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Title	Relationships of inflamm-aging with circulating nutrient levels, body composition, age, and pituitary pars intermedia dysfunction in a senior horse population
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Abstract

Similarly to aged humans, senior horses (≥20 years) exhibit chronic low-grade inflammation systemically, known as inflamm-aging. Inflamm-aging in the senior horse has been characterized by increased circulating inflammatory cytokines as well as increased inflammatory cytokine production by lymphocytes and monocytes in response to a mitogen. Little is currently known regarding underlying causes of inflamm-aging. However, senior horses are also known to present with muscle wasting and often the endocrinopathy pituitary pars intermedia dysfunction (PPID). Despite the concurrence of these phenomena, the relationships inflamm-aging may have with measures of body composition and pituitary function in the horse remain unknown. Furthermore, nutrition has been a focus of research in an attempt to promote health span as well as life span in senior horses, with some nutrients, such as omega-3 fatty acids, having known anti-inflammatory effects. Thus, an exploratory study of a population of n=42 similarly-managed senior horses was conducted to determine relationships between inflamm-aging and measures of circulating nutrients, body composition, age, and PPID. Serum was collected to determine vitamin, mineral, and fatty acid content. Peripheral blood mononuclear cells were also isolated to determine inflammatory cytokine production of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) following stimulation with a mitogen, as well as to determine gene expression of interleukin(IL)-1β, IL-6, IL-10, IFN-γ, and TNF-α. Serum IL-6 and C-reactive protein were determined by enzyme-linked immunosorbent assay. Whole blood was collected for hematological and biochemical analysis. Body composition was evaluated via ultrasound and muscle scoring for all 42 horses as well as by deuterium oxide dilution for a subset of n=10 horses. Pituitary function was evaluated by measuring basal adrenocorticotropin hormone concentrations as well as by thyrotropin releasing hormone stimulation testing (to determine PPID status). Results showed various relationships between inflammatory markers and the other variables measured. Most notably, docosadienoic acid (C22:2n6c), docosapentaenoic acid (C22:5n3c), and folate were positively associated with numerous inflammatory parameters (P≤0.05). Although no relationships were found between inflamm-aging and PPID, being positive for PPID was negatively associated with vitamin B12 (P≤0.01). No relationships between inflammation and body composition were found. Even within this senior horse population, age was associated with multiple parameters, particularly with numerous inflammatory cytokines and fatty acids. In summary, inflamm-aging exhibited relationships with various other parameters examined, particularly with certain fatty acids. This exploratory study provides insights into physiological changes associated with inflamm-aging in the senior horse.

Keywords	horse; inflamm-aging; muscle; nutrition; pituitary pars intermedia dysfunction; senior
Taxonomy	Immunology, Immunity, Immune Response

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Highlights

- Inflamm-aging in the senior horse was associated with physiological changes.
- Inflammation was positively associated with folate and certain fatty acids.
- No relationships were found between inflammation and body composition.
- Pituitary pars intermedia dysfunction was negatively associated with vitamin B12.
- Age was associated with numerous inflammatory cytokines and fatty acids.

Similarly to aged humans, senior horses (≥20 years) exhibit chronic low-grade inflammation systemically, known as inflamm-aging. Inflamm-aging in the senior horse has been characterized by increased circulating inflammatory cytokines as well as increased inflammatory cytokine production by lymphocytes and monocytes in response to a mitogen. Little is currently known regarding underlying causes of inflamm-aging. However, senior horses are also known to present with muscle wasting and often the endocrinopathy pituitary pars intermedia dysfunction (PPID). Despite the concurrence of these phenomena, the relationships inflamm-aging may have with measures of body composition and pituitary function in the horse remain unknown. Furthermore, nutrition has been a focus of research in an attempt to promote health span as well as life span in senior horses, with some nutrients, such as omega-3 fatty acids, having known anti-inflammatory effects. Thus, an exploratory study of a population of n=42 similarly-managed senior horses was conducted to determine relationships between inflamm-aging and measures of circulating nutrients, body composition, age, and PPID. Serum was collected to determine vitamin, mineral, and fatty acid content. Peripheral blood mononuclear cells were also isolated to determine inflammatory cytokine production of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) following stimulation with a mitogen, as well as to determine gene expression of interleukin(IL)-1β, IL-6, IL-10, IFN-γ, and TNF-α. Serum IL-6 and C-reactive protein were determined by enzyme-linked immunosorbent assay. Whole blood was collected for hematological and biochemical analysis. Body composition was evaluated via ultrasound and muscle scoring for all 42 horses as well as by deuterium oxide dilution for a subset of n=10 horses. Pituitary function was evaluated by measuring basal adrenocorticotropin hormone concentrations as well as by thyrotropin releasing hormone stimulation testing (to determine PPID status). Results showed various relationships between inflammatory markers and the other variables measured. Most

notably, docosadienoic acid (C22:2n6c), docosapentaenoic acid (C22:5n3c), and folate were positively associated with numerous inflammatory parameters ($P \leq 0.05$). Although no relationships were found between inflamm-aging and PPID, being positive for PPID was negatively associated with vitamin B12 ($P \leq 0.01$). No relationships between inflammation and body composition were found. Even within this senior horse population, age was associated with multiple parameters, particularly with numerous inflammatory cytokines and fatty acids. In summary, inflamm-aging exhibited relationships with various other parameters examined, particularly with certain fatty acids. This exploratory study provides insights into physiological changes associated with inflamm-aging in the senior horse.

Keywords: horse; inflamm-aging; muscle; nutrition; pituitary pars intermedia dysfunction; senior

Abbreviations:

ACTH, adrenocorticotropin hormone; alk. phosphatase, alkaline phosphatase; AHDC, Animal Health Diagnostic Center; AMV, avian myeloblastosis virus; BUN, blood urea nitrogen; BCS, body condition score; BW, body weight; CRDS, cavity ring-down spectroscopy; CBC, complete blood count; CRP, c-reactive protein; CK, creatine kinase; D_2O, deuterium oxide; D. Bili, direct bilirubin; DPA, docosapentaenoic acid; FFM, fat free mass; Hgb, hemoglobin; LDH, lactate dehydrogenase; LOA, limits of agreement; seg, mature neutrophils; MFI, mean fluorescence intensity; MAD, median absolute deviation; ppt, parts per thousand; PPID, pituitary pars intermedia dysfunction; PUFA, polyunsaturated fatty acids; RBC, red blood cells; RQ, relative quantity; SGOT/AST, serum glutamic oxaloacetic transaminase/aspartate aminotransferase; SDH, sorbitol dehydrogenase; TRH, thyrotropin releasing hormone; T. Bili, total bilirubin; TBW, total body water; VSMOW, Vienna Standard Mean Ocean Water; WBC, white blood cells

Relationships of inflamm-aging with circulating nutrient levels, body composition, age, and pituitary pars intermedia dysfunction in a senior horse population

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Abstract

Similarly to aged humans, senior horses (≥20 years) exhibit chronic low-grade inflammation systemically, known as inflamm-aging. Inflamm-aging in the senior horse has been characterized by increased circulating inflammatory cytokines as well as increased inflammatory cytokine production by lymphocytes and monocytes in response to a mitogen. Little is currently known regarding underlying causes of inflamm-aging. However, senior horses are also known to present with muscle wasting and often the endocrinopathy pituitary pars intermedia dysfunction (PPID). Despite the concurrence of these phenomena, the relationships inflamm-aging may have with measures of body composition and pituitary function in the horse remain unknown. Furthermore, nutrition has been a focus of research in an attempt to promote health span as well as life span in senior horses, with some nutrients, such as omega-3 fatty acids, having known anti-inflammatory effects. Thus, an exploratory study of a population of n=42 similarly-managed senior horses was conducted to determine relationships between inflamm-aging and measures of circulating nutrients, body composition, age, and PPID. Serum was collected to determine vitamin, mineral, and fatty acid content. Peripheral blood mononuclear cells were also isolated to determine inflammatory cytokine production of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) following stimulation with a mitogen, as well as to determine gene expression of interleukin(IL)-1β, IL-6, IL-10, IFN-γ, and TNF-α. Serum IL-6 and C-reactive protein were determined by enzyme-linked immunosorbent assay. Whole blood was collected for hematological and biochemical analysis. Body composition was evaluated via ultrasound and muscle scoring for all 42 horses as well as by deuterium oxide dilution for a subset of n=10 horses. Pituitary function was evaluated by measuring basal adrenocorticotropin hormone concentrations as well as by thyrotropin releasing hormone stimulation testing (to determine PPID status). Results showed

various relationships between inflammatory markers and the other variables measured. Most notably, docosadienoic acid (C22:2n6c), docosapentaenoic acid (C22:5n3c), and folate were positively associated with numerous inflammatory parameters ($P \leq 0.05$). Although no relationships were found between inflamm-aging and PPID, being positive for PPID was negatively associated with vitamin B12 ($P \leq 0.01$). No relationships between inflammation and body composition were found. Even within this senior horse population, age was associated with multiple parameters, particularly with numerous inflammatory cytokines and fatty acids. In summary, inflamm-aging exhibited relationships with various other parameters examined, particularly with certain fatty acids. This exploratory study provides insights into physiological changes associated with inflamm-aging in the senior horse.

1. Introduction

Senior horses (≥20 years) exhibit chronic low-grade inflammation systemically; this phenomenon is known as inflamm-aging and occurs in various species including humans (Franceschi et al., 2000). Inflamm-aging in the horse is characterized by increased pro-inflammatory cytokine production by monocytes and lymphocytes of old horses when compared to young horses. Specifically, old horses have increased levels of circulating IL-1β, IL-15, IL-18, and TNF-α in whole blood, as well as increased production of IFN-γ and TNF-α by PBMC after stimulation with a mitogen (Adams et al., 2008; Adams et al., 2009). A vast body of human literature has shown systemic inflammation to be an underlying condition predisposing humans to various diseases including Alzheimer's disease, atherosclerosis, macular degeneration, and degenerative arthritis (Franceschi and Campisi, 2014). Inflamm-aging, therefore, is considered to be a key predictor of morbidity and mortality in humans (Franceschi and Campisi, 2014); however, the implications of inflamm-aging in regards to morbidity and mortality in the horse remain

unknown. Furthermore, the causes of inflamm-aging in both humans and horses remain relatively unknown (Franceschi and Campisi, 2014).

In many species, various vitamins, minerals, and fatty acids have been associated with inflammation, whether pro- or anti-inflammatory. Omega-3 supplementation in the horse for example has demonstrated potential anti-inflammatory effects (Elzinga et al., 2019). Nutrition has implications for health span and life span; however, this relationship requires further elucidation (Dato et al., 2016), particularly in the old horse, where much currently remains unknown (Ralson and Harris, 2013; Siciliano, 2002). Furthermore, the numerous associations between inflammation and vitamins, minerals, and fatty acids in various species indicate that nutritional intervention has the potential to alter inflammatory profiles (Dasilva et al., 2016; Dato et al., 2016), which may extend to the senior horse.

In addition to inflamm-aging, old horses experience age-related muscle wasting (Lehnhard et al., 2004; Reed et al., 2015), similarly to other species including humans (Lehnhard et al., 2004; Reed et al., 2015; Schaap et al., 2009; Schaap et al., 2006). In longitudinal human studies, increased inflammatory markers, particularly TNF-α, IL-6, and C-reactive protein (CRP), were associated with decreased muscle mass and strength in the elderly (Schaap et al., 2009; Schaap et al., 2006). Given that horses are athletic animals, this muscle loss in senior horses, which may be associated with inflamm-aging, is of particular concern. The relationship between inflamm-aging and muscle wasting therefore requires further study.

While there is little evidence that senior horses have different hematological or biochemical reference ranges than adult horses (Silva and Furr, 2013), it was of interest to determine whether inflammation in the older animal was associated with any particular clinical biomarkers. For example bilirubin, a biomarker of liver function, has demonstrated variable associations with

inflammation in other species, exhibiting anti-inflammatory properties (Moreno-Otero et al., 1994) and even protecting against inflamm-aging in some studies (Zelenka et al., 2016), while exhibiting pro-inflammatory effects in other studies (Qaisiya et al., 2016). Senior horses also frequently exhibit pituitary pars intermedia dysfunction (PPID), commonly known as equine Cushing's disease. This endocrinopathy is characterized by hypertrichosis but is also associated with immunosuppression and various other clinical signs (McFarlane, 2011). Pituitary pars intermedia dysfunction has been associated with total leukocyte-mediated cytokine dysregulation as well (McFarlane and Holbrook, 2008). Therefore, it was of interest to examine whether various circulating serum and PBMC-mediated markers of inflammation would be associated with PPID.

Thus, an exploratory study to examine the potential relationships between inflammatory parameters and various vitamin, mineral, fatty acid, hematology, biochemistry, body composition, and PPID parameters in senior horses was undertaken. It was hypothesized that inflamm-aging would exhibit relationships with measures of other health parameters, providing potential areas for further study. Specifically, it was anticipated that inflamm-aging would be inversely associated with markers of muscle mass and with levels of known anti-inflammatory nutrients, while not exhibiting relationships with other parameters.

2. Methods and materials

2.1. Animals and study design

All procedures were approved by the University of Kentucky Institute of Animal Care and Use Committee. Forty-two senior horses [mean age = 24.4 ± 3.0 yr (SD); range = 18-29 yr] of mixed-breeds and sex with a mean body condition score (BCS) of 5.2 ± 0.8 and body weight (BW) of 531.2 ± 86.9 kg were used in this study. All horses were healthy with no clinical signs of infectious disease. All horses were housed at the University of Kentucky, Department of

Veterinary Science Maine Chance Farm in Lexington, Kentucky on pasture during the winter season. Horses received a diet that met NRC recommended requirements (NRC, 2007) and was comprised of 50% oats and 50% vitamin and mineral fortified alfalfa pellets (5.5 kg per day, divided equally into meals fed at 0830 and 1400 h) with mixed grass hay provided ad libitum (Table 1).

Samples and measurements collected for all (n=42) horses included the following: BW, BCS, muscle mass score, rump ultrasound, and blood samples. A subset of n=10 of these horses also had deuterium oxide assessment of percent body fat and fat free mass (FFM) performed. Horses were weighed (using a portable calibrated large-animal scale) and assessed for BCS using the Henneke scale (1-9) (Henneke et al., 1983) by two experienced assessors. Muscle mass was scored by two assessors using the previously established 1-5 scale (Graham-Thiers and Kronfeld, 2005). Blood was collected within 2 hours after horses had eaten their morning meal. Percent body fat and fat free mass were estimated using ultrasound (Kane, 1987; Lehnhard et al., 2004) and deuterium oxide (Dugdale et al., 2011) methods within one week post blood collection. Furthermore, thyrotropin releasing hormone (TRH) stimulation testing was performed on all horses within 3 hours after their morning meal, with adrenocorticotropin hormone (ACTH) being measured prior to and 10 minutes post intravenous TRH injection, to determine pituitary function, as further described below.

2.2. Blood sampling

All blood was collected from the jugular vein using aseptic technique. Heparinized blood was collected to isolate PBMC, which were processed fresh after collection. Serum was collected for analysis of vitamins, fatty acids, and inflammatory proteins [IL-6, TNF-α, and C-reactive protein (CRP)]. Serum was also collected to analyze trace mineral content [in royal blue top, serum

clot activator (silicone coated) tubes with hemogard closure to enable zinc analysis (Fisher Scientific, Waltham, MA)]. Plasma (EDTA) was collected for ACTH analysis. Serum and plasma samples were all centrifuged (800g x 10 min x 22°C) and frozen at -20°C until analysis. Lithium heparinized whole blood was collected and immediately analyzed for complete blood count with differential and blood chemistry panel analysis.

2.3. PBMC-mediated inflammatory cytokine assays

2.3.1. PBMC isolation, IFN-γ and TNF-α intracellular staining, and flow cytometry

Peripheral blood mononuclear cells were isolated from the collected heparinized blood using a Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) density gradient, as has been previously described (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). Peripheral blood mononuclear cells were counted using a VICELL™ Counter-XR (Beckman Coulter, Miami, FL) and plated at a concentration of $4x10^6$ cells/mL in complete media [RPMI 1640 (Gibco, Grand Island, NY) with 2.5% fetal equine serum (BioWest, Nuaillé, France), 55 μM 2-mercaptoethanol (Gibco), and 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/mL streptomycin (HyClone Pen/Strep/Glutamine solution; Thermo Scientific)] (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). Cells were aliquoted in duplicate into 24-well plates, with brefeldin A (10 μg/mL; Sigma) added to all wells, and PMA (25 ng/mL; Sigma) and ionomycin (1 μM; Sigma) added to one well per sample. Peripheral blood mononuclear cells were incubated 4 hours at 37°C, 5% CO_2, then fixed with 2% paraformaldehyde overnight. To determine pro-inflammatory cytokine production, PBMC were stained intracellularly with IFN-γ FITC mouse anti-bovine antibody (AbD Serotec, Raleigh, NC; 0.1 mg) and with TNF-α anti-equine monoclonal antibody (HL801; kindly provided by Dr. Rob MacKay, University of Florida) and secondary antibody FITC-conjugated goat $F(ab')_2$ anti-mouse IgG (H + L) (Invitrogen, Carlsbad, CA; 2 mg/mL)

(Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). Flow cytometry was performed using a FACS Calibur flow cytometer (Becton Dickinson) and Cell Quest software (Becton Dickinson) (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006).

2.3.2. RNA isolation and reverse transcription

Following the incubation in 24-well plates, aliquots (500 μL) of PBMC were centrifuged in 1.5 mL microcentrifuge tubes, resuspended in 1 mL of Trizol® solution, and stored at -80°C, following the manufacturer's protocol. Phenol-chloroform extraction was used to isolate RNA, which was then stored at -80°C (Breathnach et al., 2006). Using an Epoch microplate spectrophotometer (BioTek, Winooski, VT), RNA was quantified. Reverse transcription was performed on 1 μg RNA in RNase-free water (41.5 μL total) using Master Mix [16 μL avian myeloblastosis virus (AMV) buffer 5X, 16 μL $MgCl_2$, 4 μL dNTP, 1 μL RNasin, 1 μL oligo dT primer, and 0.5 μL AMV reverse transciptase per sample; Promega, Madison, WI] and a thermocycler (Bio-Rad, Hercules, CA), with samples incubated at 42°C for 15 minutes and 95°C for 5 minutes (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). Samples of cDNA were stored at -20°C for a month until PCR analysis was performed.

2.3.3. Determination of cytokine gene expression

Samples of cDNA were thawed at room temperature and loaded into the epMotion 5070 (Eppendorf) with 5 equine specific intron-spanning primers and probes including IFN-γ, IL-1β, IL-6, IL-10, and TNF-*α*, (Applied Biosystems, Foster City, CA) in addition to beta-glucuronidase, the housekeeping gene (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). Real time-PCR was performed using the 7900HT Fast RT-PCR System (Applied Biosystems), which incubated samples at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15s and 60°C for 60s (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). The reaction volume for each

sample was 10 µL of master mix, with 5 µL Sensimix II Probe Kit (Bioline), 0.5 µL assay mix for the gene of interest (primer/probe sets; Applied Biosystems), and 4.5 µL cDNA template (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). Relative changes in cytokine gene expression were quantified using the ΔΔCT method (Livak and Schmittgen, 2001). The average ΔCT of all the wells treated with media alone served as the calibrator for each cytokine. Data are reported as natural logs of relative quantity (RQ) values ($RQ = 2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001).

2.4. Inflammatory protein ELISAs

Using ELISA methods previously described in the horse, serum inflammatory protein concentrations of IL-6 (Burton et al., 2009) and CRP (Lavoie-Lamoureux et al., 2012) were quantified in duplicate. Briefly, IL-6 was measured by coating ELISA plates (Immunoplate Maxisorp, Nalge Nunc Int., Rochester, NY) with a polyclonal goat anti-horse IL-6 antibody (AF1886, R&D Systems, Inc., Minneapolis, MN), blocking plates with PBS (pH 7.2) supplemented with 0.5% BSA, washing with phosphate buffer (2.5 mmol NaH_2PO_4, 7.5 mmol Na_2HPO_4, 145 mmol NaCl, 0.1% (v/v) Tween 20, pH 7.2), and tagging with biotinylated goat anti-horse IL-6 (AF1886, R&D Systems, Inc., Minneapolis, MN) and a streptavidin–horseradish peroxidase solution (Jackson ImmunoResearch Lab., West Grove, PA) (Burton et al., 2009). A recombinant equine IL-6 (1886-EL, R&D Systems, Inc., Minneapolis, MN) in two-fold dilutions was used to create a standard curve, ranging from 500-4 ng/mL (Burton et al., 2009). Reactions were pigmented and stopped with TMB substrate solution (Thermo Scientific, Rockford, IL) and TMB stop solution (KPL, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), respectively and analyzed in duplicate colorimetrically at 450 nm of absorbance using an ELISA reader (Bio-Rad Laboratories, Inc., Philadelphia, PA). The serum sample dilution was 1:50 or 1:100, with

dilution factor taken into account when calculating protein concentrations. The mean intra-assay coefficient of variation was 6.4%.

Serum CRP was quantified using a commercially available equine-specific CRP ELISA kit (Kamiya Biomedical, Seattle, WA), according to manufacturer instructions (Lavoie-Lamoureux et al., 2012), except all serum samples were diluted 1:300 instead of 1:100. The standard curve ranged from 200-6.25 ng/mL. ELISA reader (Bio-Rad) analysis was performed at an absorbance of 450 nm. The mean intra-assay coefficient of variation was 2.5%, while the mean inter-assay coefficient of variation was 0.8%.

2.5. Circulating vitamins and minerals

Serum vitamins and minerals were analyzed at Michigan State University Diagnostic Center for Population and Animal Health, except Vitamin B12 and folate, which were analyzed at Cornell University Animal Health Diagnostic Center (AHDC) Endocrinology Laboratory using American Association of Veterinary Laboratory Diagnosticians validated assays. Serum 25-hydroxyvitamin D was analyzed via RIA using a commercially available 125 iodine-RIA kit (Dia Sorin, Stillwater, MN). Vitamins A (retinol), E (α-tocopherol), and β-carotene were measured via ultra high pressure liquid chromatography with a C18 column and photodiode array detection (Waters, Milford, MA), following liquid-liquid extraction. Fatty acids, including saturated and unsaturated, were measured via gas chromatography with a SP2556 column and flame ionization detection (PerkinElmer Inc., Waltham, MA), following methyl esterification preparation. Trace minerals including total zinc, selenium, cobalt, copper, iron, manganese, and molybdenum were measured in serum via inductively coupled plasma mass spectrometry (Agilent Technologies, Santa Clara, CA), following direct dilution preparation. Serum vitamin B12 and folate were

analyzed via chemiluminescence immunoassay using an Immulite® 2000 (Siemens, Berlin, Germany).

2.6. Muscle measures

Estimated percent body fat, fat weight, and FFM were determined using ultrasound measurements of rump fat thickness at approximately 11 cm cranial to the tail head and 10 cm off the midline. This measurement of rump fat was then used to calculate the estimated percent body fat according to the equation: % body fat = 5.4*(ultrasound rump fat thickness in cm) + 2.47 (Kane, 1987; Lehnhard et al., 2004). Estimated fat weight and FFM were calculated in turn using the equations: fat weight = body weight*(% body fat); FFM = body weight – fat weight (Lehnhard et al., 2004). Muscle mass was also determined by two assessors using the previously published scale of 1-5 (where 1=lowest; 5=highest), and scores were averaged prior to statistical analysis (Graham-Thiers and Kronfeld, 2005).

Furthermore, percent body fat and fat free mass were determined in ten horses using the deuterium oxide (D_2O) dilution method previously validated in ponies (Dugdale et al., 2011). (The horses assessed were n=5 with high inflammation and n=5 with low inflammation, as determined by %IFN-γ.) Briefly, a dose of 0.12 g/kg BW D_2O (Cambridge Isotope Laboratories, Tewksbury, MA) was administered through a temporary catheter in the left jugular vein. (All horses analyzed had a BCS of 4-6, therefore, 0.12*BW could be used for all horses.) Blood samples were collected by venipuncture of the right jugular vein immediately before and 4 hours after D_2O infusion. Deuterium oxide was administered into the catheter (16Gx5 ½”; Hospira, Inc.; Lake Forest, IL) with a 0.22 mm filter (Millex®GP Filter Unit, EMD Millipore, Darmstadt, Germany) on the syringe tip to ensure sterility of the procedure, followed by immediate administration of sterile saline (100 mL). Syringes were weighed to determine the exact weight of D_2O administered to

each animal. Blood was immediately spun at 2000xg for 10 minutes at 4°C and placed on ice. Plasma was aliquoted with limited air exposure into air-tight, o-ring screw cap vials (Fisher Scientific, Waltham, MA) and immediately placed in freezer. Samples were stored at -80°C until analysis.

Metabolic Solutions (Nashua, NH) performed D_2O content analysis in triplicate using cavity ring-down spectroscopy (CRDS) with a liquid water isotope analyzer automated injection system, version 2 upgrade (Los Gatos Research, Mountain View, CA) as has previously been published (Thorsen et al., 2011). To remove plasma proteins: zinc sulfate monohydrate (5 mg) was added to plasma (25-50 mL), samples were vortexed, and samples were spun at 6000g for 10 minutes at room temperature (22°C) to precipitate proteins. The supernatant was injected six times, with the last three being averaged to determine values. A standard curve was generated using known values of D_2O. Values were determined as deltas relative to the Vienna Standard Mean Ocean Water (VSMOW) standard in parts per thousand (ppt). Intra-run variation was <2 delta ppt/mL and inter-run variation is <3.5 delta ppt/mL. Deuterium oxide analysis was performed on samples pre and post D_2O administration as well as on D_2O infusate. The calculation of total body water (TBW) adapted from Dugdale et al 2011 (Dugdale et al., 2011) was conducted as follows:

$$\textbf{TBW (kg)} = \frac{WA}{a} \times \frac{(\text{delta of dose} - \text{delta of dilution tap water})}{(\text{delta of post} - \text{delta of pre})} \times \frac{1}{1000*1.04}$$

Here, W= water needed to dilute the D_2O infusate dose to enable CRDS measurement (58.9225 mg), A= amount of D_2O (g) administered to the horse, a= amount of D_2O infusate dose diluted and measured via CRDS (0.0276 mg), delta of D_2O infusate dose relative to VSMOW (2595.79 ppt/mL), delta of dilution tap water relative to VSMOW (-81.38 ppt/mL), 1000= necessary to convert g to kg, and 1.04= factor of overestimation of D_2O method due to deuterium binding to protein and non-exchangeable areas.

Using the adjustment factor of 0.723 adapted by Pace and Rathbun (Pace and Rathbun, 1945), equations were also used to determine percent body fat and fat free mass:

$$\% \text{ Body fat} = 100 - \frac{\text{TBW}}{0.732 \text{ BW}}$$

Fat Free Mass (FFM) = TBW / 0.732

2.7. Pituitary function

Adrenocorticotropin hormone levels were determined by Cornell AHDC Endocrinology Laboratory via chemiluminescence immunoassay using an Immulite 1000 (Siemens, Berlin, Germany), as previously published in the horse (Place et al., 2010).

As a dynamic measure of pituitary function, TRH stimulation was also performed within 3 hours after the morning meal. Thyrotropin releasing hormone (Sigma-Aldrich, St. Louis, MO) was stored at -20^{o}C until dissolved in saline. Using sterile techniques in a biochemical hood, TRH was dissolved in 0.9% saline (1 mg/mL), aliquoted into sterile microcentrifuge tubes, and immediately frozen at -80^{o}C (Beech et al., 2007; McFarlane et al., 2006). The morning of TRH testing, the aliquots were thawed at room temperature, and using sterile techniques in a chemical hood 1 mL of TRH solution was drawn into each of the syringes, which were immediately placed on ice. Blood was collected aseptically from the jugular vein in EDTA-containing tubes prior to and 10 minutes post intravenous TRH administration and placed on ice (Diez de Castro et al., 2014). The blood was then centrifuged at 800g for 10 minutes at room temperature and placed back on ice. Plasma was aliquoted and stored at -20^{o}C until shipment on dry ice to Cornell AHDC for ACTH analysis. Horses with ACTH values 10 minutes post TRH injection of >110 pg/mL were considered PPID, as recommended by the Equine Endocrinology Group (Restifo et al., 2016).

2.8. Hematological and biochemical analyses

Blood was analyzed by Rood and Riddle Equine Hospital Laboratory, Lexington, Kentucky to determine complete blood count (CBC) with differential and blood chemistry panel. A Beckman Coulter ACT/DIFF hematology machine and Beckman Coulter AU480 serum chemistry analyzer were used to determine CBC and blood chemistry, respectively. Specifically measured were: hemoglobin (Hgb), packed cell volume, red blood cells (RBC), white blood cells (WBC), total protein, mature neutrophils (seg), immature neutrophils, lymphocytes, monocytes, eosinophils, sodium, potassium, chloride, bicarbonate, albumin, serum glutamic oxaloacetic transaminase/aspartate aminotransferase (SGOT/AST), alkaline phosphatase, total bilirubin, direct bilirubin, creatine kinase (CK), creatinine, glucose, gamma-glutamyl transferase, blood urea nitrogen (BUN), phosphorus, calcium, sorbitol dehydrogenase (SDH), and lactate dehydrogenase (LDH).

2.9. Statistical analysis

Prior to data analysis, WINPEPI DESCRIBE (version 3.07) computer program for epidemiologists (Abramson, 2011) was used to determine outliers. Non-normally distributed data were natural log-transformed to achieve a normal distribution. Outliers at each end of the distribution, defined as values further than five times the median absolute deviation (MAD) from the median were then excluded prior to analysis to achieve normal distribution. (Minimal outliers were removed and are shown in S1 Table.)

Data were analyzed with SPSS version 24 (IBM Corp, Armonk, NY). Bivariate Pearson correlations were performed among the various parameter values. Partial correlations among the various parameters were also analyzed with age as a covariate. [Breed and sex were not included as covariates due to the fact that the population of horses examined were predominantly female (79%) and over half of the horses were Thoroughbreds, with quite a variety of other breeds

composing the rest of the population]. Where PPID was included as a binary variable, point-biserial correlations and partial point-biserial correlations with age as a covariate were performed. The few variables that were not normally distributed following log-transformation (serum IL-6, C22:2n6c, and C22:6n3c) were analyzed using non-parametric tests: bivariate Spearman correlations and partial Spearman correlations with age as a covariate. For comparisons of deuterium oxide method with ultrasound and muscle scoring to determine body composition, data were analyzed via partial Pearson correlations and with both age and bodyweight as covariates for n=10 horses that underwent D_2O analysis. Differences between D_2O and ultrasound methods in determining percent body fat and FFM were also evaluated using Bland-Altman plots with 95% limits of agreement.

For all analyses, data with $P \leq 0.01$ were considered significant, while $P \leq 0.05$ were considered trends. A large number of parameters were assessed in the current study, which increases the risk of Type I error. Adjustments for multiple comparisons were not undertaken in order to minimize the risk of failing to identify potentially important associations (Feise, 2002; Gelman et al., 2012; Rothman, 1990), and critical significance was set at $P \leq 0.01$ in order to achieve the best balance between Type I and Type II errors. Results tables depict significant and trending relationships between variables.

3. Results

3.1. Relationships of inflammation to serum levels of vitamins, minerals, and fatty acids

Various serum vitamins, minerals, and fatty acids were correlated with lymphocyte production of IFN-γ and TNF-α, peripheral blood mononuclear cell gene expression of inflammatory cytokines, and serum inflammatory proteins. C22:5n3c, C22:2n6c, and folate

particularly were positively associated with markers of inflammation after taking age into account as a covariate (Table 2). Docosapentaenoic acid (DPA; C22:5n3c) was positively correlated ($P \leq 0.01$) with % TNF-α, IFN-γ RQ, and IL-6 RQ, and exhibited a positive trend ($P \leq 0.05$) with % IFN-γ, IFN-γ MFI, IL-10 RQ, and TNF-α RQ (Table 2). Docosadienoic acid (C22:2n6c) exhibited a positive significant correlation ($P \leq 0.01$) with % IFN-γ, % TNF-α, TNF-α MFI, TNF-α RQ and a positive trend ($P \leq 0.05$) with IFN-γ MFI, IFN-γ RQ, and serum IL-6 (Table 2). Folate also exhibited a positive trend ($P \leq 0.05$) with % TNF-α, TNF-α MFI, IFN-γ RQ, IL-10 RQ, TNF-α RQ, and serum IL-6 (Table 2). Two fatty acids measured were correlated with two inflammatory parameters, including: C20:2n6c with % TNF-α and TNF-α RQ (positive correlation; $P \leq 0.05$) and C16:1n7c with IL-6 RQ and serum IL-6 (positive; $P \leq 0.05$ and $P \leq 0.01$, respectively) (Table 2). Serum IL-6 and C20:1n9c exhibited a significant ($P \leq 0.01$) positive correlation (Table 2).

3.2. Relationships of inflammation to hematological CBC and biochemical parameters

Measures of overall health, as determined by CBC and blood chemistry panel measures, were correlated with lymphocyte production of IFN-γ and TNF-α, peripheral blood mononuclear cell gene expression of inflammatory cytokines, and serum inflammatory proteins.

Numerous markers of inflammation were positively associated with WBC, total bilirubin, and direct bilirubin (Table 3). Specifically, WBC were positively correlated ($P \leq 0.01$) with % IFN-γ, % TNF-α, and IFN-γ RQ and exhibited a trend ($P \leq 0.05$) with IL-10 RQ and IL-6 RQ prior to age-adjustment (Table 3). After adjusting for age, the relationship of WBC with % IFN-γ remained significant ($P \leq 0.01$), while the relationship of WBC with % TNF-α, IFN-γ RQ, and IL-10 RQ became trends ($P \leq 0.05$) (Table 3). Total bilirubin was significantly correlated ($P \leq 0.01$) with % IFN-γ, IFN-γ MFI, % TNF-α, and TNF-α MFI prior to age-adjustment, while exhibiting a trend ($P \leq 0.05$) with all these inflammatory parameters following age-adjustment (Table 3). Direct

bilirubin was significantly correlated with % IFN-γ and IFN-γ RQ, while exhibiting trends ($P \leq 0.05$) with IFN-γ MFI, % TNF-α, TNF-α MFI, IL-10 RQ, and TNF-α RQ, prior to age-adjustment (Table 3). Following adjustment for age, direct bilirubin exhibited trends ($P \leq 0.05$) with % IFN-γ, IFN-γ MFI, and IFN-γ RQ (Table 3).

Inflammation was negatively correlated with eosinophils and SGOT/AST (Table 3). Specifically, eosinophils were significantly ($P \leq 0.01$) correlated with IFN-γ MFI and exhibited a trend ($P \leq 0.05$) with % TNF-α and IFN-γ RQ prior to adjustment for age (Table 3). Following age-adjustment, eosinophils likewise exhibited a significant ($P \leq 0.01$) negative correlation with IFN-γ MFI and a negative trend ($P \leq 0.05$) with % TNF-α, IFN-γ RQ, and IL-10 RQ (Table 3). Additionally, SGOT/AST was significantly ($P \leq 0.01$) correlated with TNF-α RQ and serum IL-6 and exhibited a trend ($P \leq 0.05$) with TNF-α MFI; following adjustment for age, these relationships were retained with the addition of a negative trend ($P \leq 0.05$) between SGOT/AST and serum CRP (Table 3).

Numerous other CBC and chemistry panel markers were associated individually with markers of inflammation (Table 3). Specifically, lymphocytes exhibited a positive trend ($P \leq 0.05$) with IFN-γ MFI and IL-6 RQ and a negative correlation ($P \leq 0.01$) with IL-1β, prior to age-adjustment (Table 3). Following age-adjustment, lymphocytes were positively associated with IFN-γ MFI ($P \leq 0.01$), IL-6 RQ ($P \leq 0.01$), and IFN-γ RQ ($P \leq 0.05$) and negatively correlated with IL-1β ($P \leq 0.01$) (Table 3). Neutrophils were positively correlated ($P \leq 0.01$) with IL-1β RQ regardless of age adjustment (Table 3). Hemoglobin exhibited a negative trend ($P \leq 0.05$) with IL-1β RQ, which became significant ($P \leq 0.01$) after adjusting for age (Table 3). C-reactive protein showed a significant ($P \leq 0.01$) negative correlation with BUN and creatinine, regardless of adjustment for age (Table 3). Serum IL-6 exhibited a significant ($P \leq 0.01$) negative correlation with

alkaline phosphatase, CK, LDH, and SGOT/AST and positive correlation with total protein ($P \leq 0.01$), regardless of age (Table 3). Serum IL-6 also exhibited a positive correlation that improved from a trend ($P \leq 0.05$) to significance ($P \leq 0.01$) after adjusting for age (Table 3).

3.3. Relationships of inflammation to body composition measures

Relationships between inflammation and body composition were also determined. Serum IL-6 exhibited a directly proportional relationship with body weight both prior to ($R=0.321$; $p=0.038$) and following age-adjustment ($R=0.551$; $P \leq 0.001$). Serum IL-6 also appeared to be positively correlated with fat free mass as estimated by ultrasound prior to ($R=0.322$; $p=0.037$) and following age-adjustment of the model ($R=0.534$; $P \leq 0.001$); however, upon taking bodyweight into the model as a covariate in addition to age, the association between IL-6 and FFM disappeared ($R=0.011$; $p=0.947$). No other measures of body composition (including average muscle score, BCS, body weight, % body fat via ultrasound, FFM via ultrasound, and for the subset of n=10 horses % body fat via D_2O and FFM via D_2O) exhibited relationships ($P>0.05$) with the inflammatory measures examined.

3.4. Relationships of PPID to inflammation, vitamins, minerals, fatty acids, hematological, biochemical, and body composition measures

The presence or absence of the endocrinopathy PPID was associated with various vitamin, mineral, fatty acid, CBC, chemistry panel, and body composition parameters examined (Table 4). Pituitary pars intermedia dysfunction status exhibited a significant positive association ($P \leq 0.01$) with basal ACTH and a significant ($P \leq 0.01$) negative association with vitamin B12, regardless of age adjustment (Table 4). Prior to adjustment for age, PPID status also exhibited a trend with folate, and basal ACTH exhibited a trend with vitamin B12 (Table 4). After adjusting the model

for age, PPID status yielded directly proportional trends (P≤0.05) with serum CRP and eosinophils (Table 4). After adjusting for age, PPID status also exhibited negative trends (P≤0.05) with RBC, creatinine, and fatty acid C20:4n6c (Table 4).

Basal ACTH, a known indicator of pituitary function, also exhibited some relationships with the various parameters examined. Basal ACTH exhibited a negative trend (P≤0.05) with vitamin B12; however, this relationship was not retained following adjustment for age (P=0.060) (Table 4). When age adjustment was taken into account, basal ACTH exhibited a significant (P≤0.01) positive correlation with fatty acid C16, while exhibiting a negative trend (P≤0.05) with direct bilirubin (Table 4).

3.5. Relationships of age to inflammation, vitamins, minerals, fatty acids, hematological, biochemical, body composition, and PPID measures

Even within this population of senior horses, age was associated with various vitamin, mineral, fatty acid, hematological, biochemical, body composition, and PPID parameters (Table 5). Among the inflammatory parameters, age was positively correlated (P≤0.01) with the percent of lymphocytes producing TNF-α and with gene expression of IFN-γ and IL-10, while also showing positive trends (P≤0.05) with percent of lymphocytes producing IFN-γ and with TNF-α gene expression (Table 5). Vitamin E, selenium, and fatty acids C18:2n6c and C24:1n9c also exhibited positive correlations with age, while fatty acids C16, C18:1n7c, and C18:1n9c exhibited negative correlations with age (P≤0.01) (Table 5). Age also exhibited a positive trend with C20:4n6c, and a negative trend with C16:1n7c and C20:1n9c (P≤0.05). Among CBC and chemistry panel parameters, the only relationship with age was a negative trend (P≤0.05) with SDH (Table 5). Various measures of body composition exhibited negative correlations (P≤0.01) with age including BCS, body weight, and FFM as estimated by ultrasound, as well as exhibiting

a negative trend with muscle score ($P \leq 0.05$) (Table 5). And within this group of old horses, both PPID status and basal ACTH were directly proportional to age ($P \leq 0.01$) (Table 5).

3.6. Comparison of D_2O methods with ultrasound and muscle scoring in determining body composition

When examining D_2O methods vs. ultrasound for the subset of 10 horses with age and body weight as covariates, results generally showed poor correlation ($P > 0.05$) (Table 6). However, FFM measurements via D_2O and muscle score were strongly correlated ($R = 0.895$; $p = 0.001$) (Table 6).

Bland-Altman plots were also used to visualize differences between D_2O and ultrasound methods of body composition analysis (Figure 1). The 95% limits of agreement (LOA) for percent body fat and FFM were -1.51 ± 3.90 and 7.64 ± 21.37, respectively (Figure 1). Only 5/10 and 4/10 horses fell within the 95% LOA for percent body fat and FFM, respectively (Figure 1).

4. Discussion

The occurrence of inflamm-aging in senior horses has been well-established (Adams et al., 2008; Adams et al., 2009); however, the implications of inflamm-aging on the overall health of the horse have not yet been determined. It was expected that inflamm-aging would be inversely associated with muscle measures and known anti-inflammatory nutrients, while specific markers of inflammation would be differentially associated with PPID status due to cytokine dysregulation.

This is the first study to evaluate associations between inflammatory measures and vitamin, mineral, fatty acid, hematological, biochemical, body composition, and PPID parameters in an aged horse population. The results provide novel insights into physiological changes associated with inflamm-aging in the senior horse and have identified several significant relationships that warrant further research.

Many studies have shown immune and particularly anti-inflammatory effects of various vitamins, minerals, and fatty acids in numerous species. For example, vitamin D has exhibited anti-inflammatory effects in humans and rats (Abbas, 2016; Capri et al., 2006; Moore et al., 2005). Mineral supplements including zinc (Zhu et al., 2016) and selenium (Brummer et al., 2013) in piglets and horses, respectively, have also exhibited various effects on immunity. In human patients experiencing a systemic inflammatory response, most micronutrients decreased with the exception of iron, which was shown to increase (Thurnham and Northrop-Clewes, 2016). In a human population study, many polyunsaturated fatty acids (PUFA) were associated with a lower inflammatory profile, in that a negative correlation was found between PUFA and pro-inflammatory cytokines, while a positive correlation was found between PUFA and anti-inflammatory cytokines (Ferrucci et al., 2006). Additionally, omega-3 supplementation in the horse has demonstrated potential immunomodulatory effects at a clinical level (Nogradi et al., 2015).

In this study docosadienoic acid (C22:2n6c), docosapentaenoic acid (DPA; C22:5n3c), and folate were all positively associated with numerous inflammatory parameters. Docosadienoic acid has exhibited antioxidant activity in previous *in vitro* research, exhibiting some of the highest cyclooxygenase enzyme inhibition among the numerous fatty acids examined (Henry et al., 2002). The results showing that C22:2n6c then is positively associated with inflammation is somewhat surprising, given that oxidative stress and inflammation frequently occur together; however, little research on C22:2n6c has been conducted thus far. Docosapentaenoic acid was also positively associated with various inflammatory markers despite being an omega-3 fatty acid, which are generally considered to be anti-inflammatory. In a study examining metabolic syndrome in obese adolescents, changes in DPA specifically were positively associated with changes in anti-

inflammatory adiponectin and were negatively associated with pro-inflammatory leptin and leptin/adiponectin ratio, indicating that DPA tends to be associated with an anti-inflammatory status (Masquio et al., 2016). A recent human study of ulcerative colitis (a type of inflammatory bowel disease), showed an interesting caveat in that serum DPA concentrations were higher in patients with pro-inflammatory ulcerative colitis compared to healthy controls, although the opposite relationship was found at the tissue level (Wiese et al., 2016). Similarly to these studies involving other inflammatory conditions, inflamm-aging appears to be associated with altered fatty acid metabolism. Additionally, in this study, markers of inflammation were positively associated with folate concentrations, although some previous studies have suggested folate (or its synthetic form, folic acid) to have anti-inflammatory properties (Chen et al., 2016; Cianciulli et al., 2016; Solini et al., 2006; Zheng et al., 2019), whereas other studies have found no association (Cao et al., 2016). It is necessary to note however that folic acid supplementation in the horse, at least in combination with dihydrofolate reductase inhibitors, is not recommended to correct folate deficiencies (Piercy et al., 2002; Reed et al., 2016). One possible explanation for these perhaps unexpected associations between markers of inflammation and (anti-inflammatory) folate and fatty acid concentrations is that these anti-inflammatory compounds have been released from tissues into the bloodstream in an effort to moderate the elevated systemic inflammation inherent with inflamm-aging, i.e. a compensatory effect.

Some hematological and biochemical parameters were also associated with markers of inflammation in this study. Specifically total bilirubin, direct bilirubin, and WBC were positively associated with various inflammatory parameters, while SGOT/AST and eosinophils were negatively associated with inflammatory parameters. The positive correlations between bilirubin and inflammation are somewhat expected, as some studies have shown bilirubin to be associated

with neuro-inflammation (Liu et al., 2016; Qaisiya et al., 2016). However in a rodent model examining inflamm-aging specifically, mild hyperbilirubinemia was suggested to be protective against inflamm-aging (Zelenka et al., 2016), and another study found a negative association between serum bilirubin and the number of pro-inflammatory CD8+ T cells (Moreno-Otero et al., 1994). Inflammation has also previously been associated with SGOT/AST (Tiwari et al., 2016), with experimentally-induced reductions in inflammation likewise being associated with decreases in SGOT/AST (Seif El-Din et al., 2016), which is in contrast to the negative correlation between SGOT/AST and inflammation found in this study. The relationship of inflammation to liver disease as a whole is complex in that increasing age and inflamm-aging in humans have been associated with an increased prevalence of liver disease; however, in the very elderly (>70 years), the incidence of liver disease is very low (Sheedfar et al., 2013). The complexity of the relationship between liver disease and inflammation may to some degree explain these seemingly contradictory results for bilirubin and SGOT/AST in relation to inflammation. Additionally, all inflammatory markers associated with total bilirubin (and all but one associated with direct bilirubin) were lymphocyte mediated, while those associated with SGOT/AST included serum inflammatory markers and only one lymphocyte-mediated inflammatory marker (TNF-α MFI). Furthermore, SGOT/AST is not a specific biomarker for liver damage, as it can also reflect muscle (and other soft tissue) damage, which may further explain these results, particularly since SGOT/AST was primarily associated with serum inflammation. Furthermore, SGOT/AST and CK (a common biomarker of muscle damage) values were greater than the reference range for over half of the horses sampled, suggesting that old horses may exhibit elevated levels of the muscle damage biomarkers, potentially due to age-associated muscle atrophy. White blood cell counts being positively correlated with inflammation is well-documented. Eosinophils (percentage of WBC

count) being negatively correlated with inflammation is not surprising given that elevated levels of eosinophils, which are associated with parasitic infections, elicit a T_H-2 immune response that could be decreasing the inflammatory response associated with aging.

Loss of muscle mass has long been associated with aging, and some studies suggest a relationship specifically between muscle wasting and inflamm-aging. In a study of over 3000 healthy, aged (70-79 yrs) adults, IL-6 and TNF-α were associated with decreased muscle mass and strength (Visser et al., 2002). In aged cattle, lymphocyte-mediated (primarily $CD8^+$) inflammation was also associated with muscle wasting (Costagliola et al., 2016). In the old horse, decreased muscle mitochondrial density and shifted fiber types (toward a higher percentage of myosin heavy chain types I and IIA) have been associated with aging (Li et al., 2016). Furthermore, a recent study suggests that the pro-inflammatory status associated with inflamm-aging in old horses may decrease exercise-induced satellite cell activity, thereby decreasing the normal process of hypertrophy associated with exercise (Reed et al., 2015). This, in turn, may indicate why maintaining physical fitness in senior horses is more challenging (Reed et al., 2015), a key issue in the equine industry due to the number of equine athletes competing into their senior years (Malinowski et al., 1997; McKeever, 2016).

In this study, body composition parameters showed few correlations with the systemic inflammatory markers examined. This was somewhat unexpected, given the association of both inflamm-aging and muscle atrophy with aging. No relationships between inflammation and muscle measurements remained after adjusting for age, and in the case of FFM estimated by ultrasound, adjusting for body weight as well. This indicates that some other aspect of the aging process may be contributing to muscle wasting aside from systemic inflamm-aging; however, if investigated in

a population of both old and young horses, a relationship between inflamm-aging and muscle wasting may become apparent.

Muscle composition as determined by D_2O analysis (for n=10 horses) did not exhibit strong correlations when compared to ultrasound methods after modeling with bodyweight and age as covariates. This may give further reasons for the general lack of relationships between inflammation and body composition parameters discussed previously for the n=42 horses. Additionally, Bland-Altman plots comparing differences between D_2O and ultrasound methods showed only 5/10 and 4/10 horses residing within the 95% limits of agreement for percent body fat and FFM, respectively. However, the strong positive correlation between D_2O analysis and muscle scoring even after taking bodyweight and age into account suggests muscle scoring as a valuable method of muscle assessment.

Senior horses also frequently exhibit PPID, an endocrinopathy caused by dopaminergic neurodegeneration of the hypothalamic neurons, leading to hypertrophy, hyperplasia, and often adenomas of the pituitary pars intermedia (Durham, 2016; Miller et al., 2008). This endocrinopathy, commonly known as equine Cushing's disease, frequently results in hypertrichosis, polydipsia, polyuria, hyperhidrosis, laminitis, muscle atrophy, and abnormal fat distribution (McFarlane, 2011). Endocrinologically this frequently results in increased basal levels of circulating ACTH, while also resulting in a decreased ability of horses to return to resting levels of ACTH after intravenous injection of TRH (Beech et al., 2007). Adrenocorticotropin hormone has been associated with increased gene expression of pro-inflammatory IL-6 in septic foals (Gold et al., 2012); however, the relationship of ACTH with inflammation is not well-characterized. Pituitary pars intermedia dysfunction has also been associated with cytokine dysregulation (McFarlane and Holbrook, 2008). Thus, it was thought that the PPID horse may exhibit differential

relationships with individual markers of inflamm-aging. Further, the exact mechanisms that result in this hypothalamic dopaminergic neurodegeneration found in many old horses remain unknown, and it was thought that the discovery of any specific markers of inflamm-aging being associated with PPID may yield understanding of underlying mechanisms involved in this pathology.

Pituitary pars intermedia dysfunction measures, including basal ACTH and PPID status as defined by TRH testing, were also associated with some of the various parameters analyzed in this study. Vitamin B12 exhibited a strong negative correlation with PPID status regardless of age adjustment, which was particularly noteworthy, as the non-PPID horses were younger than the PPID horses. Vitamin B12 is key to proper functioning of the brain and nervous system, with case studies showing improvements in nervous system function after treatment with B12 for those deficient in this vitamin (Kumar, 2004; Shyambabu et al., 2008). A recent human study has shown vitamin B12 to be negatively associated with inflammation (Al-Daghri et al., 2016), similarly to the current study in which vitamin B12 was negatively correlated with CRP. Studies have also found that B12 deficiencies may be associated with Parkinson's disease (Orozco-Barrios et al., 2009) and with increased risk for white matter hyperintensities (de van der Schueren et al., 2016), a predictor of Alzheimer's disease (Provenzano et al., 2013). In humans, patients with active Cushing's disease have exhibited decreased levels of vitamin B12, while cured patients did not, suggesting a role of vitamin B12 in disease state (Faggiano et al., 2005). Although the exact pathophysiology of Cushing's disease in the horse differs from that in the human, both affect the pituitary, and the results of the present study showing a negative relationship with vitamin B12 support those previously published in the human (Faggiano et al., 2005).

Age itself was examined in relation to the various parameters to determine where it might have the strongest associations. The modeling for other parameters incorporated age as a covariate

due to: 1) the number of relationships it appeared to impact and 2) the fact that inflammation was the primary focus of this study within a group of senior horses, and it was not desired to have the potentially confounding variable of age as a component of inflamm-aging. It is known that the body undergoes various changes when entering senior years, which may have effects on numerous variables. However, to better understand the process of aging in the senior horse, relationships between age and the various parameters were determined. When examining the various inflammatory measures, many parameters were positively correlated with age as expected, based on previous studies regarding inflamm-aging in the horse and other species (Adams et al., 2008; Franceschi et al., 2007). Numerous fatty acids being negatively correlated with age was also unsurprising. The positive correlation between age and both vitamin E and selenium may suggest either a protective effect in which horses with higher levels of vitamin E and selenium tend to live longer, or an inability of senescent cells to uptake these nutrients, thereby leaving them sequestered in circulation. A recent mouse model publication found that selenium deficiency was associated with longevity, despite having decreased health span (delayed wound healing as well as earlier onset of age-associated decreased glucose tolerance, decreased insulin sensitivity, and osteoporosis, etc.) (Wu et al., 2016). This demonstrates that the relationship between age and selenium may be rather complex. Numerous body composition parameters including muscle measures were negatively correlated with age as expected, since horses experience age-associated muscle wasting. Measures of PPID (including basal ACTH and PPID status, as defined by TRH testing) were also associated with increased age, which is expected, as PPID is generally an endocrinopathy of the senior horse (McFarlane, 2011), and presumably would become more common with increasing age.

The study had some limitations, particularly that it is fundamentally an exploratory study in which numerous correlations were examined in an attempt to better understand inflamm-aging of the senior horse and potential areas of further study. Additionally, the inflammatory markers were PBMC-mediated with the exception of 3 serum ELISAs, limiting the scope of application for the results. Blood was collected from horses within 2 hours after their morning meal. While blood would ideally have been collected at exactly the same time post-feeding, the animals were not grouped according to age in the feeding groups but randomly dispersed; therefore by adjusting for age, it was hoped that any post prandial effects would be taken into consideration. The horses were fed prior to blood collection on their regular schedule in order to keep them from becoming stressed, which would affect ACTH. Another limitation is that circulating levels of vitamins, minerals, and fatty acids were analyzed from a single time point, thereby not accounting for total body stores. Since circulating nutrient levels are generally maintained through homeostasis, relationships that were found are perhaps particularly worth further investigation.

5. Conclusions

This exploratory study demonstrates the complex relationships between inflammatory parameters and various vitamin, mineral, fatty acid, hematological, biochemical, body composition, and PPID parameters in senior horses. Notably, many inflammatory markers were positively associated with folate and fatty acids docosadienoic acid (C22:2n6c) and docosapentaenoic acid (C22:5n3c), while PPID status was inversely associated with vitamin B12. Further study of these relationships will aid understanding of the process of aging in the horse, with the goal of promoting longevity and health span.

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Table 1. Nutrient composition (DM basis) of feed and hay [a]

Component	Oats	Alfalfa Pellets	Grass Hay
DM, %	92	90.8	92.6
DE, Mcal/kg	3.34	3.01	1.94
CP, %	12.6	13.6	14.7
ADF, %	11.1	21.6	43.8
NDF, %	24.7	37.3	66.1
Ca, %	0.13	0.82	0.54
P, %	0.4	0.59	0.42
Mg, %	0.14	0.33	0.29
K, %	0.53	1.13	1.46
Na, %	0.028	0.206	0.078
Fe, ppm	91	313	265
Zn, ppm	35	128	36
Cu, ppm	9	56	11
Mn, ppm	49	128	115

[a] Analyzed by Equi-Analytical Laboratories (Ithaca, NY).

Table 2. Comparisons of various inflammatory measures to serum levels of vitamins, minerals, and fatty acids [a, b]

Inflammatory Parameter	Comparison Parameter	Correlations [b]		Age-adjusted [b]	
		Correlation Coefficient	p-value	Correlation Coefficient	p-value
% IFN-γ	**C16:0**	-0.343	0.028†	-0.177	0.275
	C18:1n9c	-0.327	0.048†	-0.225	0.186
	C20:2n6c	0.333	0.031†	0.276	0.081
	C20:4n6c	0.380	0.013†	0.276	0.081
	C22:2n6c	0.335	0.030†	0.407	0.008*
	C22:5n3c	0.405	0.009*	0.357	0.024†
	C24:1n9c	0.364	0.019†	0.216	0.181
	Folate	0.309	0.047†	0.222	0.164
	Iron	-0.351	0.023†	-0.281	0.075
	Selenium	0.386	0.013†	0.263	0.101
IFN-γ MFI	**C16:0**	-0.361	0.020†	-0.256	0.111
	C20:2n6c	0.320	0.039†	0.277	0.080
	C20:4n6c	0.345	0.025†	0.272	0.085
	C22:2n6c	0.286	0.066	0.349	0.025†
	C22:5n3c	0.405	0.009*	0.369	0.019†
	C24:1n9c	0.338	0.031†	0.226	0.160
	Folate	0.347	0.024†	0.290	0.066
	Selenium	0.362	0.020†	0.277	0.083
	Zinc	0.244	0.135	0.316	0.050†
% TNF-α	**C16:0**	-0.432	0.005*	-0.269	0.093
	C18:1n9c	-0.390	0.017†	-0.252	0.138
	C20:2n6c	0.368	0.016†	0.310	0.048†
	C22:2n6c	0.362	0.018†	0.445	0.004*
	C22:5n3c	0.458	0.003*	0.412	0.008*
	C24:1n9c	0.342	0.028†	0.151	0.354
	Folate	0.398	0.009*	0.313	0.046†
	Selenium	0.339	0.030†	0.182	0.262
TNF-α MFI	**C16:0**	-0.330	0.038†	-0.214	0.191
	C22:2n6c	0.366	0.019†	0.459	0.003*
	Folate	0.410	0.008*	0.357	0.024†
Ln(IFN-γ RQ)	**C16:0**	-0.377	0.015†	-0.210	0.194
	C18:1n9c	-0.381	0.020†	-0.255	0.133
	C20:4n6c	0.313	0.044†	0.182	0.254
	C22:2n6c	0.257	0.101	0.331	0.035†
	C22:5n3c	0.455	0.003*	0.409	0.009*
	Folate	0.404	0.008*	0.322	0.040†

Inflammatory Parameter	Comparison Parameter	Correlations [b]		Age-adjusted [b]	
		Correlation Coefficient	p-value	Correlation Coefficient	p-value
Ln(IL-10 RQ)	**C16:0**	-0.312	0.047†	-0.120	0.461
	C18:1n9c	-0.386	0.018†	-0.250	0.142
	C20:2n6c	0.339	0.028†	0.277	0.080
	C20:4n6c	0.322	0.038†	0.189	0.236
	C22:5n3c	0.417	0.007*	0.366	0.020†
	C24:1n9c	0.320	0.041†	0.136	0.404
	Folate	0.460	0.002*	0.384	0.013†
	Iron	-0.305	0.050†	-0.219	0.168
	Selenium	0.365	0.019†	0.215	0.183
Ln(IL-1β RQ)	**C16:0**	0.258	0.118	0.327	0.048†
	Zinc	-0.354	0.031†	-0.352	0.035†
Ln(IL-6 RQ)	**C16:0**	-0.311	0.048†	-0.212	0.189
	C16:1n7c	0.241	0.150	0.354	0.034†
	C18:0	0.364	0.018†	0.333	0.033†
	C18:1n9c	-0.484	0.002*	-0.421	0.011†
	C18:3n3c	0.300	0.060	0.356	0.026†
	C22:5n3c	0.455	0.003*	0.421	0.007*
Ln(TNF-α RQ)	**C20:2n6c**	0.407	0.008*	0.363	0.020†
	C22:2n6c	0.468	0.002*	0.537	<0.001*
	C22:4n6c	0.335	0.032†	0.305	0.056
	C22:5n3c	0.370	0.017†	0.324	0.042†
	Folate	0.401	0.008*	0.336	0.032†
	Selenium	0.356	0.023†	0.245	0.128
Ln(Serum CRP)	**Copper**	0.417	0.006*	0.368	0.018†
	Selenium	-0.338	0.031†	-0.249	0.121
	Vitamin B12	-0.321	0.038†	-0.374	0.016†
Ln(Serum IL-6)	**C16:1n7c**	0.342	0.038†	0.465	0.004*
	C18:3n6c	0.339	0.028†	0.354	0.023†
	C20:1n9c	0.361	0.020†	0.479	0.002*
	C20:3n3c	-0.222	0.164	-0.315	0.048†
	C22:2n6c	0.289	0.063	0.318	0.043†
	C22:3n3c	0.329	0.036†	0.334	0.035†
	Folate	0.351	0.023†	0.316	0.044†

[a] An asterisk (*) is used to denote P≤0.01, while a cross (†) is used to denoted P≤0.05.

[b] Pearson correlations and age-adjusted parametric analysis were performed for all data except C22:2n6c and serum IL-6, which were analyzed using Spearman correlations and age-adjusted non-parametric analysis due to non-normal distribution.

[c] IFN-γ = interferon-γ; % gated = percent of lymphocytes producing the cytokine; MFI = mean fluorescence intensity; TNF-α = tumor necrosis factor-α; RQ = relative quantity of cytokine gene expression; IL = interleukin; CRP = C-reactive protein

Table 3. Comparisons of various inflammatory measures to complete blood count and chemistry panel measures [a-c]

Inflammatory Parameter	Comparison Parameter	Correlations [c]		Age-adjusted [c]	
		Correlation Coefficient	p-value	Correlation Coefficient	p-value
% IFN-γ	**D. Bili**	0.413	0.006*	0.352	0.024†
	Hgb	0.350	0.025†	0.319	0.045†
	T. Bili	0.416	0.007*	0.352	0.026†
	WBC	0.470	0.002*	0.416	0.007*
IFN-γ MFI	**Calcium**	0.312	0.050†	0.280	0.084
	D. Bili	0.378	0.014†	0.330	0.035†
	Eosinophils	-0.419	0.006*	-0.432	0.005*
	Lymphocytes	0.377	0.015†	0.418	0.007*
	Seg	-0.293	0.063	-0.336	0.034†
	T. Bili	0.408	0.008*	0.359	0.023†
% TNF-α	**D. Bili**	0.372	0.015†	0.297	0.059
	Eosinophils	-0.329	0.033†	-0.359	0.021†
	T. Bili	0.424	0.006*	0.356	0.024†
	WBC	0.404	0.008*	0.336	0.032†
TNF-α MFI	**D. Bili**	0.358	0.022†	0.310	0.052
	Sodium	-0.319	0.042†	-0.263	0.101
	SDH	-0.374	0.025†	-0.311	0.069
	SGOT_AST	-0.345	0.027†	-0.321	0.043†
	T. Bili	0.423	0.007*	0.378	0.018†
Ln(IFN-γ RQ)	**D. Bili**	0.404	0.008*	0.336	0.032†
	Eosinophils	-0.322	0.038†	-0.348	0.026†
	Lymphocytes	0.288	0.068	0.365	0.021†
	T. Bili	0.365	0.019†	0.292	0.068
	WBC	0.452	0.003*	0.392	0.011†
Ln(IL-10 RQ)	**D. Bili**	0.368	0.017†	0.293	0.063
	Eosinophils	-0.299	0.054	-0.326	0.038†
	SDH	-0.325	0.050†	-0.181	0.290
	T. Bili	0.330	0.035†	0.251	0.119
	WBC	0.389	0.011†	0.318	0.043†
Ln(IL-1β RQ)	**CK**	-0.362	0.028†	-0.356	0.033†
	Creatinine	-0.289	0.074	-0.337	0.038†
	Hgb	-0.397	0.014†	-0.429	0.008*
	Lymphocytes	-0.448	0.004*	-0.443	0.005*
	Seg	0.431	0.006*	0.425	0.008*

Inflammatory Parameter	Comparison Parameter	Correlations [c]		Age-adjusted [c]	
		Correlation Coefficient	p-value	Correlation Coefficient	p-value
Ln(IL-6 RQ)	**Lymphocytes**	0.384	0.013†	0.422	0.007*
	SDH	-0.401	0.014†	-0.344	0.040†
	Seg	-0.350	0.025†	-0.391	0.013†
	WBC	0.321	0.038†	0.268	0.090
Ln(TNF-α RQ)	**D. Bili**	0.317	0.041†	0.254	0.109
	LDH	-0.363	0.018†	-0.307	0.051
	SDH	-0.387	0.018†	-0.291	0.086
	SGOT_AST	-0.468	0.002*	-0.446	0.004*
Ln(Serum CRP)	**BUN**	-0.447	0.003*	-0.405	0.010*
	Creatinine	-0.505	0.001*	-0.473	0.002*
	SDH	0.331	0.045†	0.205	0.230
	SGOT/AST	-0.285	0.067	-0.348	0.026†
Ln(Serum IL-6)	**Alk. Phosphatase**	-0.433	0.005*	-0.449	0.004*
	CK	-0.417	0.008*	-0.411	0.009*
	LDH	-0.426	0.005*	-0.407	0.008*
	Phosphorus	0.311	0.045†	0.410	0.008*
	RBC	-0.296	0.057	-0.337	0.031†
	Seg	0.378	0.015†	0.363	0.021†
	SGOT/AST	-0.514	0.001*	-0.511	0.001*
	Total Protein	0.401	0.008*	0.406	0.008*

[a] Complete blood count analysis by Rood and Riddle Equine Hospital (Lexington, KY).

[b] An asterisk (*) is used to denote P≤0.01, while a cross (†) is used to denoted P≤0.05.

[c] Pearson correlations and age-adjusted parametric analysis were performed for all data except serum IL-6, which was analyzed using Spearman correlations and age-adjusted non-parametric analysis due to non-normal distribution.

[d] IFN-γ = interferon-γ; % = percent of lymphocytes producing the cytokine; MFI = mean fluorescence intensity; TNF-α = tumor necrosis factor-α; RQ = relative quantity of cytokine gene expression; IL = interleukin; CRP = C-reactive protein; D. Bili = direct bilirubin; Hgb = hemoglobin; T. Bili = total bilirubin; WBC = white blood cells; Seg = mature neutrophils; SDH = sorbitol dehydrogenase; CK = creatine kinase; LDH = lactate dehydrogenase; SGOT/AST = serum glutamic oxaloacetic transaminase/aspartate aminotransferase; BUN = blood urea nitrogen; Alk. Phosphatase = alkaline phosphatase; RBC = red blood cells.

Table 4. Comparisons of measures of PPID to various inflammatory, vitamin, mineral, fatty acid, complete blood count, chemistry panel, and body composition measures [a-c]

PPID Parameter	Comparison Parameter	Pearson correlations		Age-adjusted	
		Correlation Coefficient	p-value	Correlation Coefficient	p-value
PPID Status	**RBC**	-0.205	0.193	-0.311	0.048†
	Eosinophils	0.269	0.084	0.311	0.048†
	Creatinine	-0.228	0.146	-0.383	0.013†
	Ln(Serum CRP)	0.185	0.240	0.368	0.018†
	Vitamin B12	-0.547	<0.001*	-0.556	<0.001*
	Folate	0.307	0.048†	0.201	0.208
	C20:4n6c	-0.078	0.621	-0.308	0.050†
	Ln(Basal ACTH)	0.595	<0.001*	0.482	0.002*
Ln(Basal ACTH)	**C16:0**	0.113	0.486	0.527	0.001*
	D. Bili	-0.165	0.304	-0.331	0.037†
	Vitamin B12	-0.333	0.033†	-0.300	0.060

[a] Complete blood count analysis by Rood and Riddle Equine Hospital (Lexington, KY).
[b] Pituitary pars intermedia dysfunction (PPID) status is determined by thyrotropin releasing hormone stimulation, with adrenocorticotropin hormone (ACTH) ≥110 pg/mL being considered PPID and ACTH<110 pg/mL considered non-PPID.
[c] An asterisk (*) is used to denote P≤0.01, while a cross (†) is used to denoted P≤0.05.
[d] PPID = Pituitary par intermedia dysfunction; ACTH = adrenocorticotropin hormone; RBC = red blood cells; CRP = C-reactive protein; D. Bili = direct bilirubin

Table 5. Comparisons of various inflammatory, vitamin, mineral, fatty acid, complete blood count, chemistry panel, body composition, and PPID measures to age [a-c]

Comparison Parameter	Pearson correlations with Age	
	Correlation Coefficient	p-value
% IFN-γ	0.382	0.013†
% TNF-α	0.436	0.004*
Ln(IFN-γ RQ)	0.423	0.005*
Ln(IL-10 RQ)	0.434	0.004*
Ln(TNF-α RQ)	0.330	0.033†
C16:0	-0.529	<0.001*
C16:1n7c	-0.332	0.044†
C18:1n7c	-0.410	0.008*
C18:1n9c	-0.501	0.002*
C18:2n6c	0.551	<0.001*
C20:1n9c	-0.372	0.017†
C20:4n6c	0.377	0.014†
C24:1n9c	0.454	0.003*
Selenium	0.438	0.004*
Vitamin E	0.491	0.001*
SDH	-0.410	0.012†
BCS	-0.454	0.003*
Bodyweight	-0.607	<0.001*
FFM (ultrasound)	-0.602	<0.001*
Muscle Score	-0.309	0.047†
PPID Status	0.462	0.002*
Ln(Basal ACTH)	0.454	0.003*

[a] Complete blood count analysis by Rood and Riddle Equine Hospital (Lexington, KY).

[b] All parameters with P≤0.05 are shown in this table, with P≤0.01 indicated by an asterisk (*) and P≤0.05 indicated by a cross (†).

[c] IFN-γ = interferon-gamma; % = percent of lymphocytes producing the cytokine; TNF-α = tumor necrosis factor-α; RQ = relative quantity of cytokine gene expression; IL = interleukin; SDH = sorbitol dehydrogenase; BCS = body condition score; FFM = fat free mass; PPID = pituitary pars intermedia dysfunction; ACTH = adrenocorticotropin hormone

Table 6. Comparison of deuterium oxide methods of body composition determination with body composition determinations via ultrasound and muscle scoring [a, b]

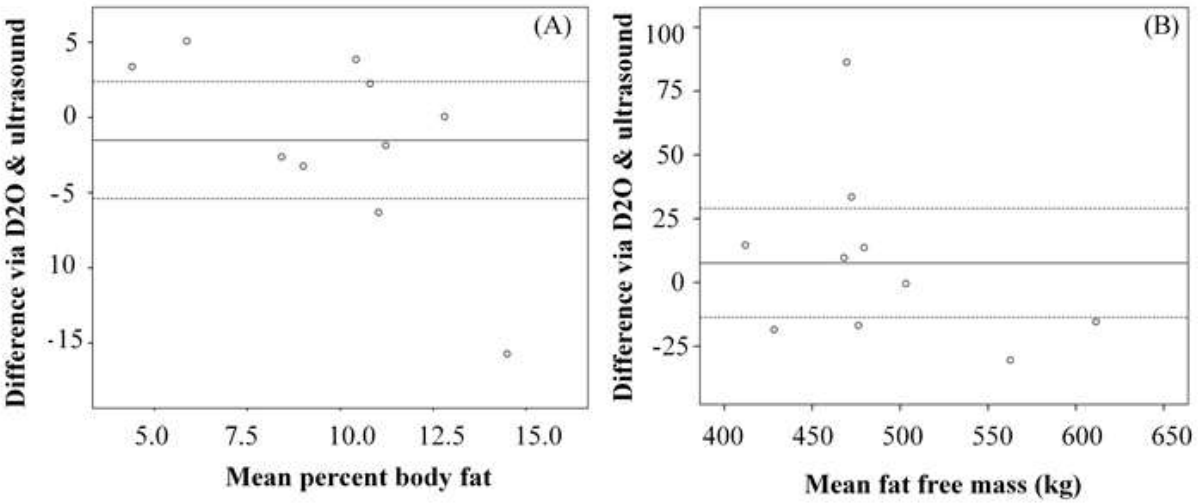
Body Composition Parameter	Comparison Parameter	Pearson correlations adjusted for Age & Body weight	
		Correlation Coefficient	p-value
Muscle Score	**% Body Fat (ultrasound)**	0.035	0.929
	% Body Fat (D_2O)	-0.529	0.143
	FFM (ultrasound)	-0.031	0.937
	FFM (D_2O)	0.895	0.001*
% Body Fat (ultrasound)	**% Body Fat (D_2O)**	-0.618	0.076
	FFM (ultrasound)	-0.651	0.057
	FFM (D_2O)	0.375	0.321
FFM (D_2O)	**% Body Fat (ultrasound)**	0.375	0.321
	% Body Fat (D_2O)	-0.683	0.043†
	FFM (ultrasound)	-0.124	0.751

[a] Correlations with age and body weight as covariates are displayed; correlation p-values denoted with (*) indicate P≤0.01, while (†) indicate P≤0.05.

[b] BCS= body condition score; D_2O = deuterium oxide; FFM = fat free mass

Figure 1. Comparison of D_2O and ultrasound methods using Bland-Altman plots. Bland-Altman plots were used to evaluate differences in D_2O infusion and rump ultrasound methods in determining (A) percent body fat and (B) fat free mass in a subset of n=10 senior (≥20 years) horses.

S1 Table. Complete dataset of inflammatory, nutritional, body composition, PPID, biochemistry, and hematology parameters.


(A)
Difference via D2O & ultrasound
5
0
-5
10
-15
5.0
7.5
10.0
12.5
15.0
Mean percent body fat
(B)
Difference via D2O & ultrasound
100
75
50
25
0
-25
400
450
500
550
600
650
Mean fat free mass (kg)