Published in final edited form as:

Chemosphere. 2019 March ; 218: 652–661. doi:10.1016/j.chemosphere.2018.11.136.

Associations between endocrine disrupting chemicals and equine metabolic syndrome phenotypes

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Abstract

Equine Metabolic Syndrome (EMS) is characterized by abnormalities in insulin regulation, increased adiposity and laminitis, and has several similarities to human metabolic syndrome. A large amount of environmental variability in the EMS phenotype is not explained by commonly measured factors (diet, exercise, and season), suggesting that other environmental factors play a role in EMS development. Endocrine disrupting chemicals (EDCs) are associated with metabolic syndrome and other endocrine abnormalities in humans. This led us to hypothesize that EDCs are detectable in horse plasma and play a role in the pathophysiology of EMS. EDCs acting through the aryl hydrocarbon and estrogen receptors, were measured in plasma of 301 horses from 32 farms. The median (range) TEQ (2,3,7,8-TCDD equivalent) and EEQ (17 β -estradiol equivalent) were 19.29 pg/g (0.59–536.36) and 10.50 pg/ml (4.35–15000.00), respectively. TEQ was negatively associated with plasma fat extracted and batch analyzed. EEQ was positively associated with pregnancy and batch analyzed, and negatively associated with being male and superfund score 100 miles of the farm. Of particular interest, serum glucose and insulin, glucose and insulin post oral sugar challenge, and leptin concentrations were associated with EEQ, and serum triglyceride concentration was associated with TEQ. Overall, we demonstrated that EDCs are present in the plasma of horses and may explain some of the environmental variability in measured EMS phenotypes. This is the first example of EDCs being associated with clinical disease phenotype components in domestic animals.

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Declarations of interest: none

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Keywords

Endocrine disrupting chemicals; equine; metabolic syndrome; environment

1. Introduction

Equine metabolic syndrome (EMS) is a common disorder of several major horse breeds, including Welsh Ponies and Morgan horses. The main features of EMS are insulin dysregulation, increased adiposity and a predisposition to develop laminitis (Johnson, 2002; Frank et al., 2010; Frank et al., 2018). Laminitis is a painful condition leading to damage to the soft tissue (laminae) that holds the pedal bone in the hoof capsule (Pass et al., 1998). Other ancillary abnormalities include: hypertriglyceridemia (Bailey et al., 2008), dyslipidemia, increased low density lipoprotein concentrations (Frank et al., 2006; Treiber et al., 2006; Carter et al., 2009), hyperleptinemia (Cartmill et al., 2003), arterial hypertension (Bailey et al., 2008; Carter et al., 2009), and altered reproductive cycling in mares (Gentry et al., 2002; Vick et al., 2006). The EMS phenotype is not a dichotomous diagnosis, and can be separated into nine individual biochemical phenotypes (table 1).

EMS has several similarities to human metabolic syndrome (MetS) (Johnson, 2002; Johnson et al., 2009). Patients with MetS typically have hyperinsulinemia, hypertension, and a predisposition to obesity, type II diabetes mellitus, and cardiovascular disease (Reaven, 2011). Both EMS and MetS are considered to be complex diseases with evidence of both genetic and environmental factors playing a role in the pathophysiology of disease (Johnson et al., 2009). In a large across-breed study of horses with EMS, we have demonstrated that there is a strong genetic contribution to variation in the EMS phenotypes (Schultz, 2016; Norton et al., *submitted 2018*). However, nearly half of the variability in the EMS phenotypes is due to environmental factors, and only a small amount of this variability can be explained by commonly measured factors such as diet, exercise and season (Schultz, 2016). This suggests that other environmental risk factors play an important role in the development of the EMS phenotypes.

Endocrine disrupting chemicals (EDCs) are found in numerous commercially produced compounds, including organochlorine pesticides such as dichlorodiphenyltrichloroethane (DDT) and as by-products during synthesis of various chlorophenols and herbicides, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Magliano et al., 2014). They also tend to be polychlorinated, lipophilic and persist in the environment (Pedersen et al., 2010). Exposure to EDCs has been associated with numerous adverse health effects in humans, including; reduced birth weight, endocrine abnormalities, and altered cognitive development (ten Tusscher and Koppe, 2004; Lundqvist et al., 2006). There are also numerous epidemiological studies that have linked EDCs to human obesity, insulin resistance and type 2 diabetes (Henriksen et al., 1997; Michalek et al., 1999; Cranmer et al., 2000; Novelli et al., 2005; Lee et al., 2006; Elobeid et al., 2010; Lee et al., 2010; Casals-Casas and Desvergne, 2011; Kim et al., 2011; Lee et al., 2011; Neel and Sargis, 2011; Magliano et al., 2014). EDCs exert their detrimental effects by binding to cellular receptors that lead to altered gene expression and effects on metabolism, cell growth and differentiation, as well as

disturbances in steroid-hormone and growth-factor signal transduction pathways (Mandal, 2005; Gregoraszczuk and Ptak, 2013; Magliano et al., 2014). The evidence linking exposure to EDCs and adverse health effects in both humans and animals has been recognized across the United States and led to the Food Quality Act and Safe Water Reauthorization Act Amendments in 1996 (Gordon, 2004).

The organochlorine pesticides and dioxins are the EDCs subclasses most commonly linked with metabolic disturbances in humans (Lee et al., 2007; Neel and Sargis, 2011; Magliano et al., 2014). Humans and livestock are exposed to EDCs by multiple routes including air, water, soil, sediments and foodstuffs (Bergman et al., 2013). Many EDCs can be taken up by plant roots and foliage and ingested by grazing livestock (Rhind, 2002). Livestock are also likely to ingest EDCs through soil and water sources particularly if the water contains organic matter (Rhind, 2002). Several surveillance studies have demonstrated uptake of EDCs by grazing animals leading to accumulation of EDCs in milk (Rychen et al., 2008) and beef (Feil and Ellis, 1998).

Until relatively recently, the measurement of EDCs was reliant on mass spectrometry, which tended to be cost prohibitive for domestic animal species. With the increasing recognition of the role of EDCs in several important disorders in humans, new *in vitro* reporter gene bioassays have become available, which provide rapid measurement of EDCs from both human and animal samples. These measurements have advantages over previous assays as they reduce exposure misclassification by taking into account variation in the uptake and affinity of the specific receptor when calculating the EDC concentration (Murk, 1997). They have been used successfully to identify associations between dioxin-like chemicals and measurements of obesity (Verhulst et al., 2009), and aryl hydrocarbon receptor (AhR)-ligands and metabolic syndrome in humans (Park et al., 2013).

To our knowledge, neither the accumulation of EDCs in horses, nor the role of EDCs in EMS has been evaluated. Our study objectives were to: 1) demonstrate that EDCs accumulate in horses similar to other grazing livestock species; 2) identify factors that affect the total EDC concentration in horses; and 3) to determine if EDCs are associated with EMS-related phenotypes in Welsh Ponies and Morgan horses.

2. Materials and Methods

2.1 Study population

All samples were collected under the University of Minnesota IACUC protocol #1109B04448. This was an observational study using 301 horses selected from a larger study population of 282 Welsh Ponies and 292 Morgan horses previously phenotyped for EMS (Schultz, 2016). To account for shared environment, animals were selected from farms with 5 horses residing on the property. Within each farm, 12 horses were selected at random using a random number generator (R Core Team, 2013) for inclusion in the study. For farms with less than 12 horses, all individuals meeting the above criteria were included. Intact males (stallions) and horses with a diagnosis of, or signs consistent with Pituitary Pars Intermedia Dysfunction were excluded. Farms were distributed across the United States

(supplementary figure 1). Only intact females (mares) or castrated males (geldings) more than 2 years of age were included in the study.

2.2 EMS phenotyping

EMS phenotyping, consisting of 9 individual biochemical measures was performed for each horse (table 1). Whole blood and serum were collected via jugular puncture into lithium heparin, ethylenediaminetetraacetic acid (EDTA), and sodium citrate blood tubes. An oral sugar test (OST) was then performed as previously described (Lindåse et al., 2016). In brief, 0.15 ml/kg body weight Light Karo syrup (ACH Food companies Inc., Cordova, TN, USA) was syringed by mouth. Blood was collected by jugular venipuncture into lithium heparin and sodium citrate blood tubes 75 minutes after syrup administration. Tubes were placed on ice immediately following sampling and were centrifuged within 6 hours of sample collection for 10 minutes at 2,700 G. Plasma and serum were stored at -80°C until hormonal and biochemical analyses were performed. Body morphometric measurements to quantify regional fat deposition in the neck (neck-circumference-to-height ratio [NH]) and generalized obesity (girth circumference-to-height ratio [GH] and body condition score [BCS]) (Henneke et al., 1983) were obtained on all horses. Other information collected included age, breed, sex, pregnancy status, laminitis history, laminitis status, hours of grazing pasture, hours of turnout, month sampled and farm location (latitude and longitude based on United States postal address and zip code). Month sampled was divided into season: spring (January, February, March), summer (April, May, June), fall (July, August, September), and winter (October, November, December). BCS and laminitis status (BCS7_LAM) were combined into four clinical categories: 1, obese (BCS $\geq 7/9$) and laminitic; 2, non-obese (BCS $< 7/9$) and laminitic; 3, obese and non-laminitic; and 4, non-obese and nonlaminitic. Sex and pregnancy status were combined into three categories: 1, pregnant female (P); 2, non-pregnant female (NP); and 3, male (M).

2.3 Analysis of blood samples

Serum high-molecular weight adiponectin concentrations were measured by ELISA (Human HMW Adiponectin ELISA [EZHMWA-64K], EMD Millipore, Massachusetts, USA) that has been previously validated for equine serum (Wooldridge et al., 2012). Serum non-esterified fatty acid (NEFA) and total serum triglyceride (TG) concentrations were measured enzymatically by use of commercial kits; NEFA-HR (FA-HR, Wako, Virginia, USA) and Serum Triglyceride Determination kit (Serum Triglyceride Determination kit [TR0100], Sigma-Aldrich Corp, Missouri, USA) respectively. Serum leptin, serum insulin pre- (INS) and post- (INS_OST) oral sugar challenge and plasma adrenocorticotrophic hormone (ACTH) concentrations were calculated using commercially available radioimmunoassays; multi-species leptin RIA (Multi-species leptin RIA [XL-85K], EMD Millipore, Massachusetts, USA), TKIN1 Insulin Coat-A-Count Kit (TKIN1 Insulin Coat-A-Count Kit, Siemens Medical Solutions, Philadelphia, USA), and LKAC1 ACTH kit (LKAC1 ACTH Kits, Siemens Medical Solutions, Philadelphia, USA) respectively. Serum glucose concentration pre- (GLU) and post-oral sugar challenge (GLU_OST) was calculated using the YSI glucose and lactate analyzer (YSI glucose and lactate analyzer [2300 STAT Plus], Marshall Scientific LLC, New Hampshire, USA). Each blood sample analysis was performed in duplicate.

2.4 Measurement of plasma EDC concentrations

EDC activity through the aryl hydrocarbon receptor (AhR) was determined in individual plasma samples using the DR CALUX[®] bioassay (BioDetection Systems B.V., Amsterdam, Netherlands [www.bds.nl]) as described previously in other species (Van Wouwe et al., 2004; Ayotte et al., 2005; Vafeiadi et al., 2013; Behnisch et al., 2018). In brief, approximately 1 gram of equine plasma was extracted by means of shake-solvent extraction (n-hexane:diethylether, 97:3). This was followed by gentle evaporation of the organic-phase and determination of the amount of fat extracted. The extractable fat was used for clean-up on an acid silica column (20% and 33% H₂SO₄), topped with sodium sulphate. Cleaned extracts were dissolved in dimethyl sulfoxide (DMSO; 8 µL) and serial dilutions in DMSO were prepared. DR CALUX[®] cells were cultured in alpha minimum essential (α-MEM) culture medium supplemented with 10% (v/v) fetal calf serum (FCS) under standard conditions (37°C, 5% CO₂, 100% humidity). Cells seeded in 96-well microtiter plates were exposed in triplicate to the prepared serial dilution series of the extracted and cleaned equine plasma samples and procedure blanks (0.8% DMSO). In addition, each 96-well microtiter plate contained the standard 2,3,7,8-TCDD-calibration range. Following a 24 hour incubation period, cells were lysed and the luciferase activity was measured by addition of a luciferine containing solution using a Berthold Technologies luminometer (TriStar LB941, Bad Wildbad, Germany) equipped with 2 dispensers. Validity of analysis results were checked (R² calibration series > 0.98; z-factor calibration series > 0.6; EC50 reference compound between preset limits; SD triplicate analysis <15%; recovery reference compounds >75%) after which sample analysis results were interpolated in the 2,3,7,8-TCDD-calibration curve and expressed in pg 2,3,7,8-TCDD toxic equivalents per gram of extractable fat from plasma (TEQ). Each sample has an individual limit of detection (LOD) and samples below the LOD were included in the analysis by dividing the LOD by 2 as previously reported (Vafeiadi et al., 2013).

The ERα CALUX[®] bioassay (BioDetection Systems B.V., Amsterdam, Netherlands [www.bds.nl]) (Sonneveld et al., 2005) was used to evaluate EDC activity through the estrogen receptor (ER) in equine plasma. Prior to extraction of estrogens from the plasma, conjugated estrogens were chemically deconjugated using hydrochloric acid (HCl). Approximately 0.5 ml of equine plasma was mixed gently with 1 ml HCl (0.25 M) and incubated for 1 hour at 40°C. Deconjugated estrogens were extracted by means of shake-solvent extraction with methyl tertiary butyl ether (MTBE). Following evaporation of MTBE under a gently stream of nitrogen, the extracts were dissolved in DMSO (20 µl) and serial dilutions were prepared. The ERα CALUX[®] bio-analyses were performed using human U2-OS cell lines stably transfected with an estrogen-controlled luciferase reporter gene construct. ERα CALUX[®] cells were cultured in nutrient mixture F12 (DMEM/F12) (with phenol red) medium supplemented with 7.5% FCS, non-essential amino-acids, antibiotics (penicillin, streptomycin) and G418 under standard conditions (37°C, 5% CO₂, 100% humidity). CALUX[®] cells were plated in 96-well plates (10,000 cells/well) in DMEM/F12 (without phenol red) medium supplemented with 5% dextran-coated charcoal-tripped FCS (DCC-FCS), non-essential amino-acids and antibiotics (penicillin, streptomycin) at a volume of 100 µl per well. After 24 hours of incubation, the medium was refreshed and cells were exposed to serial dilution series of the final extracts and procedure blanks in triplicate (0.1%

DMSO). Each 96-well microtiter plate also contained the standard 17 β -estradiol calibration range. After 24 hours the medium was removed, and cells were lysed in 30 μ l Triton-lysis buffer and measured for luciferase activity using a luminometer. Validity of analysis results were checked (R^2 calibration series > 0.98; z-factor calibration series > 0.6; EC50 reference compound between preset limits; SD triplicate analysis <15%; recovery reference compounds >75%) after which sample analysis results were interpolated in the 17 β -estradiol calibration curves and expressed in ng 17 β -estradiol equivalents per ml of plasma (EEQ). Each sample has an individual LOD and samples below the LOD were included in the analysis by dividing the LOD by 2 as previously reported (Vafeiadi et al., 2013).

The DR CALUX[®] bioassay has been validated against the gold standard mass spectrometry in sulfuric acid treated human plasma samples (Van Wouwe et al., 2004) and bovine milk samples for the measurement of EDCs acting through the aryl hydrocarbon receptor (AhR) (Chou et al., 2008). It is well known that PCDD/Fs and dioxin-like PCBs are the main contributors for the measurement of EDCs acting through the aryl hydrocarbon (AhR) (Van Wouwe et al., 2004; Chou et al., 2008). The ER α CALUX[®] bioassay (Sonneveld et al., 2005) has been validated according to the Organisation for Economic Co-operation and Development (OECD) standards and is incorporated in OECD test guideline TG455 (Besselink, 2015; OECD, 2015). The results of the characteristics for both bioassays are included in table 2.

2.5 Statistical analysis

All non-normally distributed response variables were transformed using logarithmic (TEQ, EEQ, INS, INS_OST, TG, and ACTH) or square root transformations (adiponectin, leptin, and NEFA) as appropriate based on normality of residuals. Predictor variables were not transformed for analysis. One sample failed EEQ analysis and there was insufficient sample to repeat the EEQ measurement, therefore this sample was not included in the EEQ analysis.

Associations between TEQ and EEQ concentrations and age, sex/pregnancy status, breed, amount of fat extracted from the sample (fat [g]), number of hours grazing/day, season sampled (spring, summer, fall, winter), BCS/laminitis status, NH, GH, and number of and distance from superfund sites (superfund score, supplemental information 1) were determined using multiple linear regression as described in supplemental information 2. Due to the statistically significant associations between TEQ and EEQ and batch processed, log transformed TEQ and EEQ residuals by batch were used as response variables, and TEQ and EEQ residuals by batch were utilized as predictor variables (supplemental information 2).

Associations between TEQ and EEQ residuals and EMS phenotypes (table 1) were also explored using multiple linear regression. Due to the complexity of the dataset and the number of possible variables to include in the models, small sample corrected Akaike information criterion (AICc) was used to determine the best linear regression model (supplemental information 2). Possible predictor variables included age, sex/pregnancy status, breed, amount of fat extracted from the sample, number of hours grazing/day (grazing), season sampled, BCS/laminitis status, GH, and NH (supplemental information 2). Biologically relevant interaction terms (supplemental information 3) between TEQ residuals and amount of fat extracted from the sample and EEQ residuals and sex/pregnancy status

were included as potential confounding variables. All models tested for EMS phenotypes included both TEQ and EEQ residuals. Estimated marginal means (EMMEANs [categorical predictor variables]) and estimated marginal means of linear trends (EMTRENDs [continuous predictor variables]) were calculated for statistically significant linear models and are reported with 95% confidence intervals (CI) (Lenth, 2018). Reverse transformed results are reported where appropriate. The EMMEAN demonstrates the mean response of the response variable for a predictor variable, adjusted by the other variables in the model. The EMTREND shows the mean change in the response variable for a unit change in the continuous predictor variable, adjusted for other predictor variables in the model. All statistical analysis was performed using R (R Core Team, 2013). Significance was set at the p-value of 0.05.

3. Results

Plasma samples from 140 Welsh Ponies residing on 13 farms and 161 Morgan horses residing on 19 farms were included in the analysis. Information about the horses' signalment is shown in table 3. The median (range) amount of fat extracted from each plasma sample was 5 mg/g (0.9–260). The mean and standard deviation, or median, interquartile range, and range for the nine EMS phenotypic measurements, across the entire sample population, are shown in table 4. One hundred and thirty one samples (43.67%) were below the LOD of the bioassay. TEQ concentrations varied significantly by processing batch ($p = 0.01$) (figure 1b and supplemental table 1). Therefore TEQ by batch residuals were used for further analysis. Log transformed plasma TEQ was significantly negatively associated with fat extracted (g) ($p = 3.00 \times 10^{-4}$). TEQ concentration decreased as the fat extracted from plasma increased, with an estimated marginal mean of linear trend (EMTREND) of -4.18 (confidence interval [CI] $-6.45 - -1.92$) (figure 2). No associations were identified between log transformed plasma TEQ and age, sex/pregnancy status, breed, measures of adiposity (NH, GH), hours grazing per day, season, BCS/laminitis status, EEQ, or superfund score (supplemental table 2).

3.2 Plasma EEQ concentrations

The median (range) EEQ concentration was 10.50 pg/ml (4.35–15,000.00). A histogram of the log-transformed data is shown in figure 3a. One hundred and forty six samples (48.67%) were below the LOD of the bioassay. EEQ concentrations varied significantly by processing batch ($p = 9.69 \times 10^{-5}$) (figure 3b and supplemental table 3). Therefore EEQ by batch residuals were used for further analysis. Log transformed plasma EEQ was positively associated with being female and pregnant ($p < 0.001$) and negatively associated with being male ($p = 0.01$) and superfund score (SF100, $p = 0.02$). Log transformed EEQ were increased in pregnant females, with EMMEAN of 0.90 (CI 0.62 – 1.18). Log transformed EEQ was decreased in males with an EMMEAN of -0.33 (CI $-0.59 - -0.07$). Log transformed EEQ decreased as the SF100 superfund score increased, with an EMTREND of -2.41×10^{-6} (CI $-4.45 \times 10^{-6} - -3.63 \times 10^{-7}$) (figure 4). No associations were identified between log transformed plasma EEQ and age, fat extracted from sample, breed, measures of adiposity (NH, GH), hours grazing per day, season, BCS/laminitis status, TEQ, or SF250 or SF500 superfund score (supplemental table 4).

3.3 TEQ, EEQ and equine metabolic syndrome biochemical phenotypes

Serum GLU concentration was negatively associated with EEQ ($p = 4.30 \times 10^{-3}$) and the interaction between EEQ and sex/pregnancy status (non-pregnant female $p = 3.50 \times 10^{-3}$). GLU concentrations decreased as EEQ increased, with an EMTREND of -0.18 mg/dl (CI $-0.30 - 0.06$). GLU concentrations increased as EEQ decreased in non-pregnant females (EMTREND -0.25 mg/dl, CI $-0.42 - -0.08$) (figure 5A and supplemental table 5).

Serum GLU_OST concentration was negatively associated with EEQ ($p = 0.03$) and the interaction between EEQ and sex/pregnancy status (non-pregnant female $p = 0.03$). GLU_OST concentrations decreased as EEQ increased, with an EMTREND of -0.20 mg/dl (CI $-0.38 - 0.01$). GLU_OST concentrations increased as EEQ decreased in non-pregnant females (EMTREND -0.27 mg/dl, CI $-0.51 - -0.02$) (figure 5B and supplemental table 6).

Log transformed serum INS concentration was negatively associated with EEQ ($p = 0.01$) and the interaction between EEQ and sex/pregnancy status (non-pregnant female $p = 0.01$). INS concentrations decreased marginally but significantly as EEQ concentration increased, with an EMTREND of -4.50×10^{-3} mg/dl (CI $-0.01 - -1.11 \times 10^{-3}$). Similarly, INS concentrations decreased as EEQ increased in non-pregnant females (EMTREND -0.01 mg/dl, CI $-0.01 - -1.59 \times 10^{-3}$) (figure 5C and supplemental table 7).

Log transformed serum INS_OST concentration was negatively associated with EEQ ($p = 1.40 \times 10^{-3}$) and an interaction between EEQ and sex/pregnancy status (non-pregnant female $p = 1.60 \times 10^{-3}$). INS_OST concentrations decreased as EEQ concentration increased, with an EMTREND of -0.01 mg/dl (CI $-0.01 - -2.66 \times 10^{-3}$). Similarly, INS_OST concentrations decreased as EEQ increased in non-pregnant females (EMTREND -0.01 mg/dl, CI $-0.01 - -3.45 \times 10^{-3}$) (figure 5D and supplemental table 8).

Square root transformed leptin concentration was negatively associated with EEQ ($p = 0.05$). Leptin concentrations decreased marginally but significantly as EEQ increased, with an EMTREND of -2.19×10^{-3} mEq/L (CI $-4.41 \times 10^{-3} - -4.17 \times 10^{-5}$) (figure 5E and supplemental table 9).

Log transformed TG concentration was positively associated with TEQ ($p = 0.01$) and the interaction between TEQ and plasma fat extracted ($p = 0.01$). TG concentration increased as TEQ increased, with an EMTREND of 0.03 mg/dL (CI $0.01 - 0.06$). TG concentration increased as TEQ increased as fat extracted from the sample increased with an EMTREND of 0.03 mg/dL (CI $0.01 - 0.06$) (figure 5F and supplemental table 10).

No significant associations with TEQ or EEQ were identified for ACTH (supplemental table 11), NEFA (supplemental table 12) or adiponectin (supplemental table 13).

4. Discussion

EMS is a complex phenotype with both environmental and genetic components contributing to disease (McCue et al., 2015; Norton et al., *submitted 2018*). Diet, exercise and season make up only a small proportion of the environmental component of EMS, leaving a large amount of the environmental variation in the EMS phenotype as yet unexplained (Schultz,

2016). A thorough understanding of all factors associated with the EMS phenotype is essential to improving management and decreasing the incidence of clinical disease. EMS is also a potential translational model for MetS and improving our understanding of the pathophysiology of EMS may therefore improve our understanding of MetS. This study identified associations between ligands of the AhR and ERs and multiple EMS-associated phenotypes in 301 Welsh Ponies and Morgan horses. To the authors' knowledge, this is the first report of an association between the concentrations of EDCs (AhR and ER ligands) with components of a clinical disease phenotype in domestic animals. Insulin dysregulation and increased adiposity are well recognized features of both EMS and MetS (Johnson, 2002; Johnson et al., 2009; Reaven, 2011). A large amount of the environmental variability of both EMS (Schultz, 2016) and MetS (Neel and Sargis, 2011) phenotypes are unexplained by measurable factors. In humans, EDCs have been hypothesized to explain some of that unexplained environmental variability (Henriksen et al., 1997; Michalek et al., 1999; Cranmer et al., 2000; Novelli et al., 2005; Lee et al., 2006; Elobeid et al., 2010; Lee et al., 2010; Casals-Casas and Desvergne, 2011; Kim et al., 2011; Lee et al., 2011; Neel and Sargis, 2011; Magliano et al., 2014), based on the associations identified in this study, this may also be the case in horses with EMS.

There are limited numbers of prospective studies looking at the relationship between EDCs and diabetes. One study with 20 years of follow-up identified a non-linear relationship between EDC concentration and diabetes, with individuals in the second quartile having the highest risk of developing diabetes (Lee et al., 2010). The Lee et al. 2010 study demonstrated that long-term low-dose exposure to EDCs may be more important in the development of type II diabetes than single high dose exposures. A long-term prospective study in horses is needed to determine if long term exposure to EDCs can lead to insulin dysregulation in the horse. Another consideration is that genetic variants in genes encoding receptors activated by EDCs may affect an individual's response to exposure to a certain quantity of EDCs. An association between genetic variants in xenobiotic and estrogen-metabolizing genes, EDC exposure and the risk of breast cancer has been reported in humans (Wielsøe et al., 2018), but to-date no association with these genes and endocrine disease has been reported. There is however, some evidence that variation in the AhR pathway genes in aquatic mammals may play a role in their susceptibility to the negative effects of EDCs (Zhou et al., 2010). Additionally, 23% of AhR knock-out mice have an abnormal response to glucose tolerance tests (Thackaberry et al., 2003). We are currently investigating if genetic variants in the AhR or ER play a role in EMS.

Interestingly, superfund score was not associated with TEQ, and only SF100 was marginally negatively but significantly associated with EEQ concentration in this study. As seen in supplementary figure 1 a majority of the horse farms were close to several superfund sites and so it was hard to compare horses at close to superfund sites versus far away from superfund sites. The calculations to give the superfund score (supplementary information 1) attempted to grade the 'risk' associated with being close to many superfund sites versus only close to one or two however this may not have been sufficient to show an effect. Additionally, we had incomplete information on the type of chemical predominating in each superfund site (SFS) and the classification of the SFS included in the calculation. All SFS regardless of being marked as active, inactive or pending were included in the superfund

score; therefore there is likely variability in the amount of risk with different SFS classifications. The materials that were processed at each SFS were not included in the calculation and not all sites included may have high levels of important EDCs, which may have contributed to the lack of association between SFS and plasma EDC levels in this study. The length of environmental exposure and prior exposures are also important factors to consider. Unfortunately, it was also not possible to determine how long the individual horses had lived on the farms. We attempted to control for this by only including horses >2 years of age, but not all individuals had lived on the farm for their entire lives as horses often change ownership, which may also contribute to the lack of significant associations between EEQ and TEQ concentrations and superfund score. Work in humans residing within 25 miles of a SFS demonstrated that patients with the top 10% of blood TCDD levels had hyperinsulinemia following an oral glucose tolerance test (Cranmer et al., 2000). There is also evidence that EDCs can spread by wind and can therefore be found a long way from their original source (United States Environmental Protection Agency, 2013), which could have further confounded any associations with superfund distance. To investigate the effect of distance to a SFS and the EMS phenotypes, further work needs to be performed on a larger cohort of horses housed around SFS with information about the type of disposal activity.

Another surprising finding was that EEQ concentration was negatively associated with glucose and insulin pre and post oral sugar challenge. This was the opposite effect to what we were expecting. This may be due to not having enough samples at a high EEQ concentration to see an effect; as seen in figure 3a only a few horses had very high concentrations of EEQ, therefore we may not have had the power to identify the true direction of the association. It is also possible that the association between EMS and EEQ concentrations is due to a non-monotonic dose response where the lower the concentration of EEQ, the more effect it has. This would explain the negative associations, particularly as we have very few horses with high concentrations of EEQ. There are several studies in lab animals and humans that report a non-monotonic dose response to EDCs including dioxins (Melnick et al., 2002; Birnbaum, 2012; Vandenberg et al., 2012; Vandenberg, 2014). To further explore this, we would need to measure EEQ concentrations in more horses with EMS to give power to assess if this is true for the horse.

It is important to recognize that the results of both bioassays are affected by any agonist and / or antagonist of the AhR and ER receptors. EDCs are not the only agonists in plasma that can affect these receptors; for example, amino acid metabolites have been reported as agonists for the AhR (Opitz et al., 2011). The ER α CALUX[®] bioassay measures plasma estrogenic activity (Sonneveld et al., 2005). Therefore the positive association with pregnancy and negative association in castrated males would be expected. For the first 35 days of pregnancy there is no difference in estrogen concentration between pregnancy and non-pregnant mares, but by day 85 of pregnancy there is a consistent increase in estrogen in pregnant mares compared to non-pregnant mares (Terqui and Palmer, 1979). Unfortunately, we were not able to obtain exact pregnancy dates in the mares in this study. One important advantage of the bioassays over the 'gold standard' of mass spectrometry is that both receptor agonist and antagonists affect the results, which is not the case with mass

spectrometry and is an important consideration for what may be occurring in the actual horse (Park et al., 2013).

One of the challenges with this study was that only ~1 ml of plasma was available for analysis for each horse and horses appear to have a lower level of fat in their plasma than other species. The median concentration of fat per ml of plasma in these horses was 5 mg. In humans the reported concentration of fat is ~7 mg fat per ml of plasma (Ayotte et al., 2005). Several steps in the methods were added to account for this, however there were still a good proportion of the samples (43.5% and 48.7% for TEQ and EEQ respectively) that were below the LOD of the bioassay. This led to a decreased power to detect associations, which in turn may have affected the results. While a strong correlation between EDC accumulation in the adipose tissue and circulating levels detected in the serum has been reported in humans (Pauwels et al., 2000), it is possible that low circulating lipid concentrations in the horse resulted in under-estimation of the total body EDCs accumulation in adipose tissue. Additionally, the bioassays used only detect EDCs that act through the AhR and ER. EDCs that act through other receptors were therefore not detected. However, a majority of the dioxins that have been associated with metabolic traits in humans act through the AhR (Kim et al., 2011) and several pesticides that horses are likely to be exposed to act through the ER (Lemaire et al., 2006). Therefore we considered these pathways to be two of the most important pathways to evaluate in this study.

4. Conclusion

This study has demonstrated that EDCs (AhR and ER ligands) are present in horse plasma, as previously seen in other grazing livestock species (Feil and Ellis, 1998; Rychen et al., 2008). We also identified that TEQ concentration was associated with fat extracted from the sample, and EEQ concentration was associated with sex/pregnancy status and SF100 score. Additionally, we identified associations between AhR and ER ligands with several EMS-associated phenotypes including serum glucose and insulin, glucose and insulin OST, triglyceride and leptin concentrations. The AhR and ER ligands were not associated with ACTH, adiponectin and NEFA concentrations. Overall, these results suggest that accumulation of EDCs may explain some of the previously unexplained environmental variance in the EMS phenotype, however the precise role and dose response to EDCs in horses with EMS is not clear at this time. The findings are also consistent with the associations identified between MetS in humans and EDCs, providing evidence for another similarity between EMS and human MetS. Further work, including investigation of genetic variants in the AhR and ER in horses and ponies, and possible associations with EMS, is warranted to establish if there is a gene by environment interaction that contributes to these associations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by: USDA NIFA-AFRI Project 2009–55205-05254: Integrated Research and Extension Program for Equine Metabolic Syndrome and Shivers and the Morris Animal Foundation D14EQ-033: Understanding Genetic Risk Factors for Metabolic Syndrome and D15EQ-029: Role of endocrine disrupting chemicals in equine metabolic syndrome. Salary support for SA Durward-Akhurst was provided by an American College of Veterinary Internal Medicine Foundation fellowship, and by a T32 Institutional Training Grant in Comparative Medicine and Pathology (5T320D010993–12).

The authors acknowledge the Minnesota Supercomputing Institute (MSI) at the University of Minnesota for providing resources that contributed to the research results reported within this paper. URL: <http://www.msi.umn.edu>

Abbreviations

%ile	Percentile
ACTH	Adrenocorticotrophic hormone
AICc	Small sample corrected Akaike information criterion
AhR	Aryl hydrocarbon receptor
BCS	Body condition score
BCS7_LAM	Body condition score and laminitis status
CI	95% confidence interval
DCC-FCS	Dextran-coated charcoal-tripped fetal calf serum
DMSO	Dimethyl sulfoxide
DDT	Dichlorodiphenyltrichloroethane
DMEM/F12	Nutrient mixture F-12
EDC	Endocrine disrupting chemical
EDTA	Ethylenediaminetetraacetic acid
EEQ	17 β -estradiol equivalent
EMMEAN	Estimated marginal mean
EMS	Equine Metabolic Syndrome
EMTREND	Estimated marginal mean of linear trend
ER	Estrogen receptor
Fat (g)	Fat extracted from sample (g)
FCS	Fetal calf serum
GH	Girth to height ratio
GLU	Glucose

GLU_OST	Glucose post oral sugar test
Grazing	Hours grazing in a 24 hour period
HCl	Hydrochloric acid
INS	Insulin
INS_OST	Insulin post oral sugar test
IQR	Interquartile range
LOD	Limit of detection
M	Male
MEM	Minimum essential medium
MetS	Human metabolic syndrome
MTBE	Methyl tertiary butyl ether
NEFA	Non-esterified fatty acid
NH	Neck to height ratio
NP	Non-pregnant female
OECD	Organisation for Economic Co-operation and Development
OST	Oral sugar test
P	Pregnant female
Res	Residuals
SF100	Superfund score within a 100 mile radius
SF250	Superfund score within a 250 mile radius
SF500	Superfund score within a 500 mile radius
SFS	Superfund site
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEQ	2,3,7,8-TCDD toxic equivalent
TG	Triglyceride

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Highlights

- EDCs (Ahr/ER ligands) are detectable in equine plasma
- EDCs (Ahr/ER ligands) are associated with several components of the EMS phenotype
- EDCs (Ahr/ER ligands) may explain some of the environmental variation in EMS

Figure 1.

Log 10 transformed TEQ in 301 horses (**A**) and statistically significant associations with log 10 transformed TEQ (data are reverse transformed for figure) (**B**). The horizontal lines are the 95% confidence intervals for the EMMEAN. The orange line and open triangle and green line and closed triangle represent the 25 and 75 %iles of fat (g) respectively.

Figure 2. Statistically significant associations with log10 transformed TEQ residuals (data are reverse transformed for figure). The horizontal lines are the 95% confidence intervals for the EMMEAN. The orange line and open triangle and green line and closed triangle represent the 25 and 75 %iles of fat (g) respectively.

Figure 3.

Log 10 transformed EEQ in 300 horses (A) and statistically significant associations with log 10 transformed EEQ (data are reverse transformed for figure) (B). The horizontal lines are the 95% confidence intervals for the EMMEAN. The orange line and open triangle and green line and closed triangle represent the 25 and 75 %iles of SF100 respectively.

Figure 4. Statistically significant associations with log10 transformed EEQ residuals (data are reverse transformed for figure). The horizontal lines are the 95% confidence intervals for the EMMEAN. The orange line and open triangle and green line and closed triangle represent the 25 and 75 %iles of SF100 respectively.

Figure 5.

Statistically significant associations with GLU (A), GLU_OST (B), log transformed INS (C), log transformed INS_OST (D), square root transformed LEP (E) and log transformed TG (F). Where appropriate, data are reverse transformed for figure. The horizontal lines are the 95% confidence intervals for the EMMEAN of the response variable. The orange line and open triangle and green line and closed triangle represent the 25 and 75 %iles of EEQ (res) [A, B, C, D, E] and TEQ (res) [F].

Table 1.

EMS phenotypes of interest and expected direction of change in horses with EMS.

EMS phenotype	Expected direction of change with EMS (Schultz, 2016)
Resting insulin (INS)	Increase
Resting glucose (GLU)	Increase
Insulin post oral sugar challenge (INS_OST)	Increase
Glucose post oral sugar challenge (GLU_OST)	Increase
Adiponectin	Decrease
Adrenocorticotrophic hormone (ACTH)	No change
Leptin	Increase
Non-esterified fatty acids (NEFA)	Increase
Triglycerides (TG)	Increase

Table 2.Characteristics of the DR and ER α bioassays.

	DR CALUX	ER α CALUX
Selectivity	92.3%	87.6%
Accuracy	92.6%	95.6%
Reproducibility	22%	25%
Recovery	>75%	>75%
LOD (pM/well)	0.3	0.8
Repeatability triplicate analysis	<15%	<15%

Table 3.
EMS signalment, history and morphometric measurements.

Summary of the signalment, history and morphometric data in the study cohort.

Predictor variable	Number with data	Mean	Range
Age	301	12.9	4.00–33.0
NH	301	0.67	0.53–0.83
GH	301	1.24	1.00–1.49
Predictor variable		Number	
Sex	Female (NP)	222	
	Female (P)	25	
	Male (M)	54	
Not obese (BCS < 7)	Laminitic	34	
	Non-laminitic	167	
Obese (BCS ≥ 7)	Laminitic	19	
	Non-laminitic	81	
Season sampled	Spring	53	
	Summer	143	
	Fall	55	
	Winter	50	

Table 4.
EMS biochemical measurements.

Normally distributed measurements are described as mean and standard deviation; non-normally distributed measurements are described as median and interquartile range (IQR).

EMS phenotypes	Number with data	Mean $\pm$ standard deviation	Range	Reference range or cut off for EMS
GLU (mg/dL)	301	76.67 $\pm$ 11.84	32.80–197.50	75–115 Normal (Smith, 2015)
				>150 EMS with diabetes mellitus (Johnson et al., 2012)
GLU_OST (mg/dL)	301	96.31 $\pm$ 17.27	40.70–218.50	>150 EMS with diabetes mellitus (Johnson et al., 2012)
	Number with data	Median (IQR)	Range	
INS (μ U/mL)	301	7.10 (3.89–11.61)	1.50–218.30	<20 Normal
				20–50 insulin dysregulation suspect
				>50 EMS (Frank et al., 2018)
INS_OST (μ U/mL)	300	20.94 (11.88–42.55)	1.50–308.00	<45 (Frank et al., 2018)
Adiponectin (μ g/mL)	301	4.58 (2.97–7.46)	0.10–37.00	3.2 Normal
				2.5–3.1 Metabolic derangement (Menzies-Gow et al., 2017)
				<2.5 EMS (Frank et al., 2018)
Leptin (ng/mL)	298	5.25 (3.34–8.48)	0.00–26.15	>7.3 EMS (Carter et al., 2009)
TG (mg/dL)	301	22.3 (16–32.3)	0.10–241.02	>94 EMS (Carter et al., 2009)
NEFA mEq/L	301	0.20 (0.12–0.31)	0.00–1.19	No published reference range
ACTH (pg/mL)	300	24.05 (18.88–30.95)	10–508	<58 Normal (EMS) (Carter et al., 2009)