body condition in Mexican American children (Gunanti, Marks, Al Mamun, and Long, 2014). Other authors reported no differences in serum retinol concentrations between different body mass index (BMI) groups (Mills et al., 2008). However, human studies are limited by the lack of standardization of the vitamin A intake.

In general, serum retinol concentrations are maintained within a narrow range in individuals with adequate liver retinol stores, independent of the ration (Mody, 2017). This agrees with the lack of correlation between the dietary intake of vitamin A and serum retinol concentrations in our cohort and further may explain the current findings. The serum retinol concentrations in the present study

showed some minor fluctuations over the time, but probably were without biological significance.

RBP4 is the specific transport protein for retinol in mammalian blood, and alterations of vitamin A intake affect hepatic release of RBP4 in humans (Blaner, 1989). Apart from the metabolic function as transport protein, RBP4 has been identified as an adipokine in humans and horses (Tamori et al., 2006; Ungru et al., 2012). On the one hand, it has been reported in several studies that serum RBP4 is correlated with insulin resistance, obesity measurements and oxidative stress in humans (Liu et al., 2014). On the other hand, other studies reported no difference in serum RBP4 when comparing humans with different BMI (Lewis et al., 2007). Equine studies have revealed equivocal results. Ungru et al. (2012) reported decreased serum RBP4 levels in obese ponies after a BW reduction programme. On the contrary, Selim et al. (2015) reported a negative association between tail head RBP4 gene expression and BW in grazing mares. Our results suggest an inverse relation between serum RBP4 and obesity as serum RBP4 concentrations decreased in ponies and horses with BW gain. It had been reported in mice that RBP4 contributes to insulin resistance (Yang et al., 2005). The negative correlation we found between serum insulin and RBP4 at least in ponies contradicts that RBP4 is related to insulin resistance in equines.

As mentioned above, circulating blood RPB4 concentrations are mainly influenced by retinol status in humans (Blaner, 1989). Therefore, it has been recommended to include serum retinol concentrations in the evaluation of RBP4 by calculating serum retinol/RBP4 ratio (Mills et al., 2008). In the study population of Mills et al. (2008), the differences between obese and non-obese humans became highly significant by using the retinol/RBP4 ratio, while the same population showed only moderate differences by comparing just serum RBP4 concentrations. The retinol/RBP4 ratio was shown to decrease with increasing obesity (Mills et al., 2008). In contrast to these previous results in humans and our original hypothesis, the retinol/RBP4 ratio increased in ponies and horses during the BW gain period in the present study. Additionally, we detected positive relations between the retinol/RBP4 ratio and BCS, CNS and serum insulin concentrations. As a further discrepancy, we did not find a positive correlation between serum retinol and serum RBP4 concentrations as had been described previously in humans (Mills et al., 2008). Therefore, we speculate that the interactions between serum RBP4 and serum retinol in horses are not comparable to the interactions in humans, suggesting that further research is needed to elucidate the vitamin A and RBP4 metabolism in equines.

Our results suggested that the retinol and RBP4 metabolism in obese equines is different in comparison with that reported for obese humans. There also was a strong dose–response relationship with supplementation of synthetic α-tocopherol and serum α-tocopherol in this study. Laminitic horses showed decreased serum α-tocopherol concentrations, perhaps due to higher expenditure of antioxidants in laminar tissue. This is an important finding but needs to be verified in additional studies with more laminitic equines. We did not find strong differences in serum retinol and α-tocopherol concentrations between ponies and horses, which might suggest that disturbances in fat-soluble vitamin metabolism are not the underlying cause for the higher predisposition of ponies for metabolic diseases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

ANIMAL WELFARE STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes. The Ethics Committee for Animal Rights Protection of the Leipzig District Government (No. TVV 32/15) approved the project in accordance with German legislation for animal rights and welfare. Animals were cared for according to the guidelines for the accommodation and care of animals used for experimental and other scientific purposes (2007/526/EC).

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