From the

Institute of Animal Nutrition, Nutrition Diseases and Dietetics
Faculty of Veterinary Medicine, Leipzig University

Impact of body weight gain on liver metabolism and selected fat-soluble vitamins in ponies and horses

Inaugural-Dissertation
to obtain the degree of a
Doctor medicinae veterinariae (Dr. med. vet.)
from the Faculty of Veterinary Medicine
Leipzig University

Submitted by
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from Wernigerode, Germany

Leipzig, 2020

Mit Genehmigung der Veterinärmedizinischen Fakultät der Universität Leipzig

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Tag der Verteidigung: 03.11.2020

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Abbrevations

ALP	Alkaline phosphatase
AST	Aspartate aminotransferase
BA	Bile acids
BCS	Body condition score
BMI	Body mass index
BW	Body weight
CD68	Cluster of differentiation 68
CGIT	Combined glucose-insulin test
CNS	Cresty neck score
EMS	Equine metabolic syndrome
FABP1	Fatty acid binding protein 1
GfE 2014	Society of Nutrition Physiology
GGT	Gamma-glutamyl transferase
GLUT4	Glucose transporter type 4
GLDH	Glutamate dehydrogenase
IL-1β	Interleukin-1β
IL-6	Interleukin-6
LPL	Lipoprotein lipase
mRNA	Messenger ribonucleic acid
metS	Metabolic syndrome
NAFLD	Non-Alcoholic Fatty Liver Disease
Nf-κB	Nuclear factor-κB
RBP4	Retinol-binding protein 4
TNFα	Tumor necrosis factor α

1 Introduction

Obesity is a major health problem in equines. Obesity leads to insulin dysregulation and a predisposition towards laminitis, which are key components of the equine metabolic syndrome (EMS; DURHAM et al. 2019). It is well established that human obesity is accompanied by hepatic lipid alterations, termed Non-Alcoholic Fatty Liver Disease (NAFLD; TINIAKOS et al. 2010). In contrast to that, data are missing whether equine obesity affects the hepatic metabolism as well. Various parameters have been implemented to evaluate hepatic metabolism in obese humans. Non-invasive parameters include assessing serum liver enzyme activities and bile acids (BA), which have been associated with body mass index (BMI) and obesity related metabolic disturbances, such as insulin resistance (ALI et al. 2006, LIU et al. 2014a, MA and PATTI 2014). Definitive diagnosis of NAFLD is made by light microscopy of liver biopsies (BEDOSSA 2016). Determination of hepatic enzymes mediating fatty acid influx, such as lipoprotein lipase (LPL), provide further information about hepatic lipid metabolism (PARDINA et al. 2009). Hepatic accumulation of lipids induces hepatocellular inflammation with subsequent production of pro-inflammatory cytokines (CAI et al. 2005). Several studies have detected associations of pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor α (TNFα), with obesity, insulin resistance and NAFLD (BLÜHER et al. 2005, HAUKELAND et al. 2006, KROGH-MADSEN et al. 2006, MANCO et al. 2007). In contrast to human obesity research, the effects of equine obesity with its associated consequences on hepatic alterations have been only addressed in limited studies. For example, CHAMEROY et al. (2011) reported serum liver enzyme activities exceeding the reference ranges in horses with a history of laminitis. The authors suggested hepatic steatosis as causative factor, but hepatic lipid accumulation in examined equines was not investigated. In addition to the association of obesity with hepatic inflammation, it was shown that human obesity correlates with systemic low-grade inflammation and with higher levels of oxidative stress (D'ARCHIVIO et al. 2012, ESSER et al. 2014). Human obesity research also has investigated circulating concentrations of anti-inflammatory and antioxidant factors, such as vitamin E (α-tocopherol) and vitamin A (retinol). Serum concentrations of both vitamins were often shown to be decreased in conditions of obesity (BOTELLA-CARRETERO et al. 2010, PEREIRA et al. 2012). The influence of equine obesity on serum concentrations of α-tocopherol and retinol has not been evaluated yet.

We hypothesized that body weight (BW) gain would alter hepatic metabolism of equines, with elevation of serum liver enzyme activities, BA, hepatic lipid content and hepatic inflammatory parameters. Additionally, we hypothesized that serum α-tocopherol and retinol concentrations would decrease with increasing BW. Finally, it was hypothesized that obesity leads to more profound hepatic changes in ponies compared to horses, as ponies are more prone to obesity associated metabolic diseases such as laminitis.

2 Literature

2.1 Equine obesity

Obesity is defined as the accumulation of excessive adipose tissue (GERMAN 2006). As in human obesity, equines become obese in conditions of excess energy intake and little physical exercise. Furthermore, domestic conditions prevent the natural cycle of weight loss during winter that occur under natural circumstances (DUGDALE et al. 2011). These considerations may explain the high prevalence of overweight and obese horses throughout the world (24.5-45%; WYSE et al. 2008, THATCHER et al. 2008, ROBIN et al. 2015, POTTER et al. 2016). Robust pony breeds, which are adapted to rough environmental conditions, are theorized to have developed a predisposition to insulin resistance as survival advantage in terms of famine by elevating the storage of triglycerides in adipose tissue (JEFFCOTT et al. 1986). These so-called "easy keepers" are highly predisposed to obesity and its consequences under the circumstances of continuous supply of feed.

The body condition score (BCS) system is a useful tool to assess the general body condition of horses and ponies. The scoring system of CARROLL and HUNTINGTON (1988) evaluates the shape of the neck, the back and ribs and of the pelvis on a scale from 0 to 5. Zero score points indicate emaciation and 5 score points represent an obese body condition. Horse owners often underestimate the body condition of their equines (POTTER et al. 2016). Regional adiposity in equines, especially at the neck is suspected to be correlated with an increased risk for insulin resistance (FRANK et al. 2006). Therefore, the cresty neck score (CNS) was developed by CARTER et al. (2009a). The CNS evaluates the regional adiposity on a scale from 0 to 5, with 0 score points representing no palpable crest fat and 5 score points indicating a fatty crest that droops to one side.

2.1.1 Insulin dysregulation

Generalized obesity and/or regional adiposity are often accompanied by insulin dysregulation in equines. According to FRANK and TADROS (2014), insulin dysregulation includes excessive insulin responses to oral sugars, fasting hyperinsulinemia and insulin resistance. The latter defines the failure of insulin-sensitive tissues to respond to insulin. Obesity is linked to insulin resistance by two fundamental theories: (1) adipokines and cytokines are released from adipose tissue and downregulate signaling pathways of insulin and (2) storage of intracellular lipids in tissues other than adipose tissue such as the liver (D'ADAMO et al. 2010, TADROS and FRANK 2013). Equine studies have confirmed the strong relation of obesity to insulin dysregulation. CARTER et al. (2009b) found a 71% decrease in insulin sensitivity in adult

geldings after BW increased by 20%. The importance of insulin for metabolic diseases was further highlighted by studies of ASPLIN et al. (2007) and LAAT et al. (2010), who reported the induction of laminitis by insulin infusion in healthy ponies and horses. GEOR et al. (2013) stated that pony breeds generally have lower insulin sensitivity and a higher prevalence of hyperinsulinemia in comparison to horses.

Diagnostic tools to detect insulin dysregulation include basal measurements of blood glucose and insulin concentrations and dynamic test protocols. Hyperinsulinemia has been stated to be identified by a fasting serum insulin concentration above 20 µU/mL (FRANK 2009). To obtain a more accurate impression of insulin metabolism, dynamic test protocols are preferable. For example, the combined glucose-insulin test (CGIT) developed by EILER et al. (2005) allows a more detailed insight into the glycemic and insulinemic responses of equines. After IV administration of 150 mg/kg BW glucose immediately followed by 0.1 U/kg BW fast acting insulin, blood samples are collected at 0, 1, 5, 15, 25, 35, 45, 60, 75, 90, 105, 120, 135, and 150 minutes after the insulin application. Equines are classified as insulin resistant if the plasma glucose concentrations are above baseline at 45 minutes post injection. Serum insulin concentrations above 100 µU/mL at 45 minutes post injection also were considered to indicate insulin resistance. According to FRANK (2009), elevated insulin concentrations to a standardized glucose challenge indicate that increased pancreatic insulin secretion is necessary to compensate for the insulin resistance or that insulin clearance is compromised.

2.1.2 Obesity associated consequences

Endocrinopathic laminitis is the most detrimental consequence of obesity and insulin dysregulation. While the prevalence of endocrinopathic laminitis is not well established, reports of overall laminitis frequency in the equine population have varied from as little as 1.5% to 34% (WYLIE et al. 2011). However, in an equine referral hospital, 89% of all laminitis cases in a 16-month period had underlying endocrinopathies (KARIKOSKI et al. 2011). One third of the horses suffering from endocrinopathic laminitis were diagnosed with Pituitary Pars Intermedia Dysfunction. Two thirds of the laminitic horses showed basal hyperinsulinemia and, 95% of the hyperinsulinemic equines were obese (KARIKOSKI et al. 2011). FRANK et al. (2010) hypothesized that, based on recent research, laminitis may be linked to hyperinsulinemia, insulin resistance and obesity by various mechanisms such as endothelial cell dysfunction within blood vessels of the hoof (JANSSON 2007), digital vasoconstriction (SARAFIDIS and BAKRIS 2007), impaired glucose uptake by epidermal cells (FRENCH and POLLITT 2004), altered epidermal cell function or mitosis (NOURIAN et al. 2007) and matrix metalloproteinase activation by glucose deprivation or reactive oxygen species (FRENCH and POLLITT 2004). TREIBER et al. (2006) described a prelaminitic metabolic syndrome and was able to predict the occurrence of laminitis in ponies based on body condition, plasma triglyceride concentration and proxies for insulin sensitivity and pancreatic β-cell response.

This highlights the strong interrelations between obesity, insulin dysregulation and laminitis. It has to be noted, that obesity does not necessarily lead to insulin resistance or laminitis and furthermore, insulin resistance and laminitis can occur in lean animals as well (BAILEY et al. 2008, GEOR et al. 2013).

2.1.3 Equine metabolic syndrome

The International Diabetes Federation proposed in 2006 a worldwide diagnostic tool for the human metabolic syndrome (metS). Based on this, metS is characterized by the presence of obesity, increased serum triglyceride concentrations, reduced serum high density lipoprotein–cholesterol concentrations, increased blood pressure and increased fasting plasma glucose concentrations (ALBERTI et al. 2006). EMS may be comparable to metS in humans. Obesity, insulin dysregulation and a predisposition towards laminitis constitute the three key components of EMS. Dyslipidemia, hyperleptinemia, arterial hypertension and low-grade inflammation are further possible components of EMS (FRANK et al. 2010, DURHAM et al. 2019). Pony breeds seem to be more susceptible to EMS than most horse breeds (JOHNSON et al. 2010).

2.2 Liver metabolism in equine obesity

The liver has a central role in several metabolic processes, e.g. processing absorbed nutrients, detoxification for example of ammonia, synthesis of coagulation factors and BA (GEHLEN et al. 2010). Hepatocytes constitute 70% of the cells located in the liver and the resident macrophages of the liver are named Kupffer cells (SALOMON and ACHILLES 2008).

2.2.1 Non-Alcoholic Fatty Liver Disease

Lipid infiltration of hepatocytes occurs in various species. For example, obese horses in negative energy balance undergo a fat mobilization, which leads to hyperlipemia and hepatic lipidosis (GEHLEN et al. 2010). In human medicine, hepatic steatosis in the absence of significant alcohol abuse or other known liver diseases is termed Non-Alcoholic Fatty Liver Disease (NAFLD). This disease is frequently associated with obesity (ADAMS et al. 2005) and represents the most common liver disease in developed countries, with a prevalence of up to 30% (BROWNING et al. 2004). Clinical signs range from simple steatosis to steatohepatitis, advanced fibrosis and, rarely, cirrhosis (PASCHOS and PALETAS 2009). The diagnostic basis of NAFLD is that more than 5% of hepatocytes contain lipid droplets as evaluated by light microscopy of liver samples (BEDOSSA 2016). In general, steatosis results from a dysregulation of fatty acid influx to the liver, fatty acid efflux, and hepatic de novo lipogenesis

(KOPPE 2014). Although the pathogenesis of NAFLD is not completely understood, insulin resistance accompanying obesity seems to be a crucial factor. Insulin resistance leads to an impaired inhibition of lipolysis in all adipose tissues. Together with obesity this mechanism results in elevated circulating levels of free fatty acids (KOPPE 2014) and contributes to steatosis as 60-80% of hepatic stored lipids originate from circulating free fatty acids (PASCHOS and PALETAS 2009).

In contrast to the effort in human medicine, hepatic alterations in equine obesity have not been as intensively investigated yet. Serum gamma-glutamyl transferase (GGT) and aspartate aminotransferase (AST) activities were reported to exceed the reference ranges in obese horses with a history of laminitis (CHAMEROY et al. 2011). The authors suggested that equine obesity was a possible contributing factor to the altered laboratory parameters in hepatic lipidosis. Furthermore, the toll-like receptor pathway, which is important in coordinating the innate immunity, was activated in liver tissue of horses suffering from laminitis and obesity (STOKES et al. 2010).

2.2.1.1 Liver enzyme activities and bile acids

Diagnosis of NALFD by light microscopy needs biopsy specimens of the liver and is therefore invasive and coupled to risks caused by the sampling procedure. Hence, great effort was done to implement non-invasive parameters for risk assessment or diagnosis of NAFLD. In addition to that, NAFLD constitutes the most common cause for otherwise unexplained elevated aminotransferases (CLARK et al. 2003). That led to a new focus of NALFD research, the liver enzyme activities and bile acids.

2.2.1.1.1 Alkaline phosphatase

Alkaline phosphatase is located in several tissues, including liver, bones, intestine, placenta and macrophages. It is therefore not a very specific indicator for liver diseases but it is a sensitive marker for cholestasis (PEARSON 1999). It is further proven that ALP is additionally expressed in several adipose tissue depots where it might function as modulator of adipogenesis (ALI et al. 2014). It had been shown in African volunteers that serum ALP levels were higher in obese than in lean individuals. The authors suggested the overall fat mass as origin of the majority of the ALP measured in the serum (ALI et al. 2006).

In young equines elevated serum ALP values correlated with physiological high bone turnover (GEHLEN et al. 2010). High serum ALP activities in combination with other elevated serum liver enzyme activities have been reported in equines with hepatopathies, such as liver neoplasia (GEHLEN et al. 2010). To the author´s knowledge, there have been no equine studies investigating associations of serum ALP activities and obesity.

2.2.1.1.2 Aspartate aminotransferase

Aspartate aminotransferase is present in all tissues except bones, with highest concentrations in liver and skeletal muscle (SUCKOW et al. 2012). Equivocal results exist about the association of serum AST activities and NAFLD. On one hand, serum AST activities were reported to be significantly increased in humans with moderate NAFLD compared to humans without NAFLD (WASADA et al. 2008). A recently published study reported an association of fatty liver and deregulated hepatic expression of AST and elevated serum AST activities (SOOKOIAN et al. 2016). Elevated serum AST activities were positively associated with the prevalence of metS in the Chinese population (CHEN et al. 2016). On the other hand, CORDEIRO et al. (2015) reported serum AST activities within the reference range in human subjects with different stages of NAFLD. The evaluation of serum AST activities is suspected to have a low sensitivity for NALFD, as only 50% of NAFLD patients show elevated serum AST activities (DIETRICH and HELLERBRAND 2014). Other studies reported similar serum AST activities between human cohorts with different BMI (KHAN et al. 2015, KÄLSCH et al. 2015). These opposing results might be attributable to the nearly ubiquitous occurrence of AST. Hence, the suitability of AST as biomarker for NAFLD and metS still needs to be verified.

DURHAM et al. (2003) described increased serum AST activities as good diagnostic marker for liver diseases in horses, but only in combination with other serum liver enzyme activities. Six out of 14 obese horses with a history of laminitis were reported to have plasma AST activities exceeding reference ranges (CHAMEROY et al. 2011).

2.2.1.1.3 Glutamate dehydrogenase

Glutamate dehydrogenase (GLDH) is a liver-specific key enzyme in amino acid oxidation and urea production. It is mainly present in the mitochondria of the centrolobular hepatocytes (SCHMIDT and SCHMIDT 1988, O'BRIEN et al. 2002). Serum GLDH activities were significantly increased in cases of a fatty liver syndrome in post mortem examined cows (BOGIN et al. 1988).

Serum GLDH activities are expected to increase in horses with even mild hepatocyte injury (DIVERS 2015). Furthermore, WEST (1996) reported elevated plasma GLDH activities in horses suffering from lipidosis, of which approximately 50% were obese.

2.2.1.1.4 Gamma-glutamyl transferase

Gamma-glutamyl transferase is mainly located in epithelial cells in the kidney, pancreas and the liver. Notably, high serum activities of GGT are linked with cholestasis in humans (WHITFIELD 2001). Additionally, serum GGT activities were correlated with metabolic features such as BMI, serum triglycerides and serum insulin concentrations in healthy adults

(LIU et al. 2014a). It was also suggested to include increased serum GGT activities as criterion for metS, as it may improve the predictive accuracy for cardiovascular diseases (DEVERS et al. 2008).

Plasma GGT activities were reported to exceed the reference range in 9 out of 14 obese horses with a history of laminitis (CHAMEROY et al. 2011). The authors speculated hepatic lipidosis as causative factor. The confirmation of the occurrence of hepatic lipidosis in individuals suffering from laminitis is lacking. WEST (1996) reported that horses admitted to a clinic with a purported diagnosis of lipidosis had elevated plasma GGT activities compared to clinically healthy horses. Furthermore, EMS ponies had significantly higher serum GGT activities compared to healthy control ponies. Unfortunately, differences of body condition between the EMS and the control group have not been reported (EL-SHERIF et al. 2014).

2.2.1.1.5 Bile Acids

Cholesterol, phosphatidylcholine and bilirubin constitute together with BA the components of the bile. Bile acids mediate the absorption of dietary fat and fat-soluble vitamins (MA and PATTI 2014). Elevated serum BA concentrations can originate from blockage of the bile flow, hepatocellular damage or shunts from the portal system to the vena cava (PEARSON 1999). Recently, BA have emerged as important modulators of glucose and lipid homeostasis in humans (MA and PATTI 2014). Additionally, modulation of circulating BA levels, using BA binding factors or receptor agonists, can affect glycemic control, BW and insulin sensitivity (MA and PATTI 2014). Serum BA levels were linked with disease severity of NAFLD (BECHMANN et al. 2013). Therefore, serum BA are a sensitive marker of liver diseases and, moreover, an important marker of metS and NAFLD.

Serum bile acids are also sensitive markers for dysfunctions of the equine liver. A healthy liver removes approximately 90% of BA from the enterohepatic circulation. In case of a dysfunctional liver, the circulating BA increase (GEHLEN et al. 2010). Investigations focusing BA in equine obesity with its possible consequences are missing.

2.2.2   Obesity related inflammation

Human obesity has been associated with high circulating levels of pro-inflammatory cytokines (e.g., TNFα, interleukin-1 (IL-1), and IL-6) and adipokines (e.g., chemerin; VOZAROVA et al. 2001, COTTAM et al. 2004, BOZAOGLU et al. 2007). However, equine obesity research has provided contradictory data. VICK et al. (2007), CARTER et al. (2009c) and SUAGEE et al. (2011b) all reported inflammatory correlations with obesity. Whereas other studies did not find any signs of inflammation in equines suffering from obesity or its detrimental consequences (SUAGEE et al. 2011a, HOLBROOK et al. 2012, BAMFORD et al. 2016). Therefore, the

interrelations of inflammation and equine obesity with its consequences need further elucidation.

Adipose tissue is known to contribute to chronic inflammation by secreting pro-inflammatory cytokines and adipokines in obese humans (SUGANAMI and OGAWA 2010). Adipokines such as leptin, adiponectin, resistin or vaspin are biological active substances that are secreted by adipose tissue into the circulation and act on both, local and distant tissues (BLÜHER 2012).

Kupffer cells, the resident liver macrophages, once activated, perform several reactions such as initiating a biochemical attack, phagocytosis or enhancing interactions with hepatocytes by potentiating the release of a variety of biologically active mediators (for example cytokines, chemokines, eicosanoids; BAFFY 2009). Therefore, the liver also might contribute to the chronic inflammation associated with obesity. The importance of Kupffer cells was highlighted by HUANG et al. (2010), who showed that the depletion of Kupffer cells protects the liver against the development of diet induced steatosis and hepatic insulin resistance. According to BAFFY (2009), the biological activity of Kupffer cells might be modulated by an altered abundance and composition of liver tissue lipids within steatosis by various effects. The ability of lipids to adversely affect body tissues is termed lipotoxicity. Steatosis leads to subacute hepatocellular inflammation in mice and the induced pro-inflammatory cytokines mediate hepatic and systemic insulin resistance (CAI et al. 2005). Hepatic insulin resistance leads to a disrupted inhibition of glucose and very-low-density lipoprotein production which induces hyperglycemia, compensatory hyperinsulinemia and hypertriglyceridemia in humans (YKI-JÄRVINEN 2010).

In insulin resistant and insulin sensitive horses with varying body conditions, messenger ribonucleic acid (mRNA) levels of pro-inflammatory cytokines have been investigated in different adipose tissue depots but not in the liver (BURNS et al. 2010, BRUYNSTEEN et al. 2013).

2.2.2.1 Nuclear factor-κB

Nuclear factor-κB (NF-κB) is a transcription factor for several inflammatory and immune related genes, and contributes to the production of pro-inflammatory cytokines, chemokines, inflammatory enzymes and adhesion molecules. NF-κB is a key factor for host defense and chronic inflammatory diseases (BARNES 1997). Expression of NF-κB in rodent livers was activated in two models of obesity in mice, high fat diet and genetically regulated hyperphagia. Hepatocellular activation of NF-κB in mice induced hepatic expression of IL-6 which mediates hepatic and systemic insulin resistance (CAI et al. 2005).

2.2.2.2 Interleukin-1β

One of the major pro-inflammatory cytokines is IL-1β, that impairs insulin signaling pathways (FÈVE and BASTARD 2009). Hepatic triglyceride storage is promoted by Kupffer cells via IL-1β dependent suppression of peroxisome proliferator-activated receptor α activity which leads to decreased fat oxidation (STIENSTRA et al. 2010). IL-1β was further highlighted as important mediator in the transformation from NAFLD in humans to more severe stages, namely steatohepatitis (KAMARI et al. 2011).

In horses blood mRNA expression of IL-1 was positively associated with BCS and percent body fat and negatively associated with insulin sensitivity (VICK et al. 2007). The expression of IL-1β is altered in digital laminae and lungs of horses with experimentally induced laminitis (BELKNAP et al. 2007, STEWART et al. 2009). However, in other studies, mRNA levels of IL-1β together with mRNA levels of TNFα and IL-6 of different adipose tissue depots were not different between insulin resistant and insulin sensitive horses (BURNS et al. 2010).

2.2.2.3 Interleukin-6

The cytokine IL-6 has a broad range of biological activities in immune regulation, hematopoiesis, inflammation and oncogenesis (KISHIMOTO 2010). High circulating IL-6 concentrations are associated with obesity, insulin resistance and impaired glucose metabolism in humans (BLÜHER et al. 2005). Additionally, serum IL-6 levels are elevated in patients suffering from NAFLD, supporting the link of NALFD with low-grade systemic inflammation and the strong association of metS and NAFLD (HAUKELAND et al. 2006). This cytokine is also known to induce hepatic insulin resistance in mice (KLOVER et al. 2003), probably due to the induction of suppressors of cytokine signaling which inhibits the insulin receptor signal transduction (SENN et al. 2003). Other studies have provided evidence for an ambivalent role of IL-6 in obesity associated liver diseases. For example, YAMAGUCHI et al. (2010) postulated that hepatic IL-6 signaling inhibited the progression of hepatic steatosis but enhanced liver inflammation in mice.

Equivocal results about IL-6 also have been reported in horses. VICK et al. (2007) reported that blood IL-6 expression decreased with increasing degree of obesity in younger mares. In another study, the IL-6 gene expression of peripheral blood cells was significantly decreased in obese, hyperinsulinemic horses in comparison to normal weight, normoinsulinemic horses (HOLBROOK et al. 2012). On the contrary, ponies suffering from EMS showed significantly higher serum IL-6 levels compared to obese ponies without EMS (BASINSKA et al. 2015).

2.2.2.4 Tumor necrosis factor α

The cytokine TNFα is a frequently investigated pro-inflammatory substance that is secreted by various cell types, but mainly macrophages and lymphocytes (ANTUNA-PUENTE et al. 2008). Hepatic TNFα levels are directly influenced by hepatocytes and Kupffer cells and indirectly by the abdominal adipose tissue in humans (CRESPO et al. 2001). Adipose tissue is likely to be a major contributor to the elevated serum TNFα concentrations reported in obese humans (WINKLER et al. 2003). This cytokine has key roles in insulin resistance and NAFLD. For example, TNFα has been reported to: (1) suppress the expression of the glucose transporter type 4 (GLUT 4); (2) increase serum and intrahepatic free fatty acids by inducing lipolysis; (3) trigger insulin resistance by serine phosphorylation of insulin receptor substrate 1 and 2 (POLYZOS et al. 2009). KROGH-MADSEN et al. (2006) showed that the infusion of TNFα induces insulin resistance in healthy humans. Serum TNFα levels were also reported to correlate positively with disease severity of NAFLD in children (MANCO et al. 2007).

In equine research, the link between TNFα and EMS has been reported in several studies. Blood mRNA expression of TNFα was positively linked to BCS in 60 mares with differing body conditions (VICK et al. 2007). Plasma concentrations of TNFα were significantly higher in ponies previously suffering from pasture associated laminitis compared to ponies without a history of laminitis maintained under the same management conditions (TREIBER et al. 2009). In addition to elevated circulating plasma TNFα in laminitic equines, different experimental models of laminitis (carbohydrate overload and black walnut extract administration) were reported to increase the hepatic mRNA levels of TNFα in horses (STEWART et al. 2009, TADROS et al. 2012). Additionally, ponies suffering from EMS had higher serum concentrations of TNFα compared to obese but metabolically healthy ponies (BASINSKA et al. 2015).

2.2.2.5 Cluster of differentiation 68

The transmembrane glycoprotein, Cluster of diferentiation (CD68), is highly expressed by monocytes and tissue macrophages in humans (WESTERBACKA et al. 2007). Hepatic CD68 cells correlate with the histological severity of human NAFLD (PARK et al. 2007). A genetically modified mouse model further confirmed the suitability of CD68 as marker for Kupffer cells. The authors in that study used LIKK mice, which are known to produce elevated amounts of NF-κB. In comparison to wild type mice, the hepatic mRNA levels of CD68 were upregulated in LIKK mice. However, the authors suggested that CD68 indicates the activation of resident Kupffer cells rather than the recruitment of additional macrophages, as the number of cells did not change (CAI et al. 2005). Additionally, hepatic CD68 cells were shown to increase in patients with obesity compared to patients without obesity (KRISTENSEN et al. 2017).

This glycoprotein has also been verified as suitable marker for macrophages in equines (SIEDEK et al. 2000). UNGRU et al. (2012) investigated the mRNA levels of CD68 in subcutaneous adipose tissue of the tail head of a population of ponies. The authors did not detect any differences of CD68 mRNA levels between insulin sensitive and insulin resistant obese ponies. After a BW reduction program for 14 weeks in the obese ponies, mRNA levels of CD68 seemed to decrease in subcutaneous tail head adipose tissue.

2.2.2.6 Chemerin

Chemerin is a chemoattractant protein that specifically attracts macrophages and dendritic cells through its receptor ChemR23, thereby linking innate and adaptive immunity (WITTAMER et al. 2005). In addition to its role in immune function, chemerin is recognized as an adipokine that modulates adipogenesis and adipocyte metabolism (GORALSKI et al. 2007). Besides adipocytes, hepatocytes constitute a major expression site of chemerin (BUECHLER 2014). As reviewed by YOSHIMURA and OPPENHEIM (2008), chemerin is secreted as an inactive precursor protein. Chemerin is processed by various proteases into pro-inflammatory or anti-inflammatory peptides and acts through different receptors. The multifunctional properties of chemerin with inhibitory and stimulatory abilities might be explained in this way. These varied roles of chemerin's physiology might underlie the equivocal results of research results on chemerin associations with obesity, inflammation and NAFLD.

Several studies have reported a pro-inflammatory impact of chemerin. In Mexican-American individuals with varying phenotypes, plasma chemerin levels correlated positively with BMI, fasting plasma glucose, fasting serum insulin, plasma triglycerides and total serum cholesterol concentrations (BOZAOGLU et al. 2009). Mice fed a high fat diet had increased hepatic mRNA levels of chemerin compared to animals consuming a standard diet and hepatic chemerin mRNA levels correlated with BW in the high fat diet group (KRAUTBAUER et al. 2013). Furthermore, hepatic mRNA levels of chemerin correlated positively with obesity measurements and severity of NAFLD in humans (DÖCKE et al. 2013).

Other studies have substantiated the anti-inflammatory properties of chemerin. According to DENG et al. (2013), hepatic chemerin mRNA levels were decreased in rodents with NAFLD compared to control rodents without NAFLD. Furthermore, humans suffering from a progressive form of NAFLD displayed reduced hepatic mRNA levels of chemerin compared to humans suffering from steatosis alone, as initial stage of NAFLD (POHL et al. 2017). As the expression of cytokines in the adipose tissue is known to influence the hepatic metabolism via the circulatory system, some authors have investigated associations of adipokines and hepatic alterations. In this context, negative associations of chemerin expression in visceral adipose tissue with hepatic steatosis, lobular inflammation and hepatocellular ballooning have been reported in humans (WOLFS et al. 2015, BEKAERT et al. 2016).

2.2.3   Lipid metabolism

2.2.3.1   Lipoprotein lipase

One of the rate-limiting enzymes for intravascular hydrolysis of lipoprotein-rich triglyceride particles is LPL. It therefore helps to regulate the supply of fatty acids to various tissues. This enzyme is expressed at high levels in adipose tissue, heart, skeletal muscle, kidney and the mammary gland and at lower levels in the liver, adrenal gland and brain (KIRCHGESSNER et al. 1987). Insulin supports the storage of lipids by promoting the activity of LPL (ENGELHARDT et al. 2015). It was reported in genetically modified mice, that liver specific overexpression of LPL leads to hepatocellular accumulation of triglycerides and subsequently to hepatic insulin resistance (KIM et al. 2001). PARDINA et al. (2009) reported a significant increase of hepatic LPL mRNA levels in obese humans. The authors postulated that these changes contributed to the hepatic accumulation of triglycerides, which favors steatosis. This hypothesis was supported by the results of WESTERBACKA et al. (2007), who found a strong positive correlation between hepatic mRNA levels of LPL and hepatic lipid content in humans with varying amounts of histologically determined hepatic fat.

In equine research, circulating LPL activity has been analyzed in the context of high fat feeding (GEELEN et al. 2000). It was shown that a ration high in fat induces plasma activity of LPL. This leads to decreased concentrations of circulating triglycerides and a higher availability of free fatty acids, which might be beneficial for the exercising horse. Concentrations of LPL in equine liver tissue and equine obesity have not been investigated to the author´s knowledge.

2.2.3.2   Fatty acid binding protein 1

The fatty acid binding proteins (FABP) constitute a group of proteins which are named according to the tissue in which they were first recognized, for example liver-type FABP (FABP1), intestine-type FABP (FABP2), heart-type FABP (FABP3) and adipocyte-type FABP (FABP4) (STORCH and CORSICO 2008). These proteins are abundant in the cellular cytoplasm, modulating the uptake and intracellular trafficking of long-chain fatty acids for oxidation and storage (WESTERBACKA et al. 2007). Of all FABP members, FABP1 is the most broadly distributed mammalian FABP and represents 2%−5% of the total cytosolic protein of the liver (ATSHAVES et al. 2010).

In a cross-sectional study, it was shown that serum FABP1 levels were positively associated with obesity and insulin resistance in Chinese young adults (SHI et al. 2012). Furthermore, the level of hepatic FABP1 mRNA is increased in humans suffering from NAFLD, probably as a compensatory mechanism for increased fat influx (HIGUCHI et al. 2011). Furthermore, genetically modified mice lacking FABP1, were protected from high fat, high cholesterol

western diet-induced obesity and hepatic steatosis. The authors speculated the knockout of FABP1 led to subtle changes in fatty acid availability by altering food consumption, intra- and intercellular signaling and intestinal fatty acid uptake (NEWBERRY et al. 2006).

In equine medicine, intestinal FABP in plasma and abdominal fluid has been investigated as a possible predictor of survival and need of surgical intervention in horses with colic (NIETO et al. 2005). However, FABP1 has neither been investigated in equines nor in the context of increasing equine BW.

2.3 Fat-soluble vitamins in equine obesity

Vitamins are obtained from the diet or provided by intestinal microflora and they are essential for diverse biological functions. Based on their relative solubility in water and fat, the 13 known essential vitamins are divided in two classes, fat-soluble and water soluble. The fat-soluble vitamins include vitamin A, D, E and K. Deficiencies of these vitamins cause commonly recognized clinical manifestations (vitamin A − night blindness, vitamin D − osteomalacia, vitamin E − increased oxidative stress and muscular disorders). But recently, deficiencies have been additionally associated with increased risk of cancer, type II diabetes mellitus and immune system disorders in humans (ALBAHRANI and GREAVES 2016). Furthermore, human research suggested plasma levels of fat-soluble vitamins to decrease in human obesity. Possible reasons were considered to be due to a lower intake of these nutrients or a higher deposition in adipose tissue, thus decreasing bioavailability in subjects with excess adiposity (WORTSMAN et al. 2000). Additionally, plasma concentrations of vitamin transport proteins are decreased by inflammation and therefore might diminish the bioavailability of the fat-soluble vitamins in chronic inflammation accompanying obesity (ROSALES and ROSS 1998).

2.3.1 Vitamin E

Vitamin E includes eight lipophilic variants, namely α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol of which α-tocopherol is the predominant form in whole body tissues in humans (JIANG 2014). Vitamin E has several functions, which include modulation of gene expression (e. g. scavenger receptors, α-tropomyosin, matrix metalloproteinase-19 and collagenase), inhibition of cell proliferation, platelet aggregation, monocyte adhesion (ZINGG and AZZI 2004), and regulation of bone mass (FUJITA et al. 2012). Moreover, α -tocopherol is one of the main fat-soluble antioxidants (BURTON and TRABER 1990). As major fat-soluble antioxidant, α-tocopherol combats lipid peroxidation of cell membranes and interrupts the radical chain by forming derivative that has low reactivity and is unable to attack lipid substrates (DESCAMPS-LATSCHA et al. 2001). In humans, α-tocopherol is in the group of exogenous antioxidants, such as β-carotenes, ascorbic acid and others which complement the

actions of endogenous antioxidants such as uric acid, superoxide dismutase and melatonin (PISOSCHI and POP 2015). Antioxidant defenses are necessary to fight reactive oxygen species. Reactive oxygen species are produced by aerobic processes such as cellular respiration, microbial infection and intensive physical exercise (POLJSAK et al. 2013). Imbalances between antioxidant defenses and reactive oxygen species production result in oxidative stress. Oxidative stress leads to structure modifications and function modulation in nucleic acids, lipids and proteins. Several markers of oxidative damage are known to be specific to the disturbed tissue type. Imbalances between the reactive oxygen species and the antioxidant system are involved in many diseases and can ultimately lead to cancer, neurodegeneration, cardiovascular disease, diabetes and kidney dysfunction (PISOSCHI and POP 2015). Additionally, oxidative stress has been reported to be associated with obesity in humans and is suspected to be partly responsible for comorbidities of human obesity such as atherosclerosis (D'ARCHIVIO et al. 2012, TIBAUT and PETROVIČ 2016).

As consequence of these findings, the interrelations of serum α-tocopherol concentrations and human obesity have been studied intensively. Most human studies detected negative associations between serum α-tocopherol and obesity and suggested a depletion of circulating α-tocopherol due to elevated oxidative stress, reduced dietary antioxidant intake or entrapment of vitamin E in the expanding adipose tissue as causative factors (DECSI et al. 1997, BOTELLA-CARRETERO et al. 2010, GUNANTI et al. 2014). Some opposing studies reported positive correlations of serum α-tocopherol concentrations and obesity in humans (WALLSTRÖM et al. 2001, WANIEK et al. 2017). However, most investigations of serum α-tocopherol in obese humans have been limited by the lack of the ability to standardize intake of vitamin E in the study populations.

The recommended daily dietary intake of vitamin E in adult horses is 5 IU/kg BW$^{0.75}$ per day according to the guidelines of the Society of Nutrition Physiology (GfE 2014) (FLACHOWSKY et al. 2014). Sufficient supply of vitamin E in horses is important to prevent equine degenerative myeloencephalopathy (FINNO et al. 2011), equine motor neuron disease (DIVERS et al. 2006) and nutritional myodegeneration (along with sufficient selenium supply) (LÖFSTEDT 1997). Vitamin E in equine research was frequently evaluated in the context of performance parameters during exercise which induces oxidative stress (MCBRIDE and KRAEMER 1999), although proof of beneficial effects of vitamin E supplementation in exercising horses is lacking (SICILIANO et al. 1997, WILLIAMS et al. 2005). In the context of obesity, contradictory results regarding vitamin E have been reported in horses. Markers of oxidative stress and antioxidant function were not altered in obese horses or prelaminitic ponies when compared to healthy controls (TREIBER et al. 2009, HOLBROOK et al. 2012). On the other hand, other equine studies detected reduced antioxidative capacities in obese horses (PLEASANT et al. 2013) and in horses suffering from EMS (MARYCZ et al. 2018).

2.3.2 Vitamin A

Vitamin A (retinol) is the parent compound of all bioactive retinoids and retinoic acid constitutes the active metabolite of vitamin A. Approximately 80-90% of all retinoids are stored as retinyl esters in the liver. In general, serum retinol concentrations are maintained within a narrow range in individuals with adequate liver retinol stores independent of the diet (MODY 2017). The biological effects of retinoids are mediated by two types of nuclear receptors, the retinoic acid receptor and retinoid X receptor, both mediating transcriptional activity (VILLARROYA et al. 2004). In addition to the essential function of vitamin A for vision (HYATT and DOWLING 1997), the influence of vitamin A on inflammation (e.g. in infections like measles or malaria, gastrointestinal tract and respiratory tract inflammation) has been reviewed several times in the human literature (SEMBA 1999, STEPHENSEN 2001, REIFEN 2002). Beneficial effects of a vitamin A supplementation, such as enhancement of phagocytic activity of neutrophils and macrophages have been reported as vitamin A is often decreased in inflammation in humans (SEMBA 1999, STEPHENSEN 2001, REIFEN 2002). Furthermore, ROSALES et al. (1996) reported that an injection of lipopolysaccharides, which induce systemic inflammation, decreased the serum retinol concentrations in rats. More recent studies have linked vitamin A to the regulation of adipogenesis, insulin sensitivity, and glucose homeostasis in humans, rats and mice. For example, the administration of retinoic acid prevented BW gain and improved insulin sensitivity in genetically obese mice (MANOLESCU et al. 2010). In a study population of morbidly obese human subjects, inadequate serum vitamin A levels were correlated with the presence of insulin resistance (VILLAÇA CHAVES et al. 2008). These authors concluded that it is important to maintain a sufficient supply of vitamin A to prevent a complete conversion of dietary β-carotene, an important exogenous antioxidant, into retinol since β-carotene is the precursor for hepatic synthesis of retinol. Therefore, β-carotene can fulfill its antioxidative functions instead of only converting into retinol (VILLAÇA CHAVES et al. 2008).

Several field studies reported conflicting data about the relationship between human obesity and serum retinol concentrations. However, most authors found decreased serum retinol levels in obese adults (AASHEIM et al. 2008, BOTELLA-CARRETERO et al. 2010, PEREIRA et al. 2012) and proposed several mechanisms as possible causative factors: imbalanced nutrition in obese subjects, entrapment of fat-soluble vitamins in enlarged adipose tissue depots and inflammation accompanying obesity. On the contrary, increasing amounts of serum retinol with increasing body condition were reported in children under 15 years (AEBERLI et al. 2007, GUNANTI et al. 2014). The differences in age of the study populations may have caused these discrepancies which did not take into account the impacts of growth or puberty in young humans. Of further note, the main limitation of human studies investigating circulating retinol levels is the lack of the ability to standardize vitamin A intake in the study populations.

Similar to humans, deficiencies of vitamin A in equines were described to cause loss of vision, defects in bone growth, defects in reproduction, defects in growth and differentiation of epithelial tissues, and lowered resistance to disease and infection (CRANDELL 1998). According to the GfE 2014 the recommended daily dietary intake of vitamin A in adult horses is 150 IU/kg $BW^{0.75}$ per day (FLACHOWSKY et al. 2014). However, detailed research of retinol interactions in equines are limited and have been mainly focused on performance and parturition effects on circulating vitamin A concentrations (ABRAMS 1979, BUTLER and BLACKMORE 1982, SCHWEIGERT and GOTTWALD 1999).

2.3.3   Retinol-binding protein 4

Retinol is transported in the blood by retinol-binding protein 4 (RBP4) which is primarily synthesized in the liver. Alterations of vitamin A intake also affect hepatic release of RBP4 in humans (BLANER 1989). In addition to hepatic secretion, RBP4 also is released from adipose tissue as an adipokine (TAMORI et al. 2006).

RBP4 has been suggested to play an important role in the pathogenesis of metS (KLOTING et al. 2007). This assumption was supported by YANG et al. (2005), who showed that the injection of purified RBP4 into mice or transgenic overexpression of RBP4 in mice causes insulin resistance. Adipocytes had decreased GLUT4 expression in insulin resistant humans and rodents (SHEPHERD and KAHN 1999). The study by YANG et al. (2005) suggested that adipocytes respond to decreased GLUT4 expression by secreting RBP4. Subsequently, RBP4 inhibits insulin signaling in muscles and increases circulating blood glucose by inducing hepatic gluconeogenesis, factors which promote systemic insulin resistance. However, these findings about RBP4 have been controversial. Opposing studies suggest that the observed association between plasma RBP4 concentrations and insulin resistance is less important in humans than in mice and rats (RIBEL-MADSEN et al. 2009). In addition, the metabolic syndrome is related to kidney dysfunction which results in elevated serum RBP4 concentrations. Therefore, the observed elevated serum RBP4 in patients suffering from type 2 diabetes mellitus may result from moderate renal insufficiency rather than obesity associated alterations. (HENZE et al. 2008).

Furthermore, data resulting from human clinical studies are conflicting. In several studies, serum RBP4 levels were reported to be associated with insulin resistance, obesity measurements and oxidative stress in humans (YANG et al. 2005, GRAHAM et al. 2006, AEBERLI et al. 2007, LIU et al. 2014b). However, other studies did not detect correlations between serum RBP4 levels and BMI or insulin resistance (JANKE et al. 2006, LEWIS et al. 2007, SHEA et al. 2007, YAO-BORENGASSER et al. 2007). Technical problems such as sample collection method, e.g. type of collection tubes or the antibody design for the immunoassay, may have led to inaccurate measurements of circulating RBP4 concentrations.

Additionally, the genetic background of the investigated study populations, age, sex, kidney function, retinol status and iron status are confounding factors affecting the results of the aforementioned studies and therefore perhaps contributing to the different outcomes (KOTNIK et al. 2011).

It has been recommended to include measurement of serum retinol concentrations in the studies of serum RBP4 concentrations by calculating the retinol/RBP4 ratio (MILLS et al. 2008, ERIKSTRUP et al. 2009) as circulating RPB4 concentrations are mainly influenced by retinol status (BLANER 1989). In the study population of MILLS et al. (2008), differences between obese and non-obese humans became highly significant by calculating the retinol/RBP4 ratio. The same population showed moderate differences by only comparing serum RBP4 levels. The retinol/RBP4 ratio has been reported to decrease with increasing human obesity (AEBERLI et al. 2007, MILLS et al. 2008)

Similar to human research, equine studies of RBP4 have revealed equivocal results. Horses with EMS had increased RBP4 mRNA levels in adipose tissue and liver in comparison to healthy control horses (MARYCZ et al. 2018). Similarly, UNGRU et al. (2012) reported serum RBP4 concentrations to decrease in obese ponies undergoing a BW reduction program. On the contrary, SELIM et al. (2015) detected a negative correlation between tailhead subcutaneous adipose tissue RBP4 expression and BW in grazing mares.

3   Publications

3.1   Impact of body weight gain on hepatic metabolism and hepatic inflammatory cytokines in comparison of Shetland pony geldings and Warmblood horse geldings

C. Schedlbauer, D. Blaue, M. Gericke, M. Blüher, J. Starzonek, C. Gittel, W. Brehm and I. Vervuert

Contributions

I. Vervuert created the study design. S. Klemann, J. Starzonek, D. Blaue and I fed the equines and took care for them. BW, BCS and CNS were assessed weekly by D. Blaue and me. Blood samples for assessment of liver enzyme activities and BA were collected by J. Starzonek, D. Blaue and me. Dr. W. Brehm conducted the liver tissue sampling in general anesthesia with assistance of D. Blaue, J. Starzonek and me. Organization and care for the animals before and after surgery was provided by D. Blaue and me under supervision of C. Gittel. The laboratory of the Large Animal Clinic of the Faculty of Veterinary Medicine in Leipzig analyzed liver enzyme activities and BA in provided blood samples. Preparation of histological slides of liver samples was done by D. Blaue and me. M. Gericke staged the histological liver samples according to their lipid content. The RT-qPCR of liver samples for analysis of markers for inflammation and lipid metabolism was done by me. Statistical analysis of the results was conducted by me. I wrote the first draft of all parts of the manuscript and revisions were done by D. Blaue, J. Starzonek and I. Vervuert. All coauthors revised the final manuscript and approved it for publication.

Impact of body weight gain on hepatic metabolism and hepatic inflammatory cytokines in comparison of Shetland pony geldings and Warmblood horse geldings

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ABSTRACT

Background: Non-alcoholic fatty liver disease is known as determining part of human obesity. The impact of body weight (BW) gain on liver metabolism has not been extensively investigated yet.

Objectives: To investigate hepatic alterations caused by increasing BW in ponies and horses.

Animals: A total of 19 non-obese equines (10 Shetland ponies, geldings; nine Warmblood horses, geldings).

Methods: Animals received 200% of their metabolizable maintenance energy requirements for 2 years. Serum alkaline phosphatase, glutamate dehydrogenase (GLDH), aspartate aminotransferase (AST), and gamma-glutamyl transferase activities and bile acids were analyzed several times during 2 years of hypercaloric diet. Hepatic lipid content and hepatic levels of the interleukin (IL)-6, tumor necrosis factor α (TNFα), cluster of differentiation (CD) 68, IL-1β, lipoprotein lipase (LPL), fatty acid-binding protein 1, chemerin and nuclear factor-κB mRNAs were assessed at the start of the study and after 1 and 2 years of excess energy intake.

Results: The mean ($\pm$SD) BW gain recorded during 2 years of excess energy intake was 29.9 $\pm$ 19.4% for ponies and 17 $\pm$ 6.74% for horses. The hepatic lipid content was not profoundly affected by increasing BW. Levels of the IL-6, TNFα, CD68 and IL-1β mRNAs did not change during BW gain. Levels of the chemerin mRNA increased significantly in both breeds (ponies: $P = 0.02$; horses: $P = 0.02$) in response to BW gain. Significant differences in serum GLDH and AST activities, serum bile acid concentrations and hepatic levels of the LPL mRNA were observed between ponies and horses at the end of the study.

Conclusions: Chemerin might represent an interesting marker for future equine obesity research. Interestingly, steatosis caused by increasing BW may occur later in the development of obesity in equines than in humans. Additionally, the hepatic metabolism exhibits differences between ponies and horses, which may explain in part the greater susceptibility of ponies to obesity-associated metabolic dysregulations.

INTRODUCTION

Obesity is an increasing problem in humans and companion animals, such as horses. Metabolic syndrome (metS) in humans is characterized by the accumulation of different symptoms, namely, obesity, increased circulating triglycerides, reduced high density lipoprotein–cholesterol concentrations, increased blood pressure and increased fasting glucose levels (*Alberti, Zimmet & Shaw, 2006*). Equines develop a similar but not identical symptom complex termed equine metabolic syndrome (EMS), which is defined by obesity, insulin dysregulation and a predisposition toward laminitis (*Frank et al., 2010*). EMS is furthermore linked to dyslipidemia, hyperleptinemia, arterial hypertension and low-grade inflammation (*Frank et al., 2010*). Susceptibility to EMS seems to be higher in pony breeds than in most horse breeds (*Johnson et al., 2010*).

The association between metS and the liver has been studied extensively in humans, as the livers of individuals suffering from metS exhibit frequently a form of steatosis termed nonalcoholic fatty liver disease (NAFLD). Some authors consider NAFLD to be the hepatic manifestation of metS (*Cortez-Pinto et al., 1999*). On the other hand, NAFLD also appears to be a precursor of metS and type 2 diabetes. Therefore, NAFLD seems to be a risk factor for the development of metS (*Lonardo et al., 2015*). Among other parameters, NAFLD is characterized by increased serum liver enzyme activities, such as aminotransferases (*Sookoian et al., 2016*). Hence, elevated serum liver enzyme activities emerged as potential biomarkers of an increased risk for developing metS and its related complications (*Devers et al., 2008*; *Zhang et al., 2015*). To the best of the authors' knowledge, the interrelations of equine obesity and the liver have not been studied yet. One study reported serum gamma-glutamyl transferase (GGT) and aspartate aminotransferase (AST) activities that exceeded the reference ranges in obese horses with a history of laminitis (*Chameroy et al., 2011*). The authors suggested that hepatic lipidosis caused the changes in liver metabolism.

In addition, high circulating levels of proinflammatory cytokines (e.g., tumor necrosis factor α (TNFα), interleukin-1 (IL-1), and interleukin-6 (IL-6)), markers of lipid metabolism (e.g., fatty acid-binding protein 1 (FABP1)) and adipokines (e.g., leptin, chemerin) have been reported in equine and human obesity (*Vozarova et al., 2001*; *Bozaoglu et al., 2007*; *Vick et al., 2007*; *Shi et al., 2012*; *Qu, Deng & Hu, 2013*). Adipose tissue is thought to be the main site of production of these adipokines (*Hotamisligil, Shargill & Spiegelman, 1993*; *Arner, 2005*; *Blüher, 2012*) although resident liver macrophages (Kupffer cells) may also play a prominent role (*Baffy, 2009*). Likewise, chemerin, another potent marker of inflammation, seems to be synthesized in the liver rather than in the visceral adipose tissue of human patients with liver cirrhosis (*Weigert et al., 2010*). However, researchers have not determined whether hepatic levels of the above-mentioned mediators might be affected by equine obesity.

The aim of the present study was to investigate changes in serum liver enzyme activities, serum bile acids (BA), liver lipid content and hepatic mRNA levels of several markers

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of inflammation and lipid metabolism in the course of increasing body weight (BW) in equines. The comparison of Shetland ponies and Warmblood horses should elucidate the underlying reasons for the higher predisposition of pony breeds to metabolic derangements. We hypothesized that equine obesity is associated with hepatic alterations. Furthermore, we expected that liver metabolism exhibits different responses between ponies and horses during long-term excess energy intake.

MATERIALS AND METHODS

Animals

Ten Shetland ponies (geldings; mean age 6 ± 3 years, *Equus caballus*) and nine Warmblood horses (geldings; mean age 10 ± 3 years, *E. caballus*) owned by the Institute of Animal Nutrition, Nutrition Diseases and Dietetics of the Leipzig University were included in the study. All animals were supposed to be adult, therefore we included equines older than 3 years and younger than 15 years. We decided to use only geldings to exclude the influence of gender related differences such as the sexual cycle in mares. Prior to the study, pituitary pars intermedia dysfunction was excluded by measuring adrenocorticotrophic hormone (ACTH) levels after 8 h of fasting. An experienced clinician (CG) confirmed the absence of clinical or radiological signs of previous or acute laminitis of the front feet in all animals. The animals were bedded on straw in individual box stalls and were turned out onto a dry lot for approximately 5 h a day. The animals were adapted to the experimental conditions 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).

Study design

The study was conducted from October 2015 until December 2017. Ponies and horses initially received meadow hay and a commercial mineral supplement to meet or exceed their energy and nutrient requirements during maintenance according to the guidelines of the Society of Nutrition Physiology (GfE 2014) (*Flachowsky et al., 2014*). The basal state of the animals was assessed in October 2015 (t0) by examination of serum liver enzyme activities, serum BA, serum amyloid A (SAA), plasma glucose, serum insulin, serum triglycerides (TG), serum non-esterified fatty acids (NEFA) concentrations, by conduction of a combined glucose insulin test (CGIT) (*Eiler et al., 2005*) and by liver tissue sampling. Following the collection of these initial data, the animals underwent a feeding period by receiving 200% of their metabolizable energy (ME) maintenance requirements according to the GfE (2014) under conditions of gradual adaptation (*Flachowsky et al., 2014*). A total of 60% of the energy intake was supplied by hay, and 40% was provided by a concentrate (Pavo Pferdenahrung GmbH, Vechta Langförden, Germany). For dietary intake of nutrients and intake of ME see Table 1. BW, body condition score (BCS) and cresty neck score (CNS) were monitored weekly. Energy intake was adapted monthly to the current BW. During the feeding period further data were collected in July 2016 (t1),

Schedlbauer et al. (2019), *PeerJ*, DOI 10.7717/peerj.7069

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Table 1 Estimated dietary intake per equine on a daily basis and calculated dietary composition during the whole feeding period (data are presented as mean ± SD).			
Variable		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 intake (% of dry matter intake)	Crude fat	4.42 ± 0.42	4.70 ± 0.41
	Crude protein	9.07 ± 1.85	9.20 ± 1.80
	Crude fiber	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
ME intake (% of maintenance requirements)		199 ± 0.20	185 ± 13.1

October 2016 (t2), April 2017 (t3), July 2017 (t4) and December 2017 (t5). At all time points blood samples were obtained for assessments of serum liver enzyme activities and serum BA. In addition to t0, SAA, plasma glucose, serum insulin, serum TG and serum NEFA concentrations were analyzed at t2 and t5. Additionally, at t2 and t5 the insulin sensitivity was assessed by performing a CGIT (*Eiler et al., 2005*) and a lipopolysaccharide (LPS) challenge was conducted followed by liver tissue sampling 15 h later.

Blood sampling

Blood samples for assessment of serum insulin, SAA, serum NEFA, serum TG and plasma glucose concentrations were collected at t0, t2 and t5. Blood samples for analysis of serum liver enzyme activities and serum BA concentrations were obtained at t0, t1, t2, t3, t4 and t5. After 8 h of fasting, a 14-gauge-catheter (Milacath; Mila International, Florence, KY, USA) was aseptically placed into the jugular vein of ponies and horses. Blood samples were collected in tubes containing coagulation activator (Monovette; Sarstedt AG, Nuembrecht, Germany) and centrifuged at $865 \times g$ for 10 min after 30 min of clotting time for assessments of serum insulin levels, liver enzyme activities, BA, SAA, NEFA and TG concentrations. Blood samples for plasma glucose assessments were collected in tubes containing sodium fluoride (S-Monovette; Sarstedt AG, Nuembrecht, Germany) and immediately centrifuged at $865 \times g$ for 10 min. Serum and plasma samples were gradually frozen from −20 to −80 °C and stored at −80 °C until analysis.

CGIT

The CGIT was conducted at t0, t2 and t5 and consisted of rapid IV administration of 150 mg/kg BW of glucose (40% anhydrous glucose; WDT, Garbsen, Germany) and 0.1 U/kg BW of insulin (Humulin R; Lilly USA, Indianapolis, IN, USA) mixed with three mL of 0.9% saline as adapted from *Eiler et al. (2005)*. Blood samples were collected before and 1, 5, 15, 25, 35, 45, 60, 75, 90, 105, 120, 135 and 150 min after insulin injection in tubes containing sodium fluoride (S-Monovette; Sarstedt AG, Nuembrecht, Germany) or a coagulation activator (Monovette; Sarstedt AG, Nuembrecht, Germany). Tubes containing a coagulation activator were centrifuged after a clotting time of 30 min, whereas tubes containing sodium fluoride were immediately centrifuged at

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865×g for 10 min. Plasma and serum samples were aliquoted and gradually frozen from −20 to −80 °C.

LPS challenge

The LPS challenge was performed 3–5 days after initial blood sampling. A 14-gauge-indwelling catheter (Milacath; Mila International, Florence, KY, USA) was aseptically inserted into the jugular vein. LPS (*Escherichia coli* 055:B5, 1,000 ng/mL, Sigma-Aldrich Chemie GmbH, München, Germany) (diluted in 500 mL/1,000 mL of 0.9% saline for the ponies/horses) was infused at a dosage of 10 ng/kg BW over 30 min. The animals were monitored for 3 h, using a modified pain score described by *Bussieres et al. (2008)*, which grades 13 different parameters on a scale from 0 (physiologic) to 3 (pathologic). Examined parameters were for example the rectal temperature, appetite and abdominal discomfort. Fourteen hours after the LPS infusion, blood samples were collected in tubes containing coagulation activator (Monovette; Sarstedt AG, Nuembrecht, Germany) for SAA determination. The tubes were centrifuged at 865×g for 10 min after 30 min of clotting time. Serum was harvested and gradually frozen from −20 to −80 °C.

Liver tissue sampling

The animals were sedated with 0.04 mg/kg BW romifidine (Sedivet®; Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim am Rhein, Germany) and 0.03 mg/kg BW butorphanol (Alvegesic®; CP-Pharma Handelsgesellschaft GmbH, Burgdorf, Germany) 15 h after the LPS challenge. Diazepam at a dose of 0.08 mg/kg BW (Diazepam-Lipuro®; Laboratoire TVM, Lempdes, France) and three mg/kg BW ketamine (Ursotamin®; Serumwerk Bernburg AG, Bernburg, Germany) were administered to induce general anesthesia. Inhalation anesthesia was maintained with isoflurane (CP-Pharma Handelsgesellschaft GmbH, Burgdorf, Germany). A 20-cm ventral midline incision was performed cranial to the umbilicus after aseptic preparation. Liver tissue (~two g) was collected using biopsy forceps. Additionally, adipose tissue was collected from several locations for another part of the study. One part of each tissue biopsy specimen was immediately flash frozen in liquid nitrogen (−196 °C) and stored at −80 °C. The second part was stored in formalin. The animals were orally administered 0.55 mg/kg BW flunixin twice a day for 3 days after surgery (Flunidol®; CP-Pharma Handelsgesellschaft GmbH, Burgdorf, Germany).

Determination of BW

Body weight was obtained weekly using an electronic scale system for large animals (scale system: Iconix FX 1, Texas Trading, scale precision: 0.5 kg).

BCS and CNS

Body condition score (*Carroll & Huntington, 1988*) and CNS (*Carter et al., 2009a*) were assessed on a scale ranging from 0 to 5 points. BCS and CNS were graded weekly by two independent evaluators (CS and DB). A mean of these two evaluators was calculated.

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Analysis of blood samples

Plasma ACTH levels were analyzed by a commercial laboratory using a chemiluminescence immunoassay (IDEXX GmbH, Ludwigsburg, Germany).

Serum liver enzyme activities (alkaline phosphatase (ALP), glutamate dehydrogenase (GLDH), AST, and GGT) and serum BA, TG and NEFA concentrations were analyzed using an automated chemistry analyzer (Roche Cobas C311; Roche Diagnostic GmbH, Mannheim, Germany).

SAA levels were determined by turbidimetry (ABX Pentra 400 analyzer, ABX Horiba; Axonlab, Montpellier, France).

Plasma glucose concentrations were determined using the GOD/POD method.

Serum insulin levels were analyzed using an immunoradiometric assay (IRMA, 125I; Demeditec Diagnostics GmbH, Kiel, Germany).

Histological staging of hepatic steatosis

Hematoxylin-eosin staining was routinely performed on all liver biopsies and analyzed by an experienced histologist (MG). Steatosis was graded as follows: <5% lipid content of liver parenchyma: 0; 5–33%: 1; >33–66%: 2; >66%: 3.

Analysis of hepatic mRNA levels

RNA was isolated using a commercial kit (RNeasy Lipid Tissue Mini Kit and Qiacube; Qiagen, AMBION, Inc., Germantown, MD, USA) according to the manufacturer's protocol. The RNA concentration was measured with a spectrophotometer (NanoVue® Plus; Healthcare Biosciences AB, München, Germany). RNA quality was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Two micrograms of RNA were transcribed into cDNAs in a thermocycler (Engine Peltier Thermal Cycler; Bio-Rad Laboratories GmbH, München, Germany) using two master mixes: (1) random primers and dNTP and (2) SuperScript II RT, 5x First Strand Buffer, and 0.1 M DTT (Thermo Fisher Scientific Inc., Schwerte, Germany). The genes of interest were IL-1β, IL-6, TNFα, cluster of differentiation 68 (CD68), chemerin, nuclear factor-κB (NF-κB), lipoprotein lipase (LPL) and FABP1. The 18S rRNA, hypoxanthine phosphoribosyltransferase 1 (HPRT1) and ribosomal protein L32 (RPL32) were chosen as reference genes (see Table 2 for primer sequences). Reference gene mRNAs were not altered by BW gain (Pfaffl et al., 2004). An RNA-probe was used for 18S rRNA quantification. A standard Taqman program (7500 Real Time PCR System; Thermo Fisher Scientific Inc., Schwerte, Germany) was performed for qPCR, with minor modifications. Two master mixes were utilized: Power SYBR Green PCR Master Mix for the genes detected with primers and Taqman Universal PCR Master Mix for the 18S RNA (Thermo Fisher Scientific Inc., Schwerte, Germany). The genes of interest were normalized to the geometric means of the three reference genes 18S, HPRT1 and RPL32.

Statistics

The data were analyzed using a statistical software program (STATISTICA, version 12, RRID:SCR_014213; StatSoft GmbH, Hamburg, Germany). The data were analyzed for

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Table 2 Primer sequences used to analyze the levels of the genes of interest and reference genes.

	Forward (5′-3′)	Reverse (3′-5′)
IL-6 *Ungru et al. (2012)*	CCACCTCAAATGGACCACTACTC	TTTTCAGGGCAGAGATTTTGC
TNFα *Figueiredo et al. (2009)*	AAAGGACATCATGAGCACTGAAAG	GGGCCCCCTGCCTTCT
CD68 *Ungru et al. (2012)*	CTTTGGGCCAAGTTTCTCTTGT	AAGAGGCCGAGGAGGATCAG
HPRT1 *Bogaert et al. (2006)*	GGCAAAACAATGCAAACCTT	CAAGGGCATATCCTACGACAA
RPL32 *Bogaert et al. (2006)*	AGCCATCTACTCGGCGTCA	TCCAATGCCTCTGGGTTTC
IL-1β[#]	CGGCAATGAGAATGACCTGT	GCTTCTCCACAGCCACAATG
LPL[#]	ATTGTGGTGGACTGGCTGT	GCTCCAAGGCTGTATCCCAA
FABP1[#]	CAAGATCACCATCACCACAGG	GTCACAGACTTGATGCCTTTGA
Chemerin[#]	CATGGGAGGAAGCGGAAATG	CAGCTGAGCCTGTGTCTCTA
NF-κB[#]	GCTTTGTGACAAGGTGCAGA	ACGATCATCTGTGTCTGGCA

Notes:
Five qPCR primers were newly designed and five primers were obtained from published data.
[#] Designed using http://primer3.ut.ee/. The specific equine cDNA sequences were provided by http://www.ensembl.org/index.html and the generated primers were validated in http://eu.idtdna.com/calc/analyzer to confirm the absence of hairpins, homodimers and heterodimers. The designed primers were created with two different modifications for each gene of interest and the more suitable primer was selected in preliminary tests. Primers were synthesized by biomers.net GmbH.

normal distributions using the Shapiro–Wilks test. ANOVA with repeated measurements was performed to analyze plasma glucose, serum insulin, serum TG and serum NEFA concentrations. Fisher's Least Significant Difference test was performed to identify significant differences. BCS, CNS, serum liver enzyme activities, serum BA concentrations, liver lipid content and mRNA levels of genes of interest were analyzed using nonparametric tests. Friedman's ANOVA was used to analyze the effect of time. When significant differences were observed, the Wilcoxon signed rank test with Bonferroni's correction was performed as post hoc test. The effects of the breed on nonparametric data were analyzed using the Mann–Whitney U test. Correlations among variables were examined by calculating Spearman's correlation coefficients. Pain score values of each parameter at LPS challenge were added and described descriptively. Statistical significance was set to $P < 0.05$.

RESULTS

Prior to the initiation of excess energy intake, all animals were assessed as metabolically healthy, according to the ACTH concentrations and results of the CGIT.

The 2 years of excess energy intake caused a significant increase in mean BW (± SD) of 29.9 ± 19.4% for ponies ($P = 0.0002$) and 17 ± 6.74% ($P = 0.00004$) for horses. Throughout the study, no significant differences in BW gain (%) could be found between the breeds. One pony developed an episode of laminitis during the second year of excess energy intake. Therefore, final samples were collected before the end of the study (July 2017) after complete recovery of clinical signs (pounding digital pulse, lameness). The pony received opioids once for pain relief 7 days before data collection. Laminitis occurred additionally in one horse at the end of the second year of excess energy intake. This horse received non-steroidal anti-inflammatory drugs for pain relief 20 days before sample collection. Time point of sampling was in accordance with the study design in

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Table 3 BCS and CNS in ponies and horses during 2 years of excess energy intake (data are presented as medians and 25th/75th percentiles).

Breed	Score	t0	t2	t5
Ponies	BCS	2.3 (1.2/3.4)[a]	3.6 (3.4/3.7)[a]	3.9 (3.7/4.2)[b]
	CNS	2.5 (0.8/3)[a]	2.8 (2.5/3.0)[a]	3.5 (3.3/4.0)[b]
Horses	BCS	2.7 (2.1/3.2)[ab]	3.6 (3.5/3.6)[b]	3.8 (3.7/3.9)[c]
	CNS	2 (1.8/2.3)[a]	2.8 (2.8/3.0)[b]	3.5 (3.5/4.0)[c]

Note:
Different superscript letters indicate significant differences within a row.

Table 4 Plasma glucose (mmol/L), serum insulin (µU/mL), serum NEFA (µmol/L) and serum TG (mmol/L) concentrations recorded in ponies and horses during 2 years of excess energy intake (data are presented as means ± SD).

Parameter	t0		t2		t5	
	Ponies	Horses	Ponies	Horses	Ponies	Horses
Glucose (mmol/L)	3.53 ± 0.64[a]	4.08 ± 0.21[b]	3.93 ± 0.38[ab]	4.52 ± 0.23[b]	4.34 ± 0.86[b]	4.41 ± 0.48[b]
Insulin (µU/mL)	4.26 ± 1.36[ac]	6.32 ± 2.35[c]	7.93 ± 5.75[abc]	9.3 ± 3.18[abc]	13.9 ± 14.9[b]	15.1 ± 10.3[b]
NEFA (µmol/L)	119 ± 117[a]	337 ± 381[b]	208 ± 168[ab]	211 ± 89[ab]	352 ± 141[b]	247 ± 87[ab]
TG (mmol/L)	0.49 ± 0.19[a]	0.27 ± 0.09[bc]	0.41 ± 0.31[ab]	0.24 ± 0.05[c]	0.42 ± 0.2[ab]	0.31 ± 0.08[bc]

Note:
Different superscript letters indicate significant differences within a row.

the laminitic horse. No clinical signs of laminitis were present at this point. One pony developed hyperlipemia (serum TG: 14.4 mmol/L) at the end of the second year of excess energy intake beside good appetite. The pony was carefully monitored (e.g., appetite, behavior, lameness) and the pony recovered without medication within 14 days. Samples were collected after serum TG concentrations returned to the baseline value. The animals did not suffer from additional health problems due to excess nutrition such as colic. BCS and CNS increased significantly in ponies and horses during the 2 years of excess energy intake (Table 3). No significant differences in BCS and CNS were observed between the breeds. The CNS of the laminitic pony (4.5 points) was greater than the median of the respective cohort at t5, whereas the CNS of the laminitic horse (3 points) remained within the median of the respective cohort. The BCS and CNS of the lipemic pony were within the median of the cohort of the ponies (BCS: 3.75; CNS: 4).

Plasma glucose and serum NEFA concentrations were significantly lower in ponies compared to horses at t0. With BW gain plasma glucose and serum NEFA concentrations increased in ponies, but not in horses. No significant differences between the breeds were observed at t2 and t5 concerning these parameters (Table 4). Basal serum insulin concentrations increased significantly in both breeds from t0 to t5. The ponies showed significantly higher serum TG concentrations than horses at t0 and t2 (Table 4). Mean SAA concentrations in ponies and horses were below the reference range of 2.7 µg/mL at all three data collection points.

The sum of pain score points increased subsequently to the LPS infusion in the animals from basal mean values (t0: 1.4; t2: 0.5; t5: 1.5) to mean maximum values (t0: 6.1; t2: 6.7;
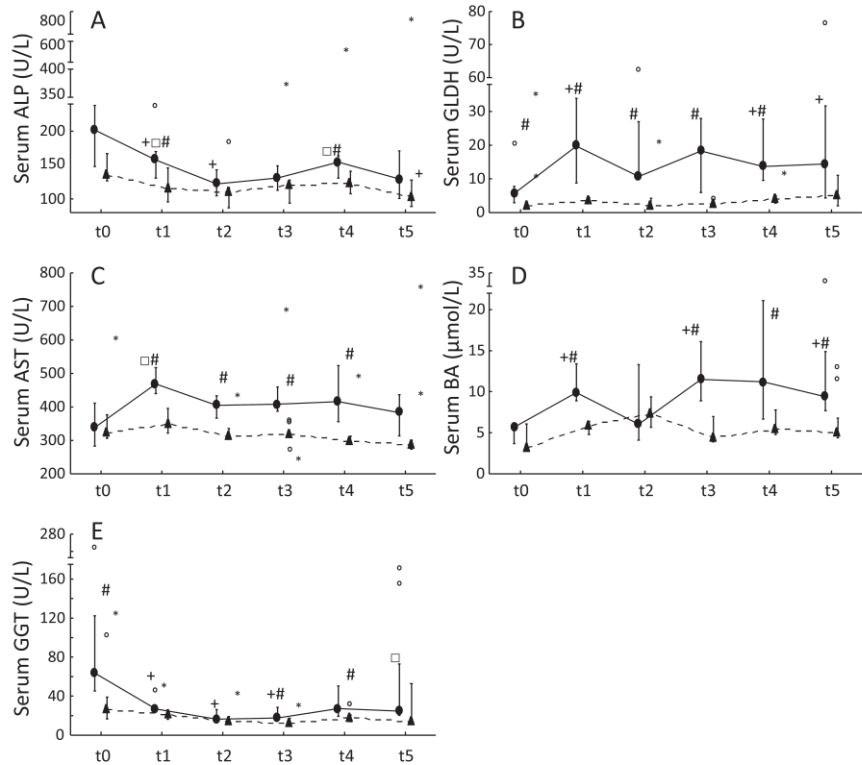
Schedlbauer et al. (2019), *PeerJ*, DOI 10.7717/peerj.7069

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Figure 1 Serum liver enzyme activities and serum BA concentrations in ponies and horses during 2 years of excess energy intake. Serum ALP activities (A), serum GLDH activities (B), serum AST activities (C), serum bile acids (D) and serum GGT activities (E) in ponies ($N = 10$) (filled circles) and horses ($N = 9$) (triangles) at t0, t1, t2, t3, t4 and t5 (reported as medians (filled circles or triangles), 25th/75th percentiles (whiskers), outliers (blank circles) and extreme values (asterisk)); + significantly different from t0; □ significantly different from t2; # significantly different between ponies and horses at the certain time point. Full-size ◩ DOI: 10.7717/peerj.7069/fig-1

t5: 6.1). Mean maximum values were reached 60 min after the LPS infusion at t0 and t2 and 30 min after the LPS infusion at t5.

Serum liver enzyme activities

Serum ALP and GGT activities were significantly decreased in ponies after 1 year of excess energy intake. In ponies, serum GLDH activities and BA concentrations increased significantly from t0 to t5. Furthermore, significantly higher serum GLDH activities (t0: $P = 0.04$; t1: $P = 0.00002$; t2: $P = 0.02$; t3: $P = 0.00002$; t4: $P = 0.001$), serum AST activities (t1: $P = 0.0007$; t2: $P = 0.003$; t3: $P = 0.001$; t4: $P = 0.02$) and BA concentrations (t1: $P = 0.0004$; t3: $P = 0.001$; t4: $P = 0.03$; t5: $P = 0.03$) were observed in ponies compared to horses. The horses showed no significant increases in serum liver enzyme activities but exhibited a significant decrease in serum ALP activity from t0 to t5 (Fig. 1). Serum ALP

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Table 5 Staging of hepatic steatosis in ponies and horses during 2 years of excess energy intake (data are presented as numbers and as percentages of the breed).

Steatosis stage	t0		t5	
	Ponies	Horses	Ponies	Horses
0	8/10 (80%)	6/9 (66.6%)	6/10 (60%)	8/9 (88.9%)
1	2/10 (20%)	3/9 (33.3%)	2/10 (20%)	1/9 (11.1%)
2	0/10 (0%)	0/9 (0%)	1/10 (10%)	0/9 (0%)
3	0/10 (0%)	0/9 (0%)	1/10 (10%)	0/9 (0%)

activity, BA concentrations and GGT activity increased in the lipemic pony during the 2 years of excess energy intake (ALP: 6.2-fold increase; BA: sixfold increase; GGT: 2.3-fold increase). Serum liver enzyme activities and BA concentrations in the laminitic equines remained within the reference ranges, (*Köller, Gieseler & Schusser, 2014*) except for serum GGT activity at t5 in the laminitic pony (GGT = 73.1 U/L).

Histological staging of steatosis

At t5 the percentage of individuals exhibiting a hepatic lipid content of more than 5% increased in ponies and decreased in horses. However, the majority of ponies and horses showed a constant steatosis grade 0 (Table 5). The pony suffering from hyperlipemia showed stage 3 steatosis, with more than 66% lipid-loaded hepatocytes at t5 (steatosis stage at t0: 0). Both laminitic equines steatosis stage 0 at t0. The laminitic horse stayed at steatosis stage 0 throughout the study, but the laminitic pony showed steatosis stage 1 at t5.

Hepatic mRNA levels of genes of interest

Levels of the TNFα, IL-6, FABP1 and CD68 mRNAs were not significantly altered in ponies and horses throughout the observation period. Hepatic levels of the chemerin mRNA remained constant from t0 to t2 in both breeds. In ponies, the hepatic level of the chemerin mRNA increased significantly from t2 to t5. The horses showed a significant increase of the level of the chemerin mRNA from t0 to t5 and from t2 to t5 (Fig. 2). Levels of the NF-κB mRNA decreased significantly in horses from t2 to t5 ($P = 0.02$) and remained unchanged in ponies throughout the study. Regarding the breed-specific differences, the ponies showed significantly higher hepatic levels of the LPL ($P = 0.005$), NF-κB ($P = 0.01$) and IL-1β ($P = 0.045$) mRNAs compared with the horses at t5. The lipemic pony showed a higher level of the LPL mRNA than the median of the pony cohort (t0: 3.1-fold higher; t2: 2-fold higher; t5: 2.5-fold higher) at all time points. Furthermore, the level of FABP1 mRNA was 2.5-fold higher at t5 compared to the median of the pony cohort. No further notable deviations in the levels of the genes of interest in the laminitic and lipemic equines from the median values of the cohort were observed.

Significant correlations between the level of the LPL mRNA and serum BA, the hepatic lipid content and level of the CD68 mRNA were analyzed. Levels of the chemerin mRNA displayed significant correlations with the BCS, CNS and level of the NF-κB mRNA (Table 6). In ponies, a negative correlation was identified between serum ALP activity and
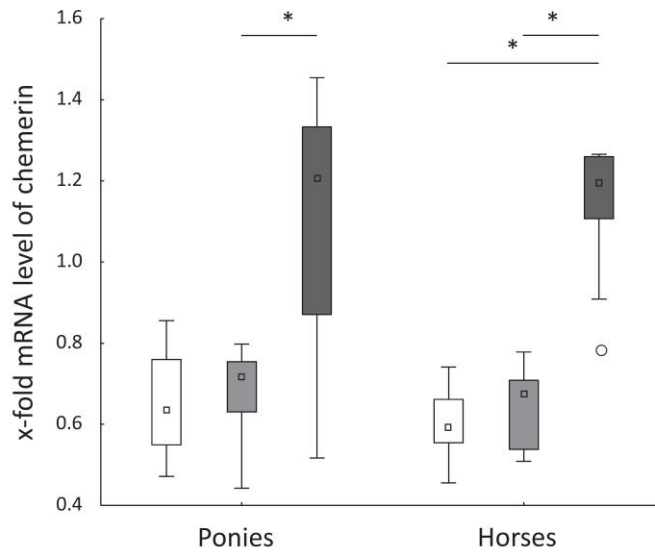
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Figure 2 Hepatic chemerin mRNA level in ponies and horses. Fold changes in the hepatic levels of the chemerin mRNA at t0 (white), t2 (light gray) and t5 (dark gray) in ponies ($N = 10$) and horses ($N = 9$) (reported as medians (squares), 25th/75th percentiles (boxes), minimum and maximum values (whiskers), and outliers (circles)); significant differences are indicated by *, no significant differences between ponies and horses were observed.   Full-size 🖼 DOI: 10.7717/peerj.7069/fig-2

Table 6 Correlations between the level of the LPL and chemerin mRNAs with serum BA concentrations, hepatic lipid contents, BCS, CNS and the levels of the CD68 and NF-κB mRNAs.

Variables	Ponies ($N = 10$; $n = 30$)		Horses ($N = 9$; $n = 27$)	
	r^1	*P*-value	r^1	*P*-value
Chemerin × BCS	0.6	<0.001	0.5	0.005
Chemerin × CNS	0.6	<0.001	0.6	0.001
Chemerin × NF-κB	−0.2	0.2	−0.6	0.002
LPL × BA	0.4	0.02	−0.3	0.2
LPL × CD68	0.4	0.02	0.6	<0.001
LPL × hepatic lipid content	0.4	0.02	0.01	0.9

Note:
r^1 = Spearman's correlation coefficient.

age ($r = -0.4$; $P = 0.01$). No significant correlation between serum ALP activity and age was detected in horses.

DISCUSSION

Previous studies of equines have been conducted to evaluate the impact of obesity on metabolic characteristics and inflammatory markers in adipose tissue (*Vick et al., 2007*; *Burns et al., 2010*). To the best of the authors' knowledge, this study is the first to investigate obesity associated hepatic alterations in equines. The study design was further

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applied to compare ponies and horses during a long-term feeding period of excess energy intake. However, in comparison with previous studies (*Carter et al., 2009b*; *Siegers et al., 2018*) our equines gained less BW during excess energy intake. In the studies of *Carter et al. (2009b)* and *Siegers et al. (2018)*, the energy intake was provided by approximately 60% concentrate and 40% roughage. In contrast, the energy intake in the present study was covered by 40% concentrate and 60% roughage. Therefore, the diet of the present study contained less starch and more fiber in comparison to the aforementioned studies. These varying diet compositions may have caused the differences in BW gain. We have used this type of diet (1) for welfare reasons during a long-term observation period and (2) its closer relation to practical feeding regimens.

In humans, steatosis is considered a hepatic manifestation of metS. A standardized procedure for assessment of liver fat content is important, as the hepatic lipid content may vary between different hepatic regions. In the present study, liver sampling was conducted at the same site at the three data collection time points by laparotomy. Using this method, a standardized follow-up of the same area of the liver was possible. But, sampling of liver tissue in vivo is always restricted to a small hepatic region. However, in cows, the liver fat content differed less than 2% between different liver lobes, suggesting a homogenous distribution of fat (*Gerspach et al., 2017*). In consequence, the used liver sampling procedure is likely to provide reliable insight in hepatic fat storing in obesity. According to our hypothesis, the percentage of ponies exhibiting a hepatic lipid content of more than 5% increased from 20% at t0 to 40% at the end of the study. But unexpected, the percentage of horses with hepatic lipid content of less than 5% increased from 67% at t0 to 89% at the end of the study. These results are in line with findings in NEFA concentrations, which increased during the study in ponies but not in horses. As 60–80% of liver stored lipid is derived by circulating free fatty acids, the differences of NEFA concentrations between ponies and horses might have contributed to breed related differences in steatosis stages. The results of serum NEFA concentrations are described in detail by *Blaue et al. (2019)*. Concluding, ponies seem to be more susceptible to hepatic lipid accumulations in early stages of obesity according to the histological evaluation. Furthermore, we speculated that horses might not develop a steatotic liver in association with early obesity in contrast to humans. Healthy humans showing a BW gain of 5–15% significantly increased the liver fat content by 2.5-fold within 4 weeks (*Kechagias et al., 2008*), highlighting the profound differences between equine and human metabolism. However, differences might be explained by the 3.5-fold higher relative daily intake of fat in humans (*Kechagias et al., 2008*) compared to our equines. Accordingly, *De Meijer et al. (2010)* showed that the dietary fat content itself, independent from caloric intake, was a detrimental factor in the development of steatosis in mice. Despite the differences between ponies and horses, the majority of our equines did not develop steatosis within 2 years of BW gain. It has been described in Shetland ponies that subcutaneous tissue expandability is limited, while the expansion of the retroperitoneally adipose tissue proceeds (*Siegers et al., 2018*). It is one possible explanation that the expandability limit of the subcutaneous adipose tissue was not reached in the present study and therefore, fat was not stored extraordinary in retroperitoneal and intraabdominal sites like the liver.

The individual pony which developed hyperlipemia in the present study increased steatosis from stage 0 at t0 to stage 3 at the end of the study. Interestingly, this pony did not show the most prominent increase in BW, BCS or CNS. However, the question of whether the liver was steatotic before the onset of hyperlipemia or whether the lipid mobilization caused the elevated hepatic lipid content remained open.

The levels of the LPL and FABP1 mRNAs in the liver were determined to elucidate the role of hepatic fat metabolism in obesity. *Pardina et al. (2009)* described a significant increase in hepatic LPL mRNA levels in obese humans compared to healthy controls. The authors postulated that these changes contributed to the hepatic accumulation of TG, which favors steatosis. Accordingly, we identified a positive correlation between the hepatic level of the LPL mRNA and the hepatic lipid content in ponies. At t5, ponies showed a 2.4-fold higher level of the LPL mRNA compared to horses. We speculated that ponies may develop an increased risk of steatosis in cases of continuing the long-term excess energy intake. Data of increasing steatosis grade in the liver of ponies supported this assumption. FABP1 is known to facilitate the intracellular trafficking of long-chain fatty acids (*Glatz, Van Der Vusse & Veerkamp, 1988*). The level of the FABP1 mRNA is increased in humans with steatosis, probably as a compensatory mechanism for increased fat influx (*Higuchi et al., 2011*). Therefore, our expectation that the level of the FABP1 mRNA in mostly nonsteatotic equines would be unaltered was confirmed. Interestingly, the lipemic pony showed a three-fold elevation in the level of the LPL mRNA at t0 compared to the median of the ponies at t0. We speculated that the pony was already predisposed to developing a steatotic liver, even in the lean body condition. In this pony, high LPL mRNA level was maintained until t5, with 2.5-fold higher levels than the median of the ponies at the end of the study. Additionally, the pony showed a 2.5-fold higher level of the FABP1 mRNA than the median of the ponies at the end of the study, probably due to metabolic demands caused by high fat influx in the liver.

Besides liver lipid content, liver enzyme activities represent useful markers for hepatic metabolism. Serum ALP and GGT activities, exhibited either a significant decrease or remained unchanged in the present study. Serum ALP activities showed a significant decrease during the study in ponies and horses. According to *Gehlen, May & Venner (2010)*, elevated serum ALP activities might be associated with increased bone turnover in young horses. Therefore, changes in ALP activities in ponies and horses might be explained by age-related effects. Serum GLDH activity has interesting properties as marker of liver diseases, as GLDH is a liver-specific enzyme that is mainly located in the mitochondria in the centrolobular hepatocytes (*Schmidt & Schmidt, 1988*). We observed a significant increase in serum GLDH activity in the ponies from t0 to t5, but not in the horses. Serum GLDH activities in the ponies were 2.4-fold higher than the upper reference range of 8.9 U/L (*Köller, Gieseler & Schusser, 2014*) at the end of the study. Similar to serum GLDH activities, the ponies showed a significant increase in serum BA concentrations from t0 to t5. However, most of the ponies showed serum BA concentrations that remained within the reference range at the end of the study. While BA have long been known to mediate nutrient absorption, BA have recently emerged as signaling molecules for lipid and glucose metabolism (*Ma & Patti, 2014*). Furthermore, plasma BA levels

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were shown to exhibit positive correlations with insulin resistance and type 2 diabetes in humans (*Haeusler et al., 2013*). Therefore, BA are not only a sensitive marker of liver diseases but also an important marker of metS and NAFLD. As further noninvasive marker, serum AST activities are increased in patients with NAFLD (*Sookoian et al., 2016*). In the present study, ponies showed significant higher serum AST activities compared to horses at four time points. As changes of serum GLDH activity and serum BA during BW gain occurred in ponies but not in horses and serum AST activities were higher in ponies than in horses, it is speculated that the liver of ponies was more affected by early obesity compared to horses.

Serum GGT activity of the laminitic pony exceeded the reference range at the end of the study. Accordingly, *Chameroy et al. (2011)* observed elevated serum GGT activities in 64.3% of obese horses with a history of laminitis. In addition, the steatosis stage of the laminitic pony increased from stage 0 at t0 to stage 1 at t5. In contrast, the laminitic horse showed neither an increase in serum liver enzyme activities nor an increase in hepatic lipid content during the study.

We determined the hepatic mRNA levels of proinflammatory cytokines to investigate whether the liver contributed to low-grade inflammation concomitant to obesity (*Vick et al., 2007*). The most prominent change in hepatic mRNA levels as BW increased was found for chemerin. Chemerin has been identified as an adipokine in mouse, rat and human adipocytes (*Bozaoglu et al., 2007*; *Goralski et al., 2007*) and has a regulatory role in adipogenesis and adipocyte metabolism (*Goralski et al., 2007*). In addition to adipose tissue, chemerin is expressed in the liver as well (*Pohl et al., 2017*). Although substantial experimental evidence supports a proinflammatory role for chemerin (*Weigert et al., 2010*; *Chakaroun et al., 2012*; *Döcke et al., 2013*), other studies have suggested that chemerin might have anti-inflammatory properties (*Cash et al., 2008*; *Luangsay et al., 2009*). Consistent with these equivocal results, discrepancies exist regarding the association of chemerin and NAFLD. According to *Deng et al. (2013)*, rodents with NAFLD displayed decreased hepatic levels of the chemerin mRNA compared to control rodents without NAFLD. In contrast, the consumption of a high-fat diet increased hepatic mRNA levels of chemerin in mice compared to animals fed a standard diet (*Krautbauer et al., 2013*). Unfortunately, the authors did not provide information about the fat content of the diet. Additionally, the chemerin mRNA levels tended to be higher in the liver of humans with NAFLD (*Krautbauer et al., 2013*). These contradictory findings might be explained by the various pathways in which chemerin is involved. After secretion, chemerin is converted into proinflammatory or anti-inflammatory peptides by different proteases, as reviewed by *Yoshimura & Oppenheim (2008)*. To date, a study investigating chemerin has not been performed in equines. In the present study, the hepatic level of the chemerin mRNA increased significantly during 2 years of excess energy intake in ponies and horses. In contrast to the upregulation of chemerin, other proinflammatory factors, such as CD68, TNFα, IL-6 and IL-1β, were not different between lean and obese equines. Notably, we observed a significant negative correlation between hepatic levels of the chemerin and NF-κB mRNAs in horses but not in ponies. NF-κB is a well-known activator of the transcription of proinflammatory cytokines. This result highlights a

possible anti-inflammatory role for chemerin. In this context, *Pohl et al. (2017)* found a downregulation of the chemerin mRNA levels in livers of humans suffering from a progressive form of NAFLD compared to humans suffering from steatosis alone. Consequently, chemerin represents a potentially interesting marker for obesity associated hepatic alterations and should be the focus of future studies in equines. A limitation of this study was that changes in chemerin levels were not verified at the protein level.

CONCLUSION

We detected significant differences in parameters such as serum GLDH and AST activities, serum BA concentrations and levels of the LPL mRNA between ponies and horses. According to our hypothesis, these differences suggested that ponies may show a more pronounced dysregulation of hepatic metabolism in reaction to the early stages of obesity compared to horses. However, in contrast to our hypothesis, liver steatosis seemed not to be an integral part of the early stages of obesity, especially in horses, and may occur in ongoing equine obesity. Liver mRNA levels of well-established proinflammatory cytokines such as TNFα or IL-6 were not significantly upregulated in response to increasing BW. However, chemerin was identified as a potentially novel marker of the hepatic changes associated with obesity in equines. A longer period of BW gain or a higher degree of obesity might be necessary to obtain more significant findings for inflammation and steatosis in the liver.

ACKNOWLEDGEMENTS

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

Grant Disclosures

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Carola Schedlbauer performed the experiments, analyzed the data, prepared figures and/ or tables, authored or reviewed drafts of the paper, approved the final draft.

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- Dominique Blaue performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Martin Gericke conceived and designed the experiments, performed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Matthias Blüher conceived and designed the experiments, performed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Janine Starzonek performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Claudia Gittel conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Walter Brehm conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Ingrid Vervuert conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

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.

Data Availability

The following information was supplied regarding data availability:

The raw data is available in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.7069#supplemental-information.

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Supplemental material

Table 1: Descriptive data of the ponies and horses at the beginning of the study

		Shetland ponies	Warmblood horses
Sex		10 geldings	9 geldings
Age, years		6 ± 3	10 ± 3
Body weight, kg		118 ± 29	589 ± 58
Body condition score (BCS), 0-5 (CARROLL and HUNTINGTON 1988)		2.3 (1.2/3.4)	2.7 (2.1/3.2)
Cresty neck score (CNS), 0-5 (CARTER et al. 2009a)		2.5 (0.8/3)	2 (1.8/2.3)
Plasma adrenocorticotropic hormone (ACTH), pg/mL Threshold: 50 pg/mL		15.9 (12.8/17.1)	16.4 (15.1/19.6)
Leukocytes (G/L)	t0	7.87 ± 1.26	5.14 ± 0.72
	t2	6.18 ± 2.23	5.58 ± 1.6
	t5	6.93 ± 2.09	5.88 ± 1.12
Erythrocytes (T/L)	t0	6.15 ± 0.6	6.52 ± 0.43
	t2	6.53 ± 0.59	7.39 ± 0.8
	t5	6.87 ± 0.96	7.92 ± 0.98
Hemoglobin (mmol/L)	t0	6.17 ± 0.67	6.93 ± 0.43
	t2	6.58 ± 0.61	7.71 ± 0.72
	t5	6.93 ± 0.77	8.13 ± 0.69

[1]Mean of two independent evaluators. Age, BW, leukocytes, erythrocytes and hemoglobin are presented as mean (± SD); BCS, CNS and ACTH are presented as median (25th/75th percentiles)

Table 2: Plasma glucose concentrations (mmol/L) and serum insulin concentrations (µU/mL) for two sampling points (0 and 45 minutes after insulin bolus) during combined glucose insulin test (CGIT) at basal measurements (t0), after one year (t2) and after two years (t5) of excess energy intake

Variable	Sampling point	Breed	t0	t2	t5
Glucose (mmol/L)	0	Ponies	3.53 ± 0.63	3.93 ± 0.38	4.34 ± 0.86
		Horses	4.08 ± 0.20	4.52 ± 0.22	4.41 ± 0.47
	45	Ponies	3.79 ± 1.24	2.4 ± 1.08	3.25 ± 1.59
		Horses	3.72 ± 1.34	3.18 ± 0.98	3.89 ± 1.94
Insulin (µU/mL)	0	Ponies	4.26 ± 1.36	7.93 ± 5.75	13.9 ± 14.9
		Horses	6.32 ± 2.35	9.3 ± 3.18	15.1 ± 10.3
	45	Ponies	26.1 ± 13.1	37.6 ± 11. 1	62.2 ± 38.4
		Horses	37.5 ± 14.1	51 ± 16.2	85.6 ± 54.4

Data are expressed as mean ± SD

Table 3: Serum amyloid A (SAA) concentrations (µg/mL) at basal measurements (t0), after one year (t2) and after two years (t5) of excess energy intake in ponies and horses

Variable	Breed	t0	t2	t5
SAA (µg/mL)	Ponies	0.35 ± 0.5	0.24 ± 0.22	0.1 ± 0
	Horses	0.1 ± 0	0.1 ± 0	0.1 ± 0

Data are expressed as mean ± SD

Table 4: Hepatic mRNA levels of TNFα, IL-6, FABP1 and CD68 (x-fold) at basal measurements (t0), after one year (t2) and after two years (t5) of excess energy intake in ponies and horses

Variable	Breed	t0	t2	t5
TNFα (x-fold)	Ponies	0.62 (0.46/0.81)	0.69 (0.45/0.79)	0.73 (0.67/1.18)
	Horses	0.45 (0.37/0.51)	0.52 (0.33/0.71)	0.64 (0.49/0.77)
IL-6 (x-fold)	Ponies	0.29 (0.25/0.8)	0.27 (0.24/0.34)	0.62 (0.4/0.79)
	Horses	0.29 (0.25/0.33)	0.27 (0.17/0.38)	0.44 (0.26/0.53)
FABP1 (x-fold)	Ponies	0.71 (0.55/0.73)	0.4 (0.36/0.6)	0.68 (0.42/0.77)
	Horses	0.71 (0.42/0.81)	0.52 (0.49/0.64)	0.71 (0.5/0.94)
CD68 (x-fold)	Ponies	0.72 (0.65/0.83)	0.77 (0.48/1.15)	0.65 (0.52/0.76)
	Horses	0.53 (0.43/0.64)	0.53 (0.47/0.55)	0.49 (0.4/0.52)

Data are presented as medians and 25th/75th percentiles

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

C. Schedlbauer, D. Blaue, J. Raila and I. Vervuert

Contributions

The study design was created by I. Vervuert. The ponies and horses were fed and groomed by S. Klemann, J. Starzonek, D. Blaue and me. Together with D. Blaue I determined the BW, BCS and CNS of all equines every week. Blood sampling for assessment of serum vitamin A, vitamin E and RBP4 concentrations was conducted by me, D. Blaue and J. Starzonek. Analysis of blood parameters was done by J. Raila and his laboratory assistance. Interpretation and statistical analysis of results was done by me. I wrote the first draft of all parts of the manuscript and it was revised by D. Blaue, J. Starzonek and I. Vervuert. All coauthors revised the final manuscript and approved it for publication.

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ORIGINAL ARTICLE

Animal Physiology and Animal Nutrition   WILEY

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

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.

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).

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$^{0.75}$ (Flachsowsky 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

Timepoint	Body weight (kg)		Intake of concentrate[a] (kg)		Intake of hay[b] (kg)	
	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
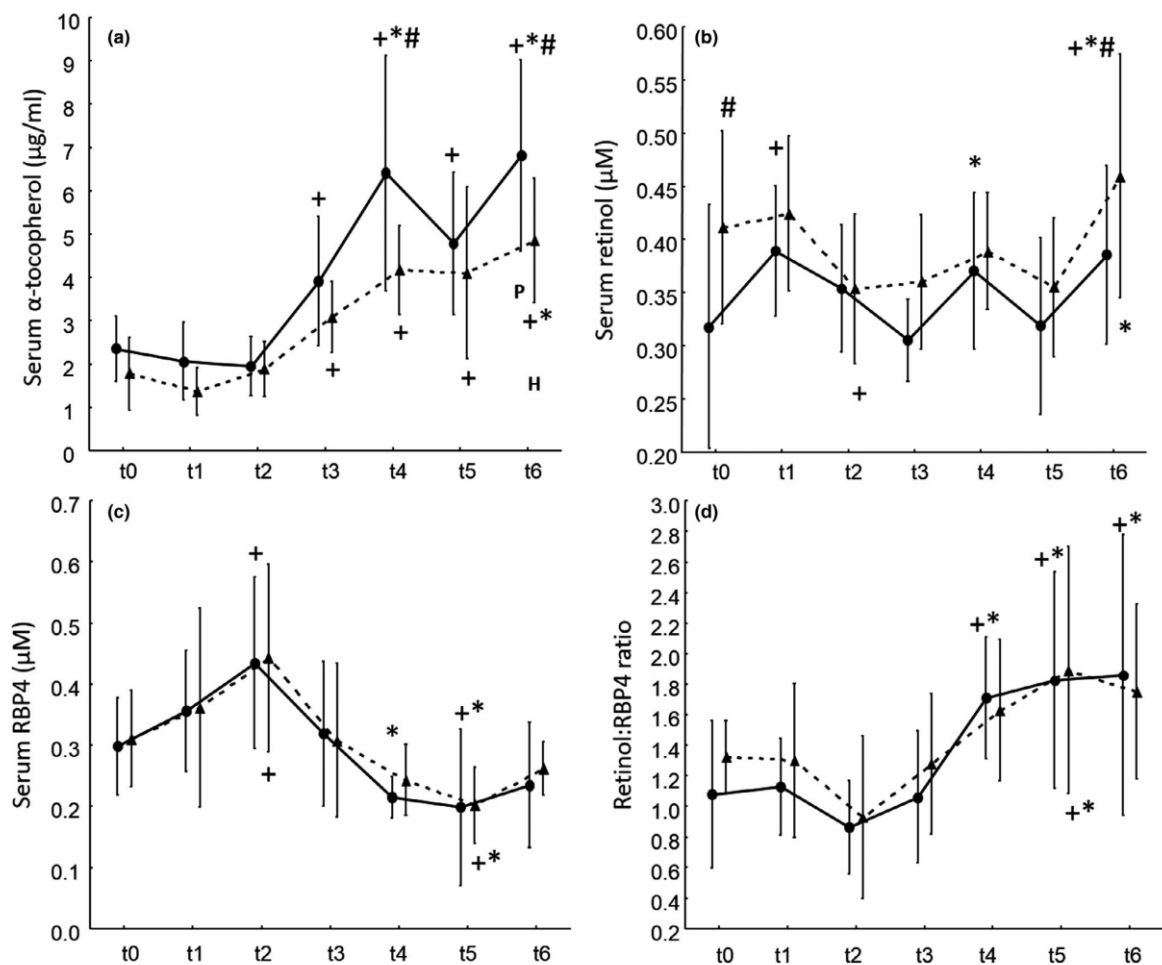
FIGURE 1 Effect of two years of 200% excess energy intake on serum α-tocopherol (a); breed: $p = .17$, time: $p < .001$, time × breed: $p = .09$; retinol (b); breed: $p = .81$, time: $p < .001$, time × breed: $p = .27$; RBP4 (c); breed: $p = .52$, time: $p < .001$, time × breed: $p = .9$; concentrations 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

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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.

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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4 Discussion

As expected, the experimental design of the present study induced a significant increase in BW, BCS and CNS in ponies and horses. Notably, the achieved BW gain of our equines was less than in previous studies (CARTER et al. 2009b, SIEGERS et al. 2018). CARTER et al. (2009) and SIEGERS et al. (2018) fed a ration in which energy intake was provided by approximately 60% concentrate and 40% roughage. In contrast, the energy intake in the present study was provided by 40% concentrate and 60% roughage. Furthermore, equines of our study were supplied with less starch and more fibre than the equines of the aforementioned studies. These differences in ration composition probably caused the lower BW gain in the present study. However, the ration was chosen out of equine welfare concerns during a long-term observation period and its closer relation to practical feeding regimens in horses. High grain rations have been associated for example with colic and laminitis (ROWE et al. 1994, LOPES et al. 2004).

4.1   Impact of body weight gain on hepatic lipid metabolism

The majority of our equines did not develop steatosis after two years of BW gain according to histological evaluation of the liver biopsies. This contrasts with a study of KECHAGIAS et al. (2008), who demonstrated a 2.5-fold increase of liver fat in lean and healthy humans following a BW gain of 5-15% within four weeks. It has to be noted that dietary fat content itself, independent from caloric intake, promotes the development of steatosis in mice (MEIJER et al. 2010). This might explain the discrepancy to the study of KECHAGIAS et al. (2008), as the humans consumed 3.5-fold the amount of fat as percent of total intake daily compared to our equines. It was further shown that the hepatic lipid metabolism of our equines was not profoundly affected by BW gain, as hepatic mRNA levels of LPL and FABP1 were unaltered. Hepatic mRNA levels of LPL were reported to increase in human obesity and thereby to promote steatosis (PARDINA et al. 2009). Increased hepatic mRNA levels of FABP1 in humans with steatosis were suggested to compensate for the increased fat influx (HIGUCHI et al. 2011). In the current study, the degree of obesity might have been too low to alter hepatic lipid accumulation and lipid metabolism. SIEGERS et al. (2018) reported a limited expandability of subcutaneous adipose tissue in Shetland ponies with excess fat storage occurring in retroperitoneal tissue instead. Therefore, BW gain of our equines might not have been sufficient to exceed the expandability of subcutaneous adipose tissue and subsequently, fat did not accumulate in other sites like abdominal adipose tissue or liver.

Despite finding no profound changes of hepatic lipid metabolism induced by increasing BW in our equines, we did detect moderate differences between ponies and horses. Ponies showed 2.4-fold higher hepatic mRNA levels of LPL compared to horses at the end of the study. According to PARDINA et al. (2009), this finding may increase the risk of the ponies to develop steatosis as consequence of enhanced hepatic uptake of circulating triglycerides. Histological evaluation of hepatic lipid content sustains this assumption. While the percentage of horses with hepatic

lipid content of more than 5% decreased marginally from the start of the study (33%) to the end of the study (11%), the percentage of ponies exhibiting a hepatic lipid content of more than 5% increased from 20% at the beginning of the study to 40% at the end of the study. The ponies therefore seemed to be more susceptible to hepatic lipid accumulation in early stages of obesity than were the horses.

4.2 Impact of body weight gain on serum liver enzyme activities and bile acids

Serum liver enzyme activities and serum BA concentrations represent useful markers for evaluation of liver metabolism. In the present study, serum AST, ALP and GGT activities were not altered by BW gain over two years in ponies and horses. However, ponies had higher serum AST activities over the course of the study compared to horses, but the serum AST activities of ponies were still within the reference range. Serum GLDH activities and serum BA increased in ponies during BW gain, but not in horses. Both parameters are specific for hepatic alterations. Bile acids are not only mediators for nutrient absorption, but also signaling molecules for lipid and glucose metabolism (MA and PATTI 2014). Plasma BA concentrations correlated positively with insulin resistance (HAEUSLER et al. 2013) and disease severity of NAFLD in humans (BECHMANN et al. 2013). We speculate that the livers of ponies were more affected by the development of obesity than the livers of horses, as BW gain did not alter any liver associated serum parameter in horses.

4.3 Impact of body weight gain on hepatic mRNA levels

Several human studies reported a link between pro-inflammatory cytokines and obesity associated NAFLD (HAUKELAND et al. 2006, MANCO et al. 2007, KAMARI et al. 2011) but this implication had not previously been investigated in equines. In the present study, hepatic mRNA levels of well-established pro-inflammatory cytokines (TNFα, IL-6, IL-1β) and macrophage marker CD68 were not affected by BW gain, either in ponies, or horses. Moreover, hepatic mRNA levels of NF-κB, decreased in horses at the end of the study. NF-κB is a transcription activator of pro-inflammatory cytokines, chemokines, inflammatory enzymes and adhesion molecules (BARNES 1997). In mice, hepatocellular inflammation with subsequent production of pro-inflammatory cytokines was induced by steatosis (CAI et al. 2005). Therefore, our findings of unaltered hepatic pro-inflammatory cytokines are in agreement with unaltered hepatic lipid content in most equines in this study. We speculate that the duration of obesity might have been too short or that the degree of obesity might have been insufficient to have detrimental effects on hepatic inflammatory pathways.

We also investigated chemerin which was described as adipokine with regulatory roles in adipogenesis and adipocyte metabolism (GORALSKI et al. 2007). The exact functions of chemerin are recently under discussion as some studies provided evidence for a pro-inflammatory role of chemerin (WEIGERT et al. 2010, CHAKAROUN et al. 2012), while

opposing studies proposed chemerin to exert anti-inflammatory properties (CASH et al. 2008, LUANGSAY et al. 2009). These discrepancies might be explained by the various pathways following the secretion of chemerin. Different proteases convert pro-chemerin into pro-inflammatory or anti-inflammatory peptides which are able to act through 3 different receptors with different functions (YOSHIMURA and OPPENHEIM 2008). According to opposing data of fundamental functions of chemerin, investigations on the associations of chemerin and NAFLD have provided equivocal results. For example, DÖCKE et al. (2013) reported a positive correlation of hepatic mRNA chemerin levels with obesity measurements, such as BMI and NAFLD severity in humans. Similarly, BW gain caused a significant increase of hepatic chemerin mRNA levels in ponies and horses in the present study. However, rodents with NAFLD had decreased hepatic chemerin mRNA levels compared to control rodents without NAFLD (DENG et al. 2013). Interestingly, horses but not ponies showed a negative correlation between hepatic mRNA levels of chemerin and NF-κB. This result implies a possible anti-inflammatory role for chemerin in conditions of increasing BW in horses. Since hepatic chemerin mRNA levels distinctly responded to BW gain in contrast to classical pro-inflammatory cytokines, chemerin may represent an interesting marker for future equine obesity research.

4.4 Impact of body weight gain on serum vitamin E (α-tocopherol) levels

The majority of previous studies in humans revealed negative associations between serum α-tocopherol levels and obesity (BOTELLA-CARRETERO et al. 2010). Authors suggested increased oxidative stress secondary to obesity as causative factor for the decrease of serum α-tocopherol. However, some contradictory studies reported positive correlations of serum α-tocopherol and obesity in humans (WALLSTRÖM et al. 2001). In our study, serum α-tocopherol concentrations had a robust positive association with α-tocopherol intake in both ponies and horses. This suggests a dietary cause for increasing serum α-tocopherol concentrations in the course of our study, rather than a BW gain impact. This statement is further strengthened as vitamin E intake of the ponies and horses was 2.5-3-fold above the maintenance requirements (FLACHOWSKY et al. 2014). The assumption of a dose-response relationship of vitamin E supplementation is supported by former studies. An almost linear dose-response relationship between vitamin E intake and blood concentrations was reported by RONÉUS et al. (1986), who supplemented different dosages of synthetic α-tocopherol over 112 days to clinically healthy Standardbreds. In another study high vitamin E supplementation resulted in higher serum α-tocopherol concentrations compared to a moderate vitamin E intake in adult equines (KIENZLE et al. 2003).

4.5   Impact of body weight gain on serum vitamin A (retinol) and RBP4 levels

Retinol, an intensively investigated parameter in human obesity research, is described as anti-inflammatory factor (REIFEN 2002) that also improves insulin sensitivity (MANOLESCU et al. 2010). Equivocal results have been found regarding the association of human obesity with serum retinol concentrations. Some studies reported decreased serum retinol concentrations in obese Caucasian individuals (BOTELLA-CARRETERO et al. 2010), whereas others described increasing levels of serum retinol with increasing body condition in Mexican-American children (GUNANTI et al. 2014). Other authors detected no differences in serum retinol concentrations between different BMI groups (MILLS et al. 2008). However, human studies are mainly limited by the lack of standardization of vitamin A intake. It has to be noted that serum retinol concentrations are maintained within a narrow range independent of the diet in individuals with adequate liver retinol stores. (MODY 2017). Serum retinol concentrations showed some minor fluctuations over the BW gaining period in ponies and horses, but changes were probably of little biological significance. Furthermore, the dietary intake of vitamin A and serum retinol concentrations were not correlated. It is likely that liver retinol stores were adequate in our ponies and horses, as supply of retinol was 1.7-2.4-fold above maintenance requirements.

Retinol is transported in the blood by RBP4, and alterations of vitamin A intake affect hepatic release of RBP4 in humans (BLANER 1989). RBP4, besides being a transport protein, has been identified as an adipokine in humans and horses (TAMORI et al. 2006, UNGRU et al. 2012). Human and equine studies provided equivocal results about the metabolic impacts of RBP4. On the one hand, a positive correlation of serum RBP4 concentrations and insulin resistance, obesity measurements, oxidative stress and the risk of developing metS has been reported in humans (LIU et al. 2014b). In agreement, a BW reduction program in obese ponies led to a decrease in serum RBP4 levels (UNGRU et al. 2012). On the other hand, human subjects with different BMI showed no differences in serum RBP4 (LEWIS et al. 2007). Furthermore, tail head RBP4 gene expression and BW were negatively associated in grazing mares (SELIM et al. 2015). In agreement with SELIM et al. (2015) we found an inverse relation between serum RBP4 and obesity in ponies and horses. Additionally, we detected a negative correlation between serum insulin and serum RBP4 in ponies. That finding is in contrast to the study of YANG et al. (2005), who stated that RBP4 favors insulin resistance in mice.

It has been recommended to include serum retinol concentrations, the most important determinant of RBP4, in the evaluation of RBP4 by calculating the serum retinol/RBP4 ratio (MILLS et al. 2008). Serum RBP4 concentrations were moderately different between obese and non-obese humans, while the differences between the groups became highly significant when using the retinol/RBP4 ratio. The retinol/RBP4 ratio decreased with increasing obesity (MILLS et al. 2008). In contrast to these previous results in humans, BW gain in the present study

induced an increase of the retinol/RBP4 ratio in equines. Furthermore, we did not detect a positive association between serum retinol and RBP4 concentrations. These findings indicate that the knowledge gained from human studies cannot be transferred to the equine. Further research is necessary to elucidate the interactions and physiological mechanisms of retinol and RBP4 in equines.

4.6   Laminitic equines

One pony and one horse developed laminitis at the end of the study. These two equines exhibited some differences compared to their respective cohorts. CARTER et al. (2009b) characterized specific cut-off values for insulin (>32 µU/mL), leptin (>7.3 ng/mL), BCS (≥7/9), CNS (≥4/5) for reproducible diagnostic accuracy for the prediction of pasture-associated laminitis. Supporting the importance of CNS, the CNS of the laminitic pony was 4.5 at the end of the study. However, the laminitic horse had a CNS of 3, which is below the cut-off value of CARTER et al. (2009b). In 64.3% of obese horses with a history of laminitis CHAMEROY et al. (2011) reported elevated serum GGT activities and speculated hepatic lipidosis as possible causative factor. In agreement with those results, serum GGT activities of the laminitic pony exceeded the reference range and furthermore, the liver biopsy showed steatosis stage 1 at the end of the study. On the other hand, liver enzyme activities and liver steatosis stage were within physiological ranges in the laminitic horse. As hepatic mRNA levels of pro-inflammatory cytokines in laminitic equines were not different in comparison to respective cohorts, it seems unlikely that hepatic inflammatory processes contributed to the development of laminitis.

Interestingly, we found 1.8 times lower- and 3.2-times lower serum α-tocopherol concentrations in the laminitic pony and the laminitic horse, respectively, compared to the mean of their respective cohorts. The equine digital laminae are highly susceptible to oxidative damage as they are lacking superoxide dismutase, an important antioxidant enzyme (LOFTUS et al. 2007). Therefore, systemic antioxidant capacity might be extraordinarily important for integrity of digital laminae. We speculated that decreased serum α-tocopherol concentrations in the affected pony and horse were induced by higher demands of antioxidants in the laminar tissue due to laminitis. This speculation agrees with the study of YIN et al. (2009), who stated that oxidative stress may play an important role in the laminar failure in laminitis. Consequently, laminitic equines might benefit from vitamin E supplementation dosages exceeding the 3-fold maintenance requirements. However, this speculation needs to be verified in further studies with more laminitic equines and a more detailed assessment of the very complex oxidant/antioxidant equilibrium.

4.7   Conclusion

In contrast to our original hypotheses, liver steatosis seemed not to be an integral part of early stages of equine obesity. However, ponies and horses exhibited breed-related differences in parameters such as steatosis stage, serum GLDH activities, serum BA concentrations and hepatic mRNA levels of LPL. In agreement with our original hypotheses, these differences might suggest that livers of ponies are more affected by early stages of obesity than in horses. These breed-related differences might be involved with the reported higher predisposition of pony breeds for metabolic disturbances. In contrast to our hypotheses, BW gain did not increase liver mRNA levels of pro-inflammatory cytokines such as TNFα or IL-6. However, chemerin significantly increased during BW gain in ponies and horses. Chemerin was therefore identified as a potentially interesting hepatic marker for equine obesity research, although it remains unknown whether chemerin may have anti- or pro-inflammatory properties. Serum α-tocopherol concentrations were less affected by BW gain but showed a strong dose-response relationship by the supplementation of synthetic α-tocopherol. Although the number of affected animals was limited, the decrease in serum α-tocopherol concentrations related to an episode of laminitis may reflect the higher demand of antioxidants in laminar tissue. This important finding needs further elucidation to specify the recommendation for vitamin E intake of laminitis prone equines.

5   Summary

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Impact of body weight gain on liver metabolism and selected fat-soluble vitamins in ponies and horses

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Introduction

Obesity is an increasing health problem in humans and domestic animals such as horses. Some equine breeds, such as pony breeds are predisposed to obesity and its consequences. The underlying reasons are not fully elucidated. It is known that human obesity goes along with fatty alterations of the liver, termed non-alcoholic fatty liver disease, which lead to hepatocellular inflammation. It is not known, however, whether equine obesity induces alterations of liver metabolism as well. In addition to hepatocellular inflammation, human obesity is accompanied by systemic inflammation and increased oxidative stress. Therefore, anti-inflammatory/antioxidant substances such as vitamin A (retinol) and vitamin E (α-tocopherol) have been intensively investigated in human research. Both vitamins were often described to decrease in obese conditions.

Aim of the study

The current investigation aimed to assess the impact of body weight (BW) gain in ponies and horses on several parameters: (1) serum liver enzyme activities and bile acids (BA), (2) hepatic lipid content, (3) hepatic messenger ribonucleic acid (mRNA) levels of markers of inflammation and lipid metabolism and (4) serum levels of retinol and α-tocopherol. Furthermore, we aimed to compare ponies and horses to identify possible reasons for the higher susceptibility of ponies for obesity related metabolic disturbances.

Materials and Methods

Ten Shetland ponies and 9 Warmblood horses, all non-obese, were fed 200% of their maintenance requirements for metabolizable energy over two years. Body weight, body condition score (BCS) and cresty neck score (CNS) were assessed weekly. Blood sampling was

Summary

conducted 6 times for analysis of serum liver enzyme activities (alkaline phosphatase (ALP), aspartate aminotransferase (AST), glutamate dehydrogenase (GLDH), gamma-glutamyl transferase (GGT)) and BA and 7 times for analysis of serum retinol and α-tocopherol during the experimental period. Three times during the study, liver tissue was sampled under general anesthesia via laparotomy. Liver biopsies were analyzed for lipid content by histological evaluation and for pro-inflammatory markers (nuclear factor-κB (NF-κB), interleukin-1β (IL-1β), IL-6, tumor necrosis factor α (TNFα), cluster of differentiation 68 (CD68), chemerin) and markers of lipid metabolism (lipoprotein lipase (LPL), fatty acid binding protein 1 (FABP1)) by real-time quantitative polymerase chain reaction (RT-qPCR). The data were analyzed using a statistical software program (STATISTICA, version 12, StatSoft GmbH, Hamburg, Germany). After analyzing normal distributions, the appropriate tests were applied with statistical significance at $P < 0.05$. 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.

Results

Ponies and horses showed a significant increase in BW (mean ± SD; ponies: 29.9 ± 19.4%; horses: 17 ± 6.74%), BCS (median (25th/75th percentiles); ponies: 157% (115/349); horses: 142% (128/192)) and CNS (median (25th/75th percentiles); ponies: 165% (123/500); horses: 200% (160/225)) due to the hypercaloric feeding over the course of the two years. Body weight gain did not induce steatosis in the majority of equines. Liver mRNA levels of IL-6, TNFα, CD68 and IL-1β did not change during BW gain. Body weight gain induced a significant increase of liver mRNA chemerin levels in ponies (x-fold increase: 1.89) and horses (x-fold increase: 2.04). Significant differences in serum GLDH activities, serum BA concentrations and hepatic mRNA levels of LPL were observed between ponies and horses at the end of the study. Serum α-tocopherol concentrations increased significantly in ponies and horses during BW gain and circulating α-tocopherol levels correlated positively with α-tocopherol intake. Serum retinol concentrations showed fluctuations during the feeding period without correlating with the intake of vitamin A.

Conclusions

Early equine obesity does not necessarily lead to steatosis and subsequent hepatocellular inflammation. Nonetheless, hepatic metabolism exhibits differences between obese ponies and horses. That might explain in part the greater susceptibility of ponies to obesity-associated metabolic disturbances. Additionally, chemerin was identified as an interesting marker for future equine obesity research. Body weight gain in equines does not seem to affect serum concentrations of retinol and α-tocopherol in equines fed above recommended intakes of these antioxidant vitamins.

6 Zusammenfassung

Carola Schedlbauer

Auswirkungen einer Körpergewichtszunahme auf den Leberstoffwechsel und ausgewählte fettlösliche Vitamine bei Ponys und Pferden

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Eingereicht im Mai 2020

60 Seiten, 3 Abbildungen, 13 Tabellen, 204 Literaturangaben

Schlüsselwörter: Leber, Steatosis, Adipositas, Chemerin, Serum Leberenzymaktivitäten, α-Tocopherol, Retinol-Binding Protein 4, Hufrehe

Einleitung

Adipositas ist ein zunehmendes Problem bei Menschen und Haustieren, z.B. in Pferden. Ponyrassen sind dabei besonders prädisponiert, wobei die Gründe bisher nicht abschließend geklärt werden konnten. Humane Adipositas geht mit einer fettigen Infiltration der Leber einher, die sogenannte Non-Alcoholic Fatty Liver Disease, welche zu einer hepatozellulären Entzündung führt. Es ist bisher nicht bekannt, ob Adipositas in Equiden auch zu hepatischen Veränderungen führt. Menschliche Fettleibigkeit ist zusätzlich mit systemischer Entzündung und gesteigertem oxidativen Stress verbunden. Das führte zu intensiven Untersuchungen von anti-inflammatorischen und antioxidativen Faktoren (z.B. Vitamin A - Retinol und Vitamin E - α-Tocopherol) in der humanen Adipositas Forschung. Viele Studien konnten ein Absinken von Vitamin A und Vitamin E in fettleibigen Menschen feststellen.

Ziele

Die vorliegende Studie sollte den Einfluss von zunehmendem Körpergewicht (KG) in Ponys und Pferden auf mehrere Parameter untersuchen: (1) Serum Leberenzymaktivitäten und Serum Gallensäuren (GS), (2) Leberfettgehalt, (3) hepatische messenger Ribonukleinsäure (mRNA) Level von Entzündungsmarkern und Markern des Lipidmetabolismus und (4) Serum Konzentrationen von Retinol und α-Tocopherol. Zusätzlich sollten Ponys und Pferde im Verlauf dieser Studie verglichen werden, um eventuelle Gründe für die Rasseprädisposition der Ponys für metabolische Störungen zu identifizieren.

Material und Methoden

Zehn Shetland Ponys und 9 Warmblut Pferde, die initial nicht adipös waren, wurden über 2 Jahre mit 200% des Erhaltungsbedarfes für umsetzbare Energie gefüttert. Die Entwicklung des

Zusammenfassung

KG, des Body Condition Scores (BCS) und des Cresty Neck Scores (CNS) wurde wöchentlich erfasst. Während der Fütterungsphase wurde zu 6 Zeitpunkten (ZP) Blut für die Bestimmung von Serum Leberenzymaktivitäten (Alkaline Phosphatase (ALP), Aspartat Aminotransferase (AST), Glutamat Dehydrogenase (GLDH), Gamma-Glutamyl Transferase (GGT)) und Serum GS entnommen und zu 7 ZP wurde Blut für die Analyse von Serum Retinol und α-Tocopherol gewonnen. An 3 ZP wurde durch Laparotomie Lebergewebe in Vollnarkose entnommen. Die Leberbiopsien wurden histologisch auf ihren Fettgehalt untersucht und mittels quantitativer Echtzeit Polymerase-Kettenreaktion (RT-qPCR) wurden die mRNA Level von Entzündungsmarkern (Nuclear Factor-κB (NF-κB), Interleukin-1β (IL-1β), IL-6, Tumor Nekrose Faktor α (TNFα), Differenzierungsgruppe 68 (CD68), Chemerin) und Lipid Metabolismus Markern (Lipoprotein Lipase (LPL), Fettsäuren Bindungsprotein 1 (FABP1)) bestimmt. Die Daten wurden mittels statistischem Software Programm ausgewertet (STATISTICA, version 12, StatSoft GmbH, Hamburg, Deutschland). Nach Prüfung auf Normalverteilung der Daten, wurden geeignete statistische Tests angewendet mit einem statistischen Signifikanzniveau bei $P < 0,05$. Die Tierschutzkommission des Bezirks Leipzig genehmigte das Projekt in Übereinstimmung mit deutschen Rechtsvorschriften (Nr. TVV 32/15).

Ergebnisse

Ponys und Pferde zeigten einen signifikanten Anstieg von KG (Mittelwert ± SD; Ponys: 29,9 ± 19,4%; Pferde: 17 ± 6,74%), BCS (Median (25./75. Perzentil); Ponys: 157% (115/349); Pferde: 142% (128/192)) und CNS (Median (25./75. Perzentil); Ponys: 165% (123/500); Pferde: 200% (160/225)) induziert durch die hyperkalorische Fütterung über 2 Jahre. Das ansteigende KG hat keine Steatosis in der Mehrheit der Equiden ausgelöst. Die mRNA Level von IL-6, TNFα, CD68 und IL-1β in der Leber wurden nicht beeinflusst. Die Leber mRNA Level von Chemerin sind signifikant angestiegen in Ponys (x-facher Anstieg: 1,89) und Pferden (x-facher Anstieg: 2,04). Signifikante Unterschiede zwischen den Rassen hinsichtlich der Serum GLDH Aktivitäten, Serum GS Konzentrationen und der hepatischen mRNA LPL Level konnten festgestellt werden. Die Serum α-Tocopherol Konzentrationen stiegen in Ponys und Pferden signifikant an und korrelierten positiv mit der Vitamin E Aufnahme. Die Serum Retinol Konzentrationen fluktuierten während der Studie, ohne mit der Aufnahme zu korrelieren.

Schlussfolgerungen

Frühe Fettleibigkeit in Equiden führt nicht zwangsläufig zu einer Steatose mit hepatozellulärer Entzündung. Gemäß der Hypothese zeigten Ponys und Pferde allerdings unterschiedliche hepatische Reaktionsmuster nach KG Zunahme. Das könnte die höhere Empfänglichkeit von Ponys für metabolische Erkrankungen erklären. Chemerin konnte als interessanter Marker für die equine Adipositas Forschung identifiziert werden. Serum Konzentrationen von Retinol und α-Tocopherol wurden durch die KG Zunahme nicht beeinflusst.

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8 Appendix

8.1 List of presentations of contents of this dissertation

2017

71st Conference of the Society of Nutrition Physiology

Comparative effects of lipopolysaccharide challenge on cytokine mRNA expression in the liver in non-obese ponies and horses

C. Schedlbauer, D. Blaue, M. Blüher, C. Gittel, W. Brehm, A. Einspanier, I. Vervuert

Göttingen, 14th-16th March 2017

published in Proc. Soc. Nutr. Physiol. 26, page 166; ISBN 978-3-7690-4110-1

2018

9th European Workshop on Equine Nutrition

Equine Obesity - Is there an impact on liver metabolism?

C. Schedlbauer, D. Blaue, M. Gericke., M. Blüher, C. Gittel, W. Brehm, I. Vervuert

Uppsala, 16th-18th August 2018

published in Proc. EWEN 2018, page 12; ISBN 978-91-576-9584-0

22nd European Society of Veterinary and Comparative Nutrition (ESVCN) Congress

Effects on the serum liver enzyme activities in equine obesity

C. Schedlbauer, D. Blaue, M. Gericke., M. Blüher, C. Gittel, W. Brehm, I. Vervuert

Munich, 6th-8th September 2018

published in Proc. ESVCN, 58; ISBN 978-3-00-058952-2

2019

73th Conference of the Society of Nutrition Physiology

Effects of equine obesity on liver metabolism

C. Schedlbauer, D. Blaue, J. Starzonek, M. Blüher, C. Gittel, W. Brehm, M. Gericke, I. Vervuert

Poster presentation: Effects of body weight gain on selected serum vitamin levels in ponies and horses

C. Schedlbauer, J. Raila, D. Blaue, J. Starzonek, I. Vervuert

Göttingen, 13th-14th March 2019

published in Proc. Soc. Nutr. Physiol. 28, page 45 and 76; ISBN 978-3-7690-4112-5

8.2   Publications related to the present study

Sonographie der Linea alba vor und nach der Laparotomie beim Warmblutpferd

D. Scharner, C. Gittel, D. Böttcher, K. Winter, D. Blaue, C. Schedlbauer, I. Vervuert, W. Brehm

Pferdeheilkunde-Equine Medicine 35(1):34-40, 2019

Comparison of incisional complications between skin closures using a simple continuous or intradermal pattern: a pilot study in horses undergoing ventral median celiotomy.

D. Scharner, C. Gittel, K. Winter, D. Blaue, C. Schedlbauer, I. Vervuert, W. Brehm

Peer J., Nov 9;6e5772, 2018

The influence of body weight gain on inflammatory cytokine expressions in adipose tissue depots of equines

S. Adolph, C. Schedlbauer, D. Blaue, A. Schöniger, C. Gittel, W. Brehm, H. Fuhrmann, I. Vervuert

PLoS ONE 14(3): e0207568

8.3   Individual values

Table 1: Crude nutrients (in % of dry matter), dry matter (%) and energy content (MJ ME/kg original substance) of the used hay

Time point	Energy content [MJ ME/kg oS]	Dry matter %	Crude ash	Crude fat	Crude protein	Crude fibre	NfE
					in % of dry matter		
28.10.2015	6.27	87.8	5.45	1.58	10.1	32	50.8
08.01.2016	6.66	91.8	3.48	0.77	14.4	31.6	49.8
04.02.2016	6.37	89.1	8.87	1.37	7.12	29.9	52.8
29.02.2016	5.87	89.6	8.92	0.83	7.61	32	50.7
30.03.2016	6.1	89.9	6.19	0.81	5.63	33.8	53.6
27.04.2016	6.7	88.7	5.12	1.41	8.87	30.8	53.8
23.05.2016	6.24	89.5	4.57	1.4	7.09	34.7	52.3
20.06.2016	6.96	89.8	4.71	1.51	9.29	30.3	54.2
19.07.2016	6.45	89.9	5.3	1.58	9	32.7	51.5
16.08.2016	6.76	90.8	5.56	1.38	8.86	30.9	53.3
15.09.2016	6.14	91.8	5.48	1.84	9.16	35.2	48.3
11.10.2016	4.26	89.2	6.37	1.88	9.11	43.8	38.8
25.11.2016	6.78	92.6	5.76	1.64	7.47	32.1	53.1
03.01.2017	5.77	86.1	3.82	1.45	7.47	36.6	50.6
14.02.2017	6.62	91.3	4.16	2.35	7.33	34.7	51.4
07.03.2017	6.51	88.3	3.68	2.04	8.35	33.9	52
08.05.2017	6.08	87.6	5.41	1.56	6.93	34.3	51.8
06.06.2017	5.63	91.7	4.62	1.98	8.23	39.1	46.1
05.07.2017	5.76	89.6	5.68	2.23	7.64	37.1	47.3
01.08.2017	6.39	89.5	5.67	1.91	7.61	33.3	51.5
29.08.2017	6.5	90.5	3.94	2.72	7.81	35.6	50

Table 2: Crude nutrients (in % of dry matter), dry matter (%) and energy content (MJ ME/kg original substance) of the used concentrate

Batches	Energy content [MJ ME/kg oS]	Dry matter [%]	Crude ash	Crude fat	Crude protein	Crude fibre	NfE[1]
				in % of dry matter			
1	13.3	90	7.89	14.6	13.3	10.4	53.8
2	13.4	90	8	14.4	13.4	9.8	54.3
3	13.1	90	7.89	14.2	13	11	53.9
4	13.2	90	7.9	14.4	13.3	10.4	54
5	14.8	90	7.44	17.3	14.2	6.89	54.1
6	14.7	91.9	5.99	15.9	13.7	8.19	56.2

[1]determined calculatory: NfE = DM − (crude ash + crude protein + crude fibre + crude fat)

Table 3: Dry matter intake in % of body weight in ponies and horses at 2015, 2016 and 2017

Ponies	2015	2016	2017	Horses	2015	2016	2017
1	2.45	2.36	2.28	4	2.43	2.37	1.83
3	2.81	2.65	2.63	6	2.42	2.25	1.9
5	2.86	2.86	2.6	8	2.38	2.36	1.79
7	2.61	2.46	2.37	10	2.37	2.34	1.88
11	3.01	2.79	2.6	12	2.39	2.37	1.65
13	2.84	2.72	2.48	14	2.33	2.24	1.8
15	2.82	2.72	2.59	16	2.36	2.33	1.9
17	2.73	2.64	2.5	20	2.44	2.44	2.04
19	2.83	2.7	2.54	22	2.4	2.33	1.92
21	2.76	2.78	2.63				

Table 4: Energy intake (% of maintenance metabolizable energy requirements) in ponies and horses at 4 time periods during the study

Ponies	05.01.2016- 22.05.2016	23.05.2016- 09.10.2016	25.12.2016- 05.06.2017	06.06.2017- 08.10.201
1	200	200	200	200
3	200	200	200	200
5	200	200	200	200
7	200	200	200	200
11	199	200	200	200
13	200	200	200	200
15	200	200	200	200
17	200	200	200	200
19	199	200	200	200
21	200	200	200	200
Horses				
4	199	195	174	162
6	199	200	185	181
8	196	195	183	157
10	200	194	178	176
12	199	192	165	156
14	200	198	176	170
16	196	190	176	174
20	198	199	184	190
22	200	198	182	173

Appendix

Table 5: Body weight, BCS and CNS of all equines at the three time points where the CGIT was conducted

Ponies	CGIT 2015 BW (kg)	BCS	CNS	CGIT 2016 BW (kg)	BCS	CNS	CGIT 2017 BW (kg)	BCS	CNS
1	186	3.15	2.5	220	4.1	3	226	4.75	3.75
3	96	1.15	0.75	134	3.6	2.5	142	4.15	4
5	99	2.8	2.5	121	3.6	2.5	132	3.9	3.5
7	145	2.5	2.5	183	3.75	4	178	4.1	4.5
11	108	3.4	3	121	3.4	2.75	130	3.65	3.25
13	95	0.65	0.5	134	3.2	2.5	158	3.75	3.25
15	120	4	3	126	3.7	2.75	132	4.15	3.5
17	119	1.2	1	146	3.7	3.5	137	3.75	4
19	95	0.75	0.25	134	3.25	2.25	144	3.7	3
21	121	3.65	3	131	3.5	3	132	3.5	3.5
Horses									
4	530	1.15	0.75	644	3.25	2	640	3.35	2.75
6	596	2.5	2	754	3.8	3	780	4.8	4.5
8	650	2.65	1.75	710	3.5	2.75	702	3.75	3.5
10	600	2.05	2	688	3.5	3.25	678	3.65	4
12	585	3.35	2	690	3.6	2.75	696	3.85	3
14	650	2.9	2.25	766	3.85	3.25	752	3.8	3.75
16	665	3.3	2.75	730	3.6	2.75	732	3.75	3.5
20	570	1.6	1.5	666	3.1	2.75	680	3.5	3.5
22	570	3.2	2.5	654	3.6	3	662	4.1	4

Values of BCS and CNS were calculated as mean of two independent evaluators

Table 6a: Plasma glucose (mmol/L), serum insulin (µU/mL), serum triglycerides (mmol/L) and serum non-esterified free fatty acids (µmol/L) in ponies and horses at 2015, 2016 and 2017

Ponies	Glucose mmol/L			Insulin µU/mL			Triglycerides mmol/L			Non-esterified free fatty acids µmol/L		
	2015	2016	2017	2015	2016	2017	2015	2016	2017	2015	2016	2017
1	3.49	3.83	4.1	5.25	6.46	9.99	0.34	0.28	0.3	60	334	277
3	3.37	3.15	4.17	6.16	5.9	12.2	0.47	0.24	0.26	111	27	348
5	3.1	4.05	5.45	5.84	8.19	16.2	0.71	0.26	0.51	58	210	166
7	3.43	3.87	6.26	3.62	7.43	55.2	0.67	0.43	0.79	77	117	332
11	4.01	3.73	3.37	3.7	5.57	10.6	0.32	0.32	0.2	422	40	107
13	3.67	4.41	4.05	2.8	5.5	10.6	0.39	0.14	0.19	15	376	567
15	5	4.57	3.73	4.68	24	5.47	0.55	0.61	0.38	137	60	488
17	2.57	3.91	4.16	2.96	5.09	6.51	0.27	0.24	0.68	48	257	425
19	3.28	3.91	4.32	2.34	4.89	6.86	0.33	0.36	0.39	81	120	444
21	3.37	3.86	3.86	5.3	6.23	5.11	0.82	1.21	0.49	183	539	364

Table 6b: Continuation of Table 6a

Horses	Glucose mmol/L			Insulin µU/mL			Triglycerides mmol/L			Non-esterified free fatty acids µmol/L		
	2015	2016	2017	2015	2016	2017	2015	2016	2017	2015	2016	2017
4	3.93	4.35	4.24	8.02	10.7	25.2	0.17	0.14	0.35	366	109	387
6	3.82	4.61	4.94	5.66	16.4	37.9	0.26	0.21	0.45	110	152	201
8	4.19	4.71	4.61	5.97	10.4	9.63	0.48	0.26	0.38	488	243	198
10	4.13	4.83	3.96	5.9	7.95	8.44	0.21	0.24	0.34	45	64	192
12	4.31	4.5	4.34	4.92	6.7	12.3	0.29	0.22	0.27	1260	327	148
14	3.83	4.25	3.85	5.14	9.75	8.94	0.24	0.28	0.24	342	262	363
16	4.11	4.46	4.07	4.73	6.82	17.3	0.28	0.3	0.26	29	260	170
20	4	4.21	4.26	12.4	11.3	7.50	0.24	0.22	0.28	282	295	257
22	4.41	4.79	5.31	5.66	8.06	8.27	0.24	0.28	0.18	113	185	309

Table 7: Serum Amyloid A concentrations (µg/mL) in ponies and horses at 2015, 2016 and 2017

Ponies	2015	2016	2017
1	0.4	0.1	0.1
3	0.1	0.1	0.1
5	0.1	0.1	0.1
7	0.1	0.6	0.1
11	0.9	0.1	0.1
13	0.1	0.1	340
15	0.1	0.1	0.1
17	0.1	0.6	0.1
19	1.5	0.4	0.1
21	0.1	187	0.1
Horses			
4	0.1	0.1	0.1
6	0.1	0.1	0.1
8	0.1	0.1	0.1
10	0.1	0.1	3.2
12	0.1	0.1	0.1
14	0.1	0.1	0.1
16	0.1	0.1	0.1
20	0.1	0.5	0.1
22	0.1	0.1	0.1

Table 8a: Serum ALP (U/L), GLDH (U/L) and AST (U/L) in ponies and horses at 6 time points during the study

Ponies	ALP (U/L)						GLDH (U/L)						AST (U/L)					
	t0	t1	t2	t3	t4	t5	t0	t1	t2	t3	t4	t5	t0	t1	t2	t3	t4	t5
1	102	99	82	80	110	80	7.5	12.2	10.3	8.4	11	7.7	445	439	430	294	304	267
3	148	135	112	124	164	112	4.1	8.8	10.4	4.5	5.1	4	340	389	381	362	269	255
5	238	170	143	173	196	171	3	18	4	6	9.6	4.1	512	489	434	442	456	314
7	225	123	105	138	161	139	3	7.7	27	27.9	12.6	6.4	270	380	366	387	356	343
11	340	165	96	113	125	79	20.3	47.5	23.9	60.7	140	31.6	337	581	505	532	587	446
13	179	171	151	149	131	124	2.6	54.2	27.2	24.5	21.4	21.2	332	518	360	411	524	423
15	154	151	124	116	146	101	2.4	7.6	1.8	12.1	4.4	4.4	283	446	326	392	379	336
17	134	131	122	113	151	836	7.3	26.1	62.1	5.8	14.8	37.8	373	497	466	406	397	437
19	230	164	123	138	158	135	7.8	33.9	10.2	27.1	27.8	24.1	255	570	421	460	435	428
21	293	237	200	377	591	199	12.4	21.5	11	255	311	76.4	411	448	389	698	629	478

Table 8b: Continuation of Table 8a

Horses	ALP (U/L)						GLDH (U/L)						AST (U/L)					
	t0	t1	t2	t3	t4	t5	t0	t1	t2	t3	t4	t5	t0	t1	t2	t3	t4	t5
4	130	96	92	93	108	81	2	3.5	3.4	2.4	5.9	1	278	322	306	309	325	247
6	167	141	110	129	154	128	35.8	5.3	2.1	1.7	3.9	13.4	605	404	317	355	295	445
8	127	98	83	94	107	89	11.6	4.5	2.2	2.1	5	3	399	321	284	257	287	269
10	135	156	114	127	136	103	2.9	2.4	2	1.6	1.7		376	416	336	359	299	278
12	170	155	184	143	146	141	2.2	4.1	4.3	3.2	3.9	7.3	307	359	337	320	303	274
14	184	96	87	113	123	112	1.9	5.2	7.6	4	2.5	8.7	305	352	312	270	262	291
16	109	116	117	120	116	99	1.9	3.5	2.2	2.3	2.9	2.7	307	317	284	310	292	300
20	156	146	141	128	141	149	2	2.8	21.9	2.8	12.7	126	349	396	435	316	503	770
22	88	77	65	76	90	70	1.5	2.9	1.9	2.6	2.8	1.3	322	339	308	322	313	286

Table 9: Serum GGT (U/L) activities and serum BA (µmol/L) concentrations in ponies and horses at 6 time points during the study

Ponies	GGT (U/L)						BA (µmol/L)					
	t0	t1	t2	t3	t4	t5	t0	t1	t2	t3	t4	t5
1	64.4	28.9	22.7	18.5	25.6	25.7	5.2	8.9	6.1	14.7	15	10.1
3	28.5	25.6	14.4	14.4	16.2	15.9	6	10.1	6.2	6.9	5.4	7.7
5	213	45.5	14.9	17	29.1	20.3	2.7	9.7	6	4.9	6.7	5.6
7	57	28.6	28.1	43.8	91.9	73.1	3.7	9.2	4.1	11	4	5.6
11	122	25.4	29.7	28.6	50.4	29.8	6	8.9	5.1	8.9	9.4	8.7
13	63	22.4	13.8	16.9	33.3	21.1	7.2	18.5	23.3	23.8	22.4	18.6
15	36	24.2	12.3	13.6	19.6	15.1	1.7	5.6	2.9	12	8	8.3
17	68	52.4	26.4	17.9	22.5	155	5.6	13.5	17.3	10.4	13	33.7
19	45.3	30.5	14.3	17.4	19.4	23.9	6.5	13.4	2.8	16.1	21.1	14.9
21	265	19.5	18.4	199	363	170	5.8	11.5	13.3	17.4	25.3	10.9
Horses												
4	10.3	17.8	17.1	17.3	20.7	15.5	3.2	6	12.4	5.4	9.4	3.8
6	103	33.9	19.3	18.4	19.2	52.9	8.1	6.4	4.6	2.4	5	5
8	39.1	16.4	10.8	12.5	13.9	14.8	2	5.9	8.8	7	8.4	5
10	26.1	16.4	11.6	12.5	11.2	11.7	2.1	2.4	2.3	2.5	3.5	2.7
12	24.6	22.2	44.2	13.7	15.3	13.8	3	5.4	9.4	4.1	4.8	6.8
14	31.9	34.4	26.3	33.8	32.5	53.6	3.2	4.8	6.3	4.5	4.3	4.4
16	16.8	20.6	12.9	12.1	12	14.9	2.9	7.2	5.7	9.4	5.5	5.9
20	128	26.3	15.1	13.2	32.5	57.7	6.1	3.3	10.4	3.9	7.8	11.5
22	6.6	12.8	9.6	9.6	17.6	12.7	8.3	7.3	7.3	7.7	6	13

Table 10: Staging of steatosis in liver tissue samples of ponies and horses at 2015 and 2017

Ponies	2015	2017
1	0	0
3	1	1
5	0	2
7	0	1
11	1	0
13	0	0
15	0	0
17	0	3
19	0	0
21	0	0
Horses		
4	0	0
6	0	0
8	0	0
10	0	1
12	0	0
14	1	0
16	0	
20	1	0
22	1	0

Table 11: x-fold mRNA levels of CD68, IL-1β, IL-6 and Nf-κB normalized to the geometric means of the three reference genes 18S, HPRT1 and RPL32 in ponies and horses at 2015,2016 and 2017

Ponies	CD68			IL-1β			IL-6			Nf-κB		
	2015	2016	2017	2015	2016	2017	2015	2016	2017	2015	2016	2017
1	0.52	0.45	0.4	0.73	0.85	2.04	0.2	0.26	1.99	0.81	0.52	0.96
3	1.04	0.57	0.51	1.55	0.35	0.39	1.51	0.2	0.45	1.21	0.57	0.53
5	0.79	0.42	0.71	1.41	1.03	1.2	0.89	0.63	0.8	1.86	0.59	0.65
7	0.7	1.35	0.62	0.86	0.37	0.7	0.25	0.27	0.38	0.73	0.76	0.47
11	1.92	1.25	0.69	0.42	1.36	1.04	0.29	0.85	0.09	0.88	0.94	0.58
13	0.64	0.83	0.8	0.79	0.42	0.74	0.53	0.35	0.77	1	0.7	0.63
15	0.56	0.7	0.77	5.05	0.94	0.57	4.81	0.3	0.82	0.82	0.76	0.55
17	0.68	0.86	0.86	0.46	0.96	0.66	0.28	0.25	0.62	0.67	0.72	0.91
19	0.75	0.18	0.56	0.41	0.48	0.65	0.16	0.2	0.62	0.78	0.88	0.62
21	0.84	1.37	0.49	0.78	0.53	0.62	0.25	0.23	0.27	0.76	0.66	0.59
Horses												
4	0.5	0.52	0.52	0.52	0.52	0.77	0.29	0.32	0.56	0.56	0.67	0.54
6	2.04	0.53	0.6	0.49	0.44	0.98	0.24	0.15	0.46	1.17	0.61	0.49
8	0.41	0.36	0.4	0.63	0.51	0.56	0.3	0.23	0.44	0.49	0.7	0.42
10	0.56	0.86	1.18	0.49	0.32	0.58	0.19	0.4	0.53	0.62	0.99	0.99
12	0.37	0.44	0.38	0.64	0.79	0.55	0.27	0.82	0.26	1.07	0.85	0.47
14	0.46	0.59	0.49	1.46	0.54	0.34	1.21	0.32	0.14	0.87	0.74	0.39
16	0.42	0.54	0.52	0.4	0.48	0.4	0.1	0.22	0.26	0.66	0.76	0.45
20	1.01	0.52	0.46	0.23	0.37	0.44	0.33	0.13	0.32	0.75	0.59	0.4
22	0.58	0.45	0.2	0.79	0.7	0.16	0.44	0.41	0.88	0.49	0.46	0.46

Appendix

Table 12: x-fold mRNA levels of TNFα, Chemerin, FABP1 and LPL normalized to the geometric means of the three reference genes 18S, HPRT1 and RPL32 in ponies and horses at 2015,2016 and 2017

Ponies	TNFα			Chemerin			FABP1			LPL		
	2015	2016	2017	2015	2016	2017	2015	2016	2017	2015	2016	2017
1	0.4	0.37	2.1	0.47	0.63	1.33	0.96	0.18	0.2	0.4	0.35	0.48
3	1.39	0.23	0.7	0.55	0.72	1.31	0.69	0.41	0.37	1.04	0.26	0.61
5	0.72	0.6	1.22	0.62	0.76	1.22	0.62	0.52	0.34	0.54	0.3	3.8
7	0.82	0.73	0.41	0.67	0.8	1.19	0.53	0.67	0.85	0.3	0.41	0.43
11	0.49	1.13	0.47	0.83	0.69	0.83	0.73	0.39	0.74	1.64	1.05	0.54
13	0.52	0.81	1.11	0.76	0.76	0.99	0.76	0.35	0.61	1.57	0.89	1.45
15	4.13	0.66	1.21	0.49	0.71	1.45	0.49	0.62	0.78	0.28	0.21	1.48
17	0.44	0.4	0.66	0.55	0.75	1.36	0.73	0.62	1.71	1.57	1.26	2.07
19	0.37	0.73	0.75	0.66	0.57	0.87	0.73	0.39	0.75	0.32	0.85	0.45
21	0.76	1.03	0.7	0.86	0.44	0.52	0.46	0.34	0.56	0.46	2.5	1.08
Horses												
4	0.47	0.47	0.64	0.55	0.68	1.27	0.75	0.51	0.7	0.5	0.36	0.76
6	0.46	0.31	0.94	0.61	0.78	1.23	0.69	0.66	0.71	0.45	0.43	0.44
8	0.39	0.21	0.49	0.66	0.73	1.27	0.73	0.67	1.01	0.2	0.12	0.13
10	0.37	0.77	0.72	0.58	0.52	1.11	0.83	0.52	1.21	0.24	0.61	0.6
12	0.45	0.91	0.77	0.46	0.51	0.91	0.38	0.42	0.5	0.4	0.24	0.24
14	1.19	0.75	0.38	0.55	0.7	1.12	0.42	0.28	0.44	0.22	0.59	0.35
16	0.31	0.38	0.32	0.59	0.71	1.26	0.41	0.49	0.19	0.41	0.18	0.34
20	0.34	0.32	0.59	0.74	0.58	1.2	1.0	0.56	0.93	1.05	0.18	0.3
22	0.53	0.58	1.5	0.67	0.54	0.79	1.03	0.68	0.94	0.53	0.21	0.22

Table 13a: Serum α-tocopherol (µg/mL), retinol (µM) and RBP4 (µM) in ponies and horses at 7 timepoints during the study

Ponies	α-tocopherol (µg/mL)							Retinol (µM)							RBP4 (µM)						
	t0	t1	t2	t3	t4	t5	t6	t0	t1	t2	t3	t4	t5	t6	t0	t1	t2	t3	t4	t5	t6
1	2.75	2.41	2.9	4.34	11.7	5.19	9.1	0.41	0.43	0.37	0.33	0.41	0.3	0.35	0.31	0.34	0.33	0.33	0.16	0.12	0.21
3	1.87	2.38	1.51	2.57	5.43	4.71	5.72	0.13	0.32	0.3	0.33	0.38	0.41	0.42	0.18	0.24	0.32	0.44	0.23	0.15	0.12
5	2.19	1.33	2.3	5.9	10.3	2.89	11.2	0.27	0.43	0.26	0.26	0.31	0.39	0.29	0.23	0.28	0.5	0.55	0.26	0.14	0.16
7	3.69	1.76	1.11	1.44	2.78	6.96	3.87	0.26	0.41	0.41	0.35	0.37	0.25	0.41	0.26	0.5	0.57	0.19	0.2	0.12	0.26
11	3.44	3.77	2.94	3.95	5.7	2.08	6.24	0.27	0.29	0.31	0.25	0.28	0.33	0.28	0.31	0.22	0.73	0.35	0.24	0.19	0.19
13	1.45	1.16	1.54	3.1	4.84	3.5	4.16	0.25	0.33	0.39	0.33	0.43	0.3	0.49	0.41	0.42	0.49	0.22	0.25	0.17	0.24
15	2.5	1.82	1.5	4.27	4.81	4.33	6.38	0.38	0.44	0.39	0.28	0.37	0.27	0.36	0.31	0.4	0.42	0.27	0.2	0.18	0.33
17	2.18	1.38	1.62	4.64	6.11	6.08	8.39	0.39	0.45	0.29	0.31	0.33	0.21	0.47	0.44	0.3	0.33	0.27	0.18	0.18	0.13
19	1.64	1.32	1.42	2.69	4.84	5.19	6.34	0.22	0.31	0.3	0.26	0.25	0.2	0.26	0.31	0.39	0.37	0.17	0.18	0.19	0.23
21	1.79	3.31	2.68	6.22	7.65	6.98	6.81	0.51	0.38	0.42	0.28	0.48	0.43	0.43	0.22	0.48	0.28	0.39	0.24	0.56	0.47

Table 13b: Continuation of Table 13a

Horses	α-tocopherol (µg/mL)							Retinol (µM)							RBP4 (µM)						
	t0	t1	t2	t3	t4	t5	t6	t0	t1	t2	t3	t4	t5	t6	t0	t1	t2	t3	t4	t5	t6
4	2.52	1.69	1.43	2.16	3.42	6.08	1.64	0.33	0.43	0.33	0.35	0.44	0.39	0.41	0.21	0.29	0.15	0.6	0.3	0.16	0.24
6	2.22	1.54	2.17	2.5	3.64	2.68	4.4	0.56	0.46	0.27	0.46	0.41	0.43	0.71	0.41	0.41	0.64	0.28	0.27	0.12	0.23
8	3.16	2.43	3.29	4.39	5.58	3.16	6.31	0.44	0.43	0.43	0.34	0.41	0.41	0.42	0.33	0.75	0.47	0.21	0.25	0.17	0.22
10	0.73	1	1.57	3.39	5.1	5.04	5.59	0.38	0.53	0.44	0.4	0.4	0.34	0.47	0.27	0.25	0.59	0.34	0.15	0.28	0.26
12	1.98	1.44	2.04	4.38	5.81	8.3	5.92	0.27	0.34	0.32	0.31	0.3	0.32	0.34	0.17	0.17	0.51	0.21	0.22	0.32	0.31
14	1.98	1.21	1.47	2.54	3.3	2.32	4.37	0.5	0.4	0.39	0.39	0.36	0.31	0.44	0.31	0.34	0.32	0.21	0.19	0.15	0.24
16	1.47	1.61	2.22	3.02	3.57	3.38	4.51	0.38	0.45	0.38	0.35	0.44	0.38	0.47	0.39	0.38	0.32	0.22	0.34	0.2	0.26
20	1.47	0.79	1.62	2.94	3.75	2.95	6.24	0.38	0.4	0.28	0.26	0.33	0.28	0.43	0.34	0.33	0.44	0.33	0.25	0.2	0.36
22	0.52	0.58	1.15	2.44	3.33	3.1	4.69	0.37	0.28	0.26	0.29	0.32	0.24	0.33	0.36	0.34	0.55	0.37	0.23	0.21	0.24

9 Acknowledgments