EVALUATION OF AMINO ACID SYNTHESIS *IN VITRO* BY EQUINE ENTEROCYTES AND THE ABILITY OF DIETARY ARGININE SUPPLEMENTATION TO IMPROVE GESTATIONAL PERFORMANCE OF AGED MARES

A Dissertation

by

RAFAEL ERNESTO MARTINEZ

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Chair of Committee,	Jessica L. Leatherwood
Committee Members,	Carolyn E. Arnold Fuller W. Bazer Carolyn J. Hammer James C. Heird Guoyao Wu
Head of Department,	Andy Herring

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ABSTRACT

Arginine and its metabolites are essential for optimal growth and development as well as reproductive efficiency, with a specific interest to improve placental development and fetal survival. Currently classified as a conditionally essential amino acid, little information exists about arginine synthesis in the horse and thus dietary requirements specific to the horse. Therefore, a series of studies were conducted to test the hypothesis that horses of different age groups can utilize glutamine and proline for the *de novo* synthesis of citrulline and arginine using jejunal enterocytes from horses of three different age groups as well as the dietary supplementation of arginine (50 mg/kg BW/d) can be used as a potential strategy to enhance placental vascularity and nutrient transport throughout gestation in aged mares. The initial study demonstrated that incubated equine enterocytes were able to utilize glutamine as a major metabolic fuel, similar to the pig and sheep, (*in vitro*) except equine enterocytes did not oxidize proline, possibly due to the absence or limited enzyme activity. Furthermore, regardless of age, equine enterocytes synthesized ornithine from glutamine and proline, but failed to convert ornithine into citrulline and arginine. Further studies were conducted in aged pregnant mares to determine the effects of arginine supplementation (50 mg/kg BW/d) on mare performance throughout gestation, placental characteristics, and the impacts on the resulting foal. Dietary supplementation of arginine increased mobilization of adipose tissue throughout pregnancy when compared to control mares, and increased blood perfusion to the uterus in pre-partum mares. At parturition, foaling and placental variables did not differ between the control and arginine-supplemented mares. This study had a small population which is a common challenge for equine

studies; however, more sensitive molecular changes are valuable for use in smaller populations that may be indicative of maternal nutrition affecting the cellular and molecular mechanisms of placental function. Currently, more information is needed in order to understand arginine metabolism in the horse, regardless of age or physiological status.

DEDICATION

I would like to dedicate this to mi papa Quirino y mama Susana, and to my mom Irene. Gracias por haberme dado la dicha de ser tu hijo.

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NOMENCLATURE

AAs – amino acids

ASL - arginosuccinate lyase

BCS – body condition score

BSA – bovine serum albumin

BCS – body condition score

BW – body weight

CDE - chronic degenerative endometrial disease

CP - crude protein

DE - digestible energy

EED - early embryonic death

GUA - gravid uterine artery

HEPES – *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid

HPLC – high-performance liquid chromatography

IUGR - intrauterine growth restriction

KHB – Krebs bicarbonate buffer

mTOR - mammalian target of rapamycin

NO – nitric oxide

OCT – ornithine carboxyltransferase

OPA – *o*-phthaldialdehyde

P5C – Δ^1-pyrroline-5-carboxylate

PI - pulsatility index

RI - resistance index

SLC7A1 - High affinity cationic amino acid transporter 1 of lysine and arginine

VEGF - Vascular Endothelium Growth Factor

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Nutritional guidelines for horses are limited and in many cases are based on standards published for swine; however, differences in enzymatic activities, rate of passage, presence, and number of amino acid transporters in the gastrointestinal tract warrant further investigation in the horse. Since horses are non-ruminants, there are 10 amino acids presumed to be essential: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (NRC 2007). Six amino acids considered conditionally essential are arginine, cysteine, glycine, glutamine, proline, and tyrosine. The six amino acids considered as nonessential are alanine, aspartic acid, asparagine, glutamic acid, serine. Historically, more focus has been paid to the supplementation of essential amino acids because of the inability of mammals to synthesize these endogenously. More recent evidence, however, elucidates the importance of conditionally essential amino acids to a multitude of physiological processes, and endogenous production of these amino acids may be insufficient to maintain these processes, such as pregnancy and lactation, during various stages of life. Furthermore, amino acids are absorbed solely by the small intestine; therefore, appropriate supplementation of both essential and conditionally essential amino acids is imperative for maintenance of normal physiological processes.

The small intestine is unique in the versatility of its functions and responsibilities for digestion and absorption, but it also plays a vital role in the metabolism of amino acids. Intestinal amino acid metabolism is necessary for the maintenance of intestinal integrity, muscle mass, physiological functionality, and whole-body metabolic homeostasis. The columnar epithelial absorptive cells (enterocyte cells) embody >80% of the mucosal cell population and possess an

elevated rate of intracellular protein turnover and cell proliferation. Enterocytes rely upon dietary amino acids as a metabolic fuel for the intestinal mucosa, and as fundamental precursors for physiological metabolites such as intestinal proteins, polyamines, and nitric oxide. Specifically, the amino acids, glutamine, glutamate, and proline are substrates for the production of citrulline and arginine in the neonates of many mammalian species, and primarily of citrulline in adults (Bertolo and Burrin 2008; Wu and Morris 1998). In post-natal pigs, arginine is formed in the small intestine by enterocytes from both glutamine and proline (Wu et al., 1994). Post-feeding, the small intestine releases amino acids and products of amino acid metabolism into the portal vein through the mucosal vasculature where they will then be delivered to the liver.

Currently, there is no known dietary arginine requirement in the horse at any stage of development. This conditional amino acid is considered essential for gestating mammals (pigs, rats, and sheep) (Bazer et al. 2012; Wu 2013). In most mammals, arginine serves not only as a substrate for protein synthesis, but also as a precursor for the synthesis of multiple biologically active molecules including nitric oxide (**NO**), polyamines, ornithine, creatine, and agmatine (Wu and Morris, 1998). Polyamines and NO stimulate cell proliferation and migration, cellular remodeling, and angiogenesis, allowing for the development and maintenance of reproductive functions such as implantation and placentation (Wu 2013; Wu et al. 2004). Arginine and its metabolites also play critical role in the detoxification of ammonia via the urea cycle, and dilation of blood vessels to increase blood flow (Wu et al., 2008).

Arginine Synthesis

Enterocytes of the small intestine play a pivotal role in the whole-body synthesis of citrulline, which is the immediate precursor for the endogenous synthesis of arginine (Wu and Morris 1998). Citrulline is derived from glutamine/glutamate and proline which has been

confirmed through the use of incubated enterocyte cells from pigs, sheep, and cattle (Wu and Morris. 1998). *In vivo* experiments confirm *in vitro* evidence of citrulline and arginine synthesis from enteral proline and glutamate in pigs (Wu et al., 1997). In post-weaned pigs, the synthesis of arginine from glutamine is accomplished through various enzymatic reactions (Figure 1). Enzymes involved include: glutaminase, Δ^1-pyrroline-5-carboxylate (**P5C**) synthase, ornithine aminotransferase (OAT), carbamoylphosphate synthase I CPS-I), ornithine carbamoyltransferase (**OCT**), argininosuccinate synthase (ASS) and argininosuccinate lyase (**ASL**) (Wu et al., 1994). Glutaminase, OAT, ASS and ASL are widely distributed in mammalian tissues. The enzymes CPS-I and OCT are exclusively situated in the liver and small intestine (Curthoys and Watford 1995, Wakabayashi 1995). On the other hand, P5C synthase, is almost exclusively located in enterocytes of many mammals, resulting in the small intestine as the exclusive organ for the synthesis of P5C from arterial glutamine and dietary glutamate/glutamine (Flynn and Wu 1996, Wakabayashi, 1995; Wu et al. 1994). Concerning the pig's small intestine, it is suggested that glutamine/glutamate are the only source of P5C for the synthesis of citrulline (Wakabayashi 1995).

There is also evidence that proline is an important source of P5C (via proline oxidase) and citrulline in pig enterocytes (Wu et al., 1997). P5C is necessary for the synthesis of citrulline from both glutamine and proline through interconversion into ornithine by OAT in enterocytes (Wu et al., 1997). The resulting citrulline from ornithine may then be used for arginine production in enterocytes. The liver is also capable of synthesizing arginine through the urea cycle, but there is no net synthesis of arginine because of the exceedingly high activity of the enzyme arginase resulting in its rapid hydrolysis. Unfortunately, there is a paucity of knowledge

on the synthesis of arginine and its role in protein synthesis, creatine, urea, polyamines, proline, and glutamate in the horse.

Arginine Supplementation

Currently, there is no known dietary arginine requirement in the horse at any stage of development. Because of its many versatile roles, the interest in arginine is growing and there is increasing evidence that dietary supplementation of arginine is not only beneficial but may be necessary for young and gestating swine (Wu et al., 2008, 2018). To date, dietary supplementation with arginine has been shown to improve cardiovascular, reproductive, pulmonary, renal, digestive, and immune functions in various species. Specifically, arginine prevented fetal growth retardation in rats, and increased birth weights in rats and pigs (Mateo et al., 2007; Vosatka et al., 1998). These data, along with others, suggest an elevated requirement for arginine in young animals as they undergo rapid growth. Thus, arginine is nutritionally essential for young mammals including humans, pigs, and rats.

Recent studies have identified various benefits and drawbacks from dietary supplementation of arginine to horses. Specifically, supplementation of 100 g arginine to pre- and post-partum mares increased uterine blood flow but beneficially decreased gestation length (336.8 ± 2.4 days) without affecting the birth weight of foals, compared to horses receiving no supplemental arginine (345.8 ± 2.8 days; Mortensen et al., 2011). These results indicate that arginine supplementation enhanced fetal growth in horses. Because of the long gestation period in mares, a reduction in gestation length ensures that mares foal closer to their expected foaling date, which is extremely valuable to horse breeders of all disciplines. Primiparous mares supplemented with arginine during late gestation produced foals with heavier birthweights than primiparous mares not supplemented with arginine, which may be a result of enhanced uterine

blood flow allowing for increased nutrient transfer through the maternal-fetal interface (Chavatte-Palmer et al., 2018). Investigation into arginine supplementation in the horse is in its infancy; however, if similar benefits were to occur as has been observed in other species, this simple nutritional treatment holds great promise in improving the physiological state of the equine maternal and fetal membranes.

As previous studies with different dosages (Table 1) have demonstrated benefits from the supplementation of arginine, an appropriate dosage has yet to be identified for the horse to avoid potentially undesired effects on amino acid metabolism. For example, the supplementation of arginine at 0.025% BW (Kelley et al., 2014) decreased concentrations of lysine in plasma, as that high dose of arginine most likely suppressed the absorption of lysine, as both amino acids (**AAs**) share the same transporter. In the second part of this study, Kelley (2014) reported no differences in the concentrations of other AAs in plasma when arginine was supplemented at 0.0125% of BW, which was half the previous reported dosage rate that had negative implications. However, the timing of blood sampling relative to the last feeding was not reported. Based on studies with pigs, sheep, and rats the half-life of arginine in blood is approximately 60 to 70 min (Wu et al. 2007). The supplementation of 100 g arginine described by Mortensen (2011) led to mares having a shorter gestation period, and this finding is advantageous to the industry. However, the study did not report if all foals were born completely normal and/or healthy from a shorter gestation period. Arginine and its metabolites have essential roles in reproduction, particularly in improving embryonic and fetal survival; however, this has not been investigated in the aged mare.

Nutrition of the Gestating Mare

In the pregnant mare, nutrient requirements are allied to support fetal growth and development throughout gestation. Fetal growth can be allocated into two different developmental phases including the initial portion of gestation, in which the fetus undergoes slow growth, as this stage is primarily designated for the development of the placenta. Throughout this initial phase, the non-lactating mare's energy and protein requirements can be fulfilled at maintenance levels (NRC 2007). Nutrient balance is critical, particularly throughout the last four months of gestation, as most fetal growth occurs during this phase of gestation.

As gestation progresses from mid gestation until parturition, the fetus undergoes rapid growth and development (60% development), especially from the 8th month to parturition at 11-12 months (Martin-Rosset, 2012). In order fulfill the needs of the mare and the rapidly growing fetus the nutritional demand of the mare drastically increases. It is estimated that the digestible energy (**DE**) requirements during late gestation increase by 1.3 to 1.5 times that the level of maintenance (NRC 2007). During this time the uterus and placenta (fetal-maternal tissues) are known to be more metabolically active (66.6 kcal/kg) when compared the rest of the body (33.3 kcal/kg) (Coenen at al., 2011), reflecting the importance of meeting those nutritional demands during the different stages of gestation.

Crude protein (**CP**) requirements for the pregnant mare follow a comparable trajectory to that of DE as gestation progresses. Similarly, the non-lactating mare's CP demand throughout early gestation can be met by feeding at a maintenance level(e.g. 0.64 kg/d for a 500 kg mare), only to dramatically increase throughout the last trimester of gestation and lactation (e.g. 0.77 to 1.36 kg/d for a 500 kg mare).

Unfortunately, the digestibility and amino acid composition varies across different protein sources, of which impacts the overall quality of the protein. In order to improve the mare and fetus' efficiency in utilizing amino acids throughout pregnancy, it is currently recommended to offer broodmares a protein of higher quality (NRC 2007). However, available information should expand beyond individual protein requirements by instead considering optimum levels of indispensable amino acids required in the diet in order to meet the demand of tissue development throughout gestation. Lysine, also known as the first limiting amino acid, is the only required amino acid, and is required to make up 4.3% of the CP requirement (NRC 2007). However, lysine requirements were not measured, but were extrapolated from the crude protein requirements and nitrogen retention (Urschel et al., 2010). Therefore, continued efforts along with novel and improved experimental approaches are needed in order to continue towards the progression of optimizing amino acid requirements for the horse, with a particular interest in the gestating mare.

Calcium, phosphorus, and Vitamin A are critical nutrients of particular importance throughout gestation. The requirements of these nutrients, similar to that of DE and CP, continuously increase as gestation progresses. It is during the tenth month that the largest amount of mineral retention occurs in the unborn foal. Therefore, adequate nutrition of the mare is critical for normal fetal development.

Equine Placenta and Fetal Nutrient Delivery

The placenta is known to dictate not only birthweight, but also post-natal growth and development as well as life-long health of its offspring by providing for the fetal metabolic demands. The mare is known to develop a non-invasive placenta which allows for the uterine lumen of the mare and fetal trophectoderm and chorion to remain intact while having an

intimately close attachment. This type of placentation is known as a diffuse epitheliochorial placenta, which begins to occur as late as day 40 following ovulation as the fetal trophoblast's microvilli commence their non-invasive interdigitation attachment to the luminal epithelial cells of the uterus. This intimate relationship remains stable throughout gestation, as it is continuously developing the placental vascular architecture necessary for the haemotropic exchange of nutrients, gases, hormones, and waste products between mother and fetus (Faber et al., 1983; Dunlap et al., 2015).

The developing fetus requires amino acids for metabolism, protein accretion, and other biological pathways that are attained from the placenta. For instance, in most species (pig, sheep, guinea pig, and humans) the concentration of amino acids is higher in fetal plasma when compared to maternal circulation, especially glutamine, clearly indicating the priority of amino acid delivery to the fetus (Chung et al., 1998; Cetin 2001; Parimi et al. 2002; and Wu et al. 1995). Glutamine is one of the most abundant free alpha amino acids in the circulation of most mammals, including the horse (Curthoys and Watford 1995; Newsholme et al., 2003; Young et al., 2003) and it serves an important role in the transport of nitrogen, carbon, and energy between tissues, including a large uptake across the placenta during pregnancy. Young et al. (2003) reported that the concentrations of these free amino acids in the allantochorion were higher than those in the maternal and fetal plasmas, with glycine, glutamate, and glutamine having the highest concentrations (e.g., 1804, 1468, and 965 μM, respectively). Similarly, this has also been observed in the pig which has a diffuse epitheliochorial placenta comparable with that of the horse (Wu et al., 1998).

Maternal undernutrition, results in the impairment of the placenta's ability to provide the optimum presence and concentration of amino acids (arginine, glutamine, and leucine) to the

fetus throughout gestation, and is associated with intrauterine growth restriction (**IUGR**) (Wu et al., 1998; Kwon et al., 2004). Foals that are diagnosed with IUGR often exhibit skeletal and respiratory problems and reduced immune function, due to impaired pulmonary and metabolic functions (Rossdale and Ousey, 2002) as a result of underdevelopment while in utero. IUGR foals often have a compromised early perinatal life and have an increased risk in developing chronic diseases later in life. However, it has been reported that a moderate case of maternal undernutrition does not affect placental size, and foal birthweight (Henneke et al. 1984, Peugnet et al. 2015, Robles et al. 2018). It was observed by Robles et al. (2018) that the placenta produced from mares that were moderately underfed (forage only diet) had the capability to adapt to this diet, by having an increased development in gene expression for amino acids, folate and anions transport, and amino acids, fatty acids, cholesterol, and folate catabolism when compared to mares fed at 100% recommendation (forage plus concentrate). This further elucidates the importance of amino acids, as the placenta relies heavily on amino acid catabolism throughout fetal development.

Placental Vascularization

There is a total of six cell layers separating fetal and maternal blood, including the maternal endothelium, endometrial connective tissue, endometrial epithelium, trophoblast, allantochorion connective tissue and fetal vessels. Therefore, the efficiency of nutrient transfer from the mother to fetus is highly dependent on placental vascularization and on the uteroplacental blood flow. The development of new blood vessels from pre-existing ones (angiogenesis) is fundamental for placental growth and development (Borowicz et al. 2007; Reynolds et al. 2010; Reynolds and Redmer 1992), as well as for increased efficiency in the

transfer of nutrients and gasses from mother to fetus and for fetal survival (Grazul- Bilska et al. 2010; Liu et al. 2012).

Placental vascularization and angiogenesis are controlled by the pro-angiogenic factors Vascular Endothelium Growth Factor (**VEGF**) as well as the endothelial Nitric Oxide Synthase (**eNOS**), an enzyme that catalyzes the production of Nitric Oxide (**NO**), considered as the main vasodilator agent in feto-placental vessels (Krause et al., 2011). These factors are critical for embryonic survival throughout implantation and placentation (Bazer et al. 2012; Wu et al. 2017) in order to achieve the successful development of healthy offspring. VEGF and its receptors are present at the maternal–conceptus interface in pigs between early and late gestation (Vonnahme et al. 2001; Winther et al. 1999) to regulate the synthesis of NO and utero-placental blood flow (Reynolds et al. 2010), through the dilation of placental blood vessels. Furthermore, fetal protein accretion is regulated by the signaling mechanism of the mammalian target of rapamycin (**mTOR**; Du et al., 2005). Arginine, glutamine as well as leucine, are known to regulate the activation of the mTOR signaling pathway.

Microcotyledons are the primary unit responsible for the non-invasive haemotropic exchange of nutrients, by maximizing the area of contact between the fetal and maternal epithelial layers and blood capillaries of both fetal and maternal origin (Samuel et al., 1976). Each microcotyledon unit is equipped with a sizable artery of maternal origin, as well as an equivalent placental vein of fetal origin which is the architecture responsible for the fetomaternal exchange of the comprehensive biological molecules. The volume of nutrient delivery through the exchange between maternal and fetal membranes is highly influenced by the size of the microcotyledons. It has been previously reported that the integrity of microcotyledon development appeared to be reduced in older mares when compared to younger mares at the

same gestational ages (Bracher et al., 1996). Furthermore, the reduction in fetomaternal contact caused by the reduced development of microcotyledons in aged mares, results in the production of foals with lower birthweights when compared to younger mares (Wilsher and Allen 2003). It has been established that foal birthweight is directly proportional to both the weight and area of fetomaternal contact in the term placenta (Allen et al., 2002; Wilsher and Allen 2003). Further elucidating that the efficacy of uteroplacental blood flow and nutrient transfer from the mother to fetus is highly associated with the development of the placental vascularization network.

Age-Related Changes in the Uterine Endometrium and Placental Development

A mare needs to produce a foal six out of seven years to be financially viable (Bosh et al., 2009). However, increasing age negatively impacts the reproductive capacity of these valuable mares, as their uterine endometrium undergoes degeneration hindering placental development. Reproductive efficiency in horses tends to be lower when compared to other domestic livestock species. Infertility or low reproductive efficiency in mares is predominantly due to early embryonic death (**EED**) which has a detrimental effect on the profitability of equine breeders throughout the equine industry, due to decreased foal production.

In horses, the frequency of EED is greater (70%; $\geq$ 12 yr of age) in older mares when compared to younger mares ($\leq$ 10%; $\leq$ 11 yr of age). The greater frequency of early embryonic loss in aged mares has been correlated with poor uterine blood flow and associated with endometrial degeneration. There is an undisputable requirement for arginine to meet demands for growth and survival of the conceptus (embryo/fetus and placenta). The nutritional status of mares at the time of conception and throughout gestation will enhance cellular development of blastocysts, fetal-placental growth and development, a successful outcome of pregnancy, and long-term health of the foal into adulthood.

The entire endometrial surface is necessary for nutrient and oxygen delivery to the foal, through maternal to fetal blood exchange. Impaired placental development in older broodmares leads to suboptimal maternal-fetal transfer of nutrients. Ricketts and Alonso (1991) reported that mares of the age 17 yr or older are more susceptible to chronic degenerative endometrial disease (**CDE**), which disrupts the uterine endometrium from functioning properly. Decreases in endometrial function in aged mares is considered to impair implantation of the blastocyst and subsequent placental development. This decreases the area of the functional utero-placental interface required for adequate exchange of nutrients and gases essential for optimum fetal development. Specifically, fewer chorionic villi develop on the placenta of aged mares in which their functional endometrial surface area is reduced (Wilsher and Allen, 2003). Delay and abnormal development of the microcotyledons can reduce the surface area available for hematotrophic exchange across the utero-placental interface.

A healthy placenta also provides growth factors that regulate fetal development and progestamedins, such as fibroblast growth factors 7 and 10 and hepatic growth factor from the endometrium (Bazer et al., 2015). These contribute to placental development and functions which are essential for maintenance of pregnancy until parturition. Age-related degeneration of the endometrium thereby limits placentation by decreasing the surface area of contact between the mare's uterus and the developing placenta, inhibiting proper transfer of nutrients and oxygen from mare to fetus along with decreased transfer of fetal waste products, and negatively affecting long term health of the foal (Wilsher and Allen, 2003). Thus, the intrauterine development of the fetus and the resulting size of the foal are closely related to the total area of contact of the allantochorion.

The general perception throughout the equine industry is that larger yearlings usually sell for higher prices than smaller yearlings, because of expected superior athletic performance. Unfortunately, insufficient placental development in aged mares will lead to suboptimal fetomaternal transfer of nutrients resulting in foals with lower birthweights, a significant factor affecting future athletic performance and health of foals. Low birth weights among foals produced from aged mares presents a major economic challenge for horse breeders, as foal birth weight is commonly thought to be positively associated with size as yearlings. Most of a breeder's financial well-being relies on foal production due to the high cost of maintenance for mares. Therefore, a mare needs to produce a foal six out of seven years to be financially viable.

Influence of Dietary Arginine Supplementation on Maternal-Fetal Nutrient Transfer

Dietary arginine supplementation has demonstrated enhanced placental growth and reproductive performance in sows (Mateo et al., 2007; Gao, 2012). Supplementation with 0.80% L-arginine (16 g/sow per day) between days 14 and 25 of gestation or with 0.83% L-arginine (16.6 g/sow per day) between days 30 and 114 of gestation enhanced litter size and litter weight in gilts (Mateo et al., 2007; Li et al., 2014). In gestating sows supplemented with dietary arginine, expression of VEGF, a signaling protein that enhances angiogenesis, was greater in their placentae compared to placentae of non-supplemented sows (Wu et al., 2012), and enhanced placental angiogenesis (Wu et al., 2017).

In a similar study, larger fetuses from arginine-supplemented sows expressed more VEGF than IUGR fetuses (Liu et al., 2011). Most recently, Zhu et al. (2021) and Elmetwally et al. (2022) demonstrated that dietary arginine enhances placental water transport and NO synthesis, as well as embryonic and fetal survival in gestating swine. Interestingly, the sow develops a placenta that has a superficial form of implantation better described as a diffuse

epitheliochorial placenta. Because of the similarity in placenta types, (epitheliochorial) between the sow and the mare, the dietary supplementation of arginine has the potential to improve placental growth and development in aged mares.

In mares, Köhne et al. (2018) demonstrated that arginine supplementation at 0.0125% BW supports embryonic growth before and during the initial stages of implantation. Fetal size from Days 25 to 45 after ovulation was greater in both younger and older mares (young: 12.4 ± 0.8 mm and old: 14.3 ± 1.4 mm) supplemented with arginine compared to unsupplemented control mares (young: 10.6 ± 0.6 mm and old: 11.4 ± 0.8 mm) (Köhne et al., 2018). However, this study lacked an isonitrogenous control group, but the findings support our hypothesis regarding the potential benefits of dietary arginine supplementation in mares.

Primiparous mares supplemented with 100 g/d of L-arginine during late gestation produced foals with heavier birthweights than primiparous mares that were not supplemented with L-arginine (Chavatte-Palmer et al., 2018). To date, increased *VEGF* has been detected in multiparous mares supplemented with L-arginine compared to primiparous mares (Robles et al., 2019), but no studies reflect expression of this protein in supplemented, aged mares. There are limited studies concerning the role of maternal dietary L- arginine supplementation on placental vascularity, and the expression of placental nutrient transporters in aged mares.

CONCLUSION

Classified as a conditionally essential amino acid, little information exists about arginine synthesis and thus dietary requirements specific to the horse. Arginine is a precursor for the synthesis of many metabolites necessary for whole-body homeostasis, including ornithine, polyamines, proline, glutamine, creatine, agmatine, and nitric oxide (Wu et al., 2013). These biological molecules are essential for stimulating cell proliferation, cell migration, cellular

remodeling, angiogenesis, and dilation of blood vessels, as well as stimulation of various cell signaling pathways. Unfortunately, to date there is no known dietary arginine requirement in the horse at any stage of development. Therefore, to establish a specific dietary requirement of arginine one must first quantify the endogenous synthesis of arginine in the small intestine (enterocytes) of the horse at different stages of life.

The conceptus requires amino acids for protein accretion, metabolic processes, and other biosynthetic pathways that are only attainable from the placenta through maternal and fetal blood exchange. Impaired placental development in older broodmares along with inability for the horse to endogenously synthesize the conditionally amino acid L-arginine along with its unknown dietary requirement warrants scientific importance for equine researchers, nutritionist, and producers alike.

Arginine and its metabolites are very active throughout gestation, however there are no published results concerning the role of maternal dietary arginine supplementation on placental vascularity, and the expression of placental transporters of nutrients in aged mares. Previous studies may not have detected beneficial effects from L-arginine supplementation to horses. This could be due to unbalanced supplementation of this amino acid (in excess) which has antagonistic effects on the absorption of other essential amino acids. Therefore, continued efforts utilizing innovative and interdisciplinary research techniques focused on nutrition and reproductive physiology are necessary for obtaining knowledge to better refine and design gestational nutrient balanced diets to improve the maternal-fetal vascular interface. This will enhance the economic efficiency of equine reproduction, health, and performance across the equine industry.

CHAPTER II

IN VITRO SYNTHESIS OF ARGININE BY THE EQUINE ENTEROCYTE[*]

Currently, there is no known dietary requirement for arginine in the horse at any stage of development or physiological state (NRC 2007), and little is known about its ability to endogenously synthesize arginine *de novo*. In other mammalian species, such as pigs, humans, rats, cattle, and sheep, arginine is formed from glutamine and proline via the generation of citrulline in the enterocytes of the small intestine (Wu et al., 1994; Wu and Morris 1998; Bertolo and Burrin 2008; Wu 2022). Accordingly, in contrast to the milk of most mammals that is deficient in arginine (e.g., pigs 40, 36, 34, and 34 mg/g of total amino acids in the milk of sows, humans, cows, and sheep, respectively; Wu and Knabe 1994; Davis et al., 1994a,b; Wu 2022), cats have a limited ability to synthesize arginine *de novo* due to a deficiency of pyrroline-5-carboxylate (**P5C**) synthase in enterocytes (Rogers and Phang 1985). However, feline milk contains a high concentration of arginine; 64 mg/g of total amino acids; Davis et al., 1994a,b) to compensate for the limited endogenous synthesis of this amino acid (Rogers and Phang 1985).

In animals, arginine serves not only as a building block for proteins, but also as a precursor for the synthesis of many biologically active molecules including nitric oxide (**NO**), polyamines, creatine, homoarginine, creatinine, and agmatine (Wu and Morris 1998; Wu et al., 2013; Hou et al., 2016). Additionally, arginine supplementation reduced white fat gain, increased skeletal muscle, decreased serum triglycerides, enhanced insulin sensitivity, and increased brown fat tissue in obese rats (Jobgen et al., 2009), and improved cardiovascular, reproductive, pulmonary, renal,

[*] Reprinted with permission from "Equine enterocytes actively oxidize l-glutamine, but do not synthesize l-citrulline or l-arginine from l-glutamine or l-proline in vitro" R. E. Martinez, J. L. Leatherwood, A. N. Bradbery, B. L. Silvers, J. Fridley, C. E. Arnold, E. A. Posey, W. He, F. W. Bazer, and G. Wu., 2022. Journal of Animal Science, 100, 4, Copyright 2022 by Oxford University Press.

digestive, and immune functions in humans (Wu et al., 2021). Of particular note, arginine prevented fetal growth retardation in rats, and increased birth weights of rats and pigs (Vosatka et al., 1998; Mateo et al., 2007).

Because of the versatile roles of arginine, there is a growing interest in its nutritional value and metabolism in animals. Increasing evidence shows that dietary supplementation of arginine to animals is not only beneficial for health and productivity under certain physiological conditions such as growth, lactation, and pregnancy (Wu et al., 2009; Wu et al. 2021, 2022). Interestingly, arginine is abundant in the milk of mature mares (60 mg/g of total amino acids) (Davis et al., 1994a), suggesting a possibility that, as for cats, there is little or no synthesis of arginine in horses (Wu 2022). However, experimental evidence is required to support this proposition. Therefore, we conducted the present study to test the hypothesis that *de novo* synthesis of citrulline and arginine is limited or absent in enterocytes of young and adult horses.

MATERIALS AND METHODS

Animal Use Protocol was not required by the Institutional Animal Care and Use Committee at Texas A&M University as no manipulation of live animals were performed, and animals were not euthanized for the purpose of this study.

Chemicals

L-proline, L-glutamine, *o*-phthaldialdehyde (**OPA**), bovine serum albumin (**BSA**; fraction V, essentially fatty acid free), *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid (**HEPES**), and EDTA (disodium) were obtained from Sigma Chemicals (St. Louis, MO). L-[U-^{14}C]glutamine and L-[U-^{14}C]proline were purchased from American Radiolabeled Chemicals (St. Louis, MO). Before use, the radioactive tracers were purified by anion-exchange chromatography as previously

described (Wu 1997). High-performance liquid chromatography (**HPLC**)-grade methanol and water were obtained from Fisher Scientific (Houston, TX).

Collection of samples from the equine jejunum

Jejunum samples were harvested from 19 stock-type horses of three different age groups (Table 2). All horses were euthanized for reasons outside the scope of this current study and for reasons not pertaining to small-intestinal function and health. Tissues were obtained from the necropsy laboratory at the Texas A&M Large Animal Hospital (College Station, TX) and the Veterinary Medical Park at Texas A&M University (College Station, TX).

Preparation and incubation of enterocytes from the equine jejunum

In the horse, the jejunum represents the longest portion (~80%) of the small intestine. Therefore, the jejunum was selected for the isolation of enterocytes in this study. Enterocytes were isolated from the equine jejunum as described for the porcine jejunum (Wu et al., 1994). A mid-jejunal segment (50 cm) was obtained from each horse immediately following euthanasia and rinsed thoroughly with 0.9% saline. The jejunum was filled with calcium-free oxygenated (95% O_2/5% CO_2) Krebs-Henseleit bicarbonate (**KHB**) buffer (119 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4 and 25 mmol/L $NaHCO_3$, pH 7.4) containing 20 mmol/L HEPES (pH 7.4), 5 mmol/L EDTA (disodium), and 5 mmol/L D-glucose, and was incubated for 30-min at 37°C in a shaking water bath (70 oscillations/min). Thereafter, the jejunum was gently patted with fingertips for 1 min and its luminal fluid was drained into a 50-ml polystyrene tube. Enterocyte pellets were then obtained by centrifugation (400 × *g*, 2 min) and washed three times with 20 mL oxygenated KHB buffer (95% O_2/5% CO_2) containing 2.5 mmol/L $CaCl_2$ (no EDTA), 20 mmol/L HEPES (pH 7.4), and 5 mmol/L D-glucose, and then suspended in fresh KHB buffer. Examination of the isolated equine enterocytes under a dissecting 40× microscope (Fisher

Scientific, Houston) showed that they were predominantly (> 90%) enterocytes of columnar shape. The viability of prepared enterocytes was > 95%, as assessed by trypan blue exclusion analyses (Wu et al., 1994; Baldwin and McLeod 2000).

Incubation of equine enterocytes

Incubations of equine enterocytes were performed in 15-ml polypropylene tubes (round-bottom, 17×120 mm; Fisher Scientific, Houston, USA) placed in a shaking water bath (70 oscillations/min). Cells ($5 \times l0^6$ cells/ml) were incubated at 37°C for 0 or 30-min in 2 ml of KHB buffer (pH 7.4, saturated with 95%O_2/5% CO_2, vol/vol) containing 20 mM HEPES, 1% BSA, 5 mM glucose, and either 0 mM (control medium), 2 mM L-[U-^{14}C]glutamine, or 2 mM L-[U-^{14}C]proline plus 2 mM L-glutamine (150 dpm/nmol). Glutamine (2 mM) was added to the 2 mM L-[U-^{14}C]proline medium to provide both ammonia and glutamate to convert the proline-derived P5C into ornithine (Wu, 1997). No tubes with 2 mM L-[U-^{14}C]proline alone were included in the study. The cell incubations were replicated in three tubes within animal. A center-well, which was connected to the rubber stopper of a flask, was suspended within the flask for the collection of $^{14}CO_2$. At the end of the 30-min incubation, 0.2 ml of Soluene was added through the rubber stopper into the suspended center-well, followed by the addition, through the sealed rubber stopper, of 0.2 mL of 1.5 mol/L $HClO_4$ to the incubation medium. The radioactivity of $^{14}CO_2$ trapped into Soluene was determined using a liquid scintillation counter (Zhu et al., 2021). We adopted a 30-min period for the incubation of equine enterocytes because the results of our preliminary experiment indicated that the oxidation of 2 mM L-[U-^{14}C]glutamine to $^{14}CO_2$ by these cells was linear during this time period, as reported for porcine enterocytes (Wu et al., 1995).

Preparation and incubation of enterocytes from the porcine jejunum

To ensure that a lack of synthesis of citrulline and arginine from glutamine and proline in equine enterocytes was not due to an artifact of our cell incubation system, we isolated enterocytes from the jejunum of 7-day-old pigs as previously described (Wu et al., 1994). Incubations of porcine enterocytes in the presence of 2 mM L-[U-^{14}C]glutamine (150 dpm/nmol) and the collection of $^{14}CO_2$ were the same as described above for equine enterocytes.

Amino acid analysis by HPLC

Acidified medius plus cell content was neutralized with 0.1 mL of 2 mol/L K_2CO_3 and used for amino acid analysis by HPLC (Wu 1993; Wu et al., 1994). The HPLC apparatus and precolumn derivatization of amino acids with OPA were as previously described (Wu and Meininger, 2008). Amino acids (except proline and cysteine) were separated on a Supelco 3-μm reversed-phase C18 column (4.6 x 150 mm, I.D.) guarded by a Supelco 40-μm reversed-phase C18 column (4.6 x 50 mm, I.D.). The HPLC mobile phase consisted of solvent A (0.1 mM sodium acetate-0.5% tetrahydrofuran-9% methanol; pH 7.2) and solvent B (methanol), with a combined total flow rate of 1.1 ml/min. A gradient program with a total running time of 49 min (including the time for column regeneration) was developed for satisfactory separation of amino acids (0 min, 14% B; 15 min, 14% B; 20 min, 30% B; 24 min, 35% B; 26 min, 47% B; 34 min, 50% B; 38 min, 70% B; 40 min, 100% B; 42 min, 100% B; 42.1 min, 14% B; 48.5 min, 14% B). Proline was measured by an HPLC method involving oxidation of proline to 4-amino-1-butanol and precolumn derivatization with OPA (Wu, 1993). For cysteine analysis, 100 μl sample was mixed with 50 μl of 50 mM iodoacetic acid (an alkylating agent) for 5 min at room temperature, to convert cysteine to S-carboxymethylcysteine. The latter then reacts with OPA to form a highly fluorescent derivative (Wu et al., 1997). For cystine analysis, 100 μl sample was mixed with 100 μl of 28 mM 2-mercaptoethanol (a reducing agent) for 5 min at room temperature, to convert cystine to cysteine,

and the latter was then analyzed as described above. Amino acids were quantified on the basis of authentic standards (Sigma Chemicals, St. Louis, MO) using the Millennium™ workstation (Waters Inc., Milford, MA). Differences in the concentrations of amino acids in incubation medium plus cell extracts between 0- and 30-min incubation periods in the presence of 2-mM glutamine or 2 mM proline plus 2 mM L- glutamine were used to determine the production of ornithine, citrulline and arginine by enterocytes.

Statistical Analysis

Data were analyzed using the one-way ANOVA in JMP Pro 15 (SAS Inst., Inc., Cary, NC), with the Student-Newman-Keuls multiple comparison test for identifying significant differences among means. For statistical analysis of data from cells without incubation and cells incubated for 0 or 30 min, values were normalized to the baseline (no incubation) before performing one-way analysis of variance, as described previously (Lee et al., 2019). Because data for CO_2 production from glutamine were not normally distributed, they were log-transformed for one-way analysis of variance **(**Assaad et al., 2014). P-values ≤ 0.05 were taken to indicate statistical significance.

RESULTS

Oxidation of glutamine and proline by equine enterocytes

Jejunal enterocytes from horses of all age groups oxidized glutamine to CO_2 (Table 3). The rate of glutamine oxidation was high in enterocytes from neonatal horses, but low in cells from adult and aged horses. Interestingly, enterocytes from aged horses oxidized more glutamine to CO_2 than cells from adult horses ($P < 0.05$). There was no production of CO_2 from proline by enterocytes from any age group of horses (Table 3).

Synthesis of arginine, citrulline, and ornithine by equine enterocytes in the presence of glutamine or proline

Regardless of age, equine enterocytes synthesized ornithine from glutamine and proline, but failed to convert ornithine into citrulline and arginine (Table 4). The production of ornithine by equine enterocytes incubated in the presence of 2 mM proline plus 2 mM glutamine was greater ($P < 0.05$) than that in the presence of 2 mM glutamine. The rate of formation of ornithine from glutamine or proline was greater ($P < 0.05$) in enterocytes from neonatal horses than those from adult or aged horses (Table 4). The rate of formation of ornithine from glutamine or proline was greater ($P < 0.05$) in enterocytes from aged horses than young horses (Table 4).

Metabolism of glutamine by porcine enterocytes

Jejunal enterocytes from 7-d-old pigs extensively oxidized glutamine to CO_2 (Table 5). In contrast to equine enterocytes, porcine enterocytes converted glutamine into ornithine, citrulline, and arginine. The rate of net formation of arginine by porcine enterocytes was greater ($P < 0.05$) than the rate of net formation of ornithine and citrulline.

DISCUSSION

Understanding the equine enterocyte's ability to endogenously produce arginine is of physiological and nutritional importance with respect to the horse's physiological demands such as athletic performance, reproduction, growth, and longevity. The present study investigated age-related effects on the ability of enterocytes to synthesize arginine, citrulline, and ornithine from glutamine and proline. Similarly, ovine enterocytes are able to oxidize glutamine to CO_2 (Oba et al., 2004). Thus, equine enterocytes, as reported for porcine (Wu et al., 1994) and ovine (Oba et al., 2004) enterocytes, used glutamine as a major metabolic fuel. In contrast, equine enterocytes did not oxidize proline to CO_2 possibly due to the absence or limited activity of P5C dehydrogenase in their enterocytes (Wu 2022).

Enterocytes of the small intestine are exclusively responsible for the synthesis of P5C from glutamine from blood, as well as dietary glutamine and proline in mammals (e.g., pigs, humans, and rats) that are capable of the endogenous synthesis of arginine (Flynn and Wu 1996; Wakabayashi 1995; Wu 2022). In these cells, P5C undergoes transamination with glutamate to generate ornithine. Enzymes responsible for the conversion of glutamine into ornithine are glutaminase, P5C synthase, and ornithine aminotransferase (**OAT**), whereas proline is oxidized to ornithine via proline oxidase and OAT. We found that equine enterocytes produced ornithine from glutamine and proline, suggesting that those cells likely express glutaminase, P5C synthase, proline oxidase, and OAT. Results of the current work provides a foundation for future studies, particularly those involving the intravenous administration of ^{13}C- and ^{15}N-labeled glutamine and ornithine into young and adult mares.

In mammalian enterocytes that can produce citrulline from glutamine and proline, ornithine carboxyltransferase (**OCT**; a mitochondrial enzyme) catalyzes the formation of citrulline from ornithine and carbamoylphosphate. The latter is generated from ammonia and bicarbonate by carbamoylphosphate synthase-I; **CPS-I**), which is allosterically activated by N-acetylglutamate. Interestingly, enterocytes from young and adult horses failed to convert ornithine into citrulline and arginine. This finding was not due to deficiencies in our incubation system for equine enterocytes, because enterocytes from 7-d-old pigs in the same system produced ornithine, citrulline, and arginine from glutamine in the present study, as previously reported for neonatal pigs (Wu et al., 1994). It is possible that equine enterocytes do not express CPS-I and/or OCT. Thus, there is a species difference in the ability of mammalian enterocytes for endogenous synthesis of citrulline (the immediate precursor of arginine).

All mammalian cells can form arginine from citrulline (Wu and Morris 1998). The lack of intestinal production of citrulline from glutamine and proline is the metabolic basis for the absence of *de novo* synthesis of arginine in horses. Thus, the diets of young and adult horses must provide an adequate amount of arginine or citrulline. Because arginine is required for the synthesis of both proteins and many physiologically vital molecules, including polyamines for many cellular functions, creatine (essential for energy metabolism in the skeletal muscle and brain, as well as exercise performance) and nitric oxide (essential for blood flow; Wu 2022), results of the present work have important implications for the nutrition and feeding of horses during their life cycle. Furthermore, our findings explain why the milk of horses, as for milk of cats, contains unusually large amounts of arginine (Davis et al. 1994a, b) to support the growth, development, and survival of foals during the neonatal period.

Findings from the current work can guide future *in vivo* studies of arginine nutrition and metabolism in growing, gestating, lactating, and exercising horses. In the absence of de novo synthesis of arginine, the provision of this amino acid in young and adult horses must depend on their diets or arginine administration. Studies with pigs have shown that dietary supplementation with arginine to neonatal pigs, gestating swine, and lactating sows can enhance their growth rate, embryonic survival, and milk production, respectively (Wu et al., 2009, 2018). Köhne et al. (2018) and Aurich et al. (2019) demonstrated that dietary L-arginine supplementation to mares before and during the initial stages of implantation improved embryonic growth. For example, fetal size from days 25 to 45 after ovulation was greater in mares of reproductive age (young mares: 12.4 ± 0.8 mm and old mares: 14.3 ± 1.4 mm) supplemented with L-arginine compared to unsupplemented control mares (young mares: 10.6 ± 0.6 mm and old mares: 11.4 ± 0.8 mm). In addition, Hunka et al. (2016) reported that dietary supplementation with arginine to lactating mares increased the

concentration of glutamine in their milk by ~15%, but these authors did not determine either the milk yield of the mares or the growth performance of foals due to a limited number of animals. At present, we are not aware of any published study concerning the role of dietary arginine supplementation on neonatal growth, milk production, and exercise performance in horses.

In conclusion, the current findings indicated that the enterocytes of horses, regardless of age of horse, did not synthesize citrulline and arginine from glutamine or proline due to the lack of conversion of ornithine into citrulline (Figure 1). As a result of this preliminary study, arginine may be considered a nutritionally essential amino acid for horses, especially during physiological states such as performance, pregnancy, and lactation. Future replicated studies are warranted to further investigate the equine enterocyte's ability to synthesize arginine *in vitro* and *in vivo* as well as to determine the expression of all enzymes involved in glutamine and proline catabolism in equine enterocytes.

CHAPTER III

EVALUATION OF ARGININE SUPPLEMENTATION TO AGED MARES ON GESTATIONAL AND FOAL PERFORMANCE

Aged broodmares that are desired for reproduction generally possess an impressive black-type pedigree, and impeccable genetics. However, advancing age negatively impacts the reproductive capacity of these valuable mares, as their uterine endometrium undergoes degeneration hindering placental development (Robles et al., 2022, Wilsher and Allen 2003, and Bracher et al., 1996). The conceptus requires amino acids for protein accretion, metabolic processes, and other biosynthetic pathways that are only attainable from the placenta through maternal and fetal blood exchange. However, increases of age negatively impacts the reproductive capacity of these valuable mares, as their uterine endometrium undergoes degeneration hindering placental development.

Even with improved breeding management techniques, there has been little progress made to improve placental development in aged mares. The dietary supplementation of L-arginine has been shown to enhance placental development and reproductive performance in other species. Unfortunately, there is limited research in amino acid requirements of the horse, particularly of L-arginine. Kelley et al. (2013) revealed that mares supplemented with 100 g of L-arginine increased ovarian blood flow and observed larger dominant follicles 10 d after the preceding ovulation and a reduction in uterine fluid accumulation post-breeding. Köhne et al. (2018) reported that arginine supplementation at 0.0125% BW supports embryonic growth as fetal size from Days 25 to 45 after ovulation was greater in both younger and older mares (young: 12.4 ± 0.8 mm and old: 14.3 ± 1.4 mm) supplemented with arginine compared to non-

supplemented control mares (young: 10.6 ± 0.6 mm and old: 11.4 ± 0.8 mm) (Köhne et al., 2018). Furthermore, Primiparous mares supplemented with 100 g/d of L-arginine during late gestation produced foals with heavier birthweights than primiparous mares that were not supplemented with L-arginine (Chavatte-Palmer et al., 2018).

To date, increased placental expression of Vascular Endothelium Growth Factor ***VEGF*** has been detected in multiparous mares supplemented with L-arginine compared to primiparous mares (Robles et al., 2019), but no studies reflect expression of this protein in placentas from aged mares. Allen and Wilsher (2007) reported that VEGF facilitates the development of the extensive fetal and maternal capillary networks that are such prominent features within the microcotyledons of the diffuse, epitheliochorial equine placenta. Which further reflects the importance of VEGF and its ability to facilitate the development of maternal and fetal vascular networks for the interchange of gases, nutrients, and waste products throughout gestation. Furthermore, presence of VEGF has also been reported to increase the arginine transport via modulation of **SLC7A1** (transporter of lysine and arginine) in endothelial cells (Shashar et al., 2017).

Impaired placental development in older broodmares along with inability for the horse to endogenously synthesize the conditionally amino acid L-arginine along with its unknown dietary requirement warrants scientific importance for equine researchers, nutritionist, and producers alike. L-arginine is of particular interest due to its multiple biological responsibilities that are critical for adequate placental growth and fetal development throughout pregnancy. Interestingly, there is not *de novo* synthesis of L-arginine by the enterocytes and there is no established dietary L-arginine requirement for the aged pregnant mare or any horse during their various stages of life (Martinez et al., 2022). In summary, there are limited studies concerning the role of maternal

dietary arginine supplementation on placental vascularity, and the expression of placental nutrient transporters in aged mares. Therefore, we conducted the present study to test the hypothesis that dietary supplementation of arginine (50 mg/kg BW/d) to aged mares throughout gestation would improve the utero-placental environment and mare performance characteristics

MATERIALS AND METHODS

All care, handling, and sampling of horses was reviewed and approved by the Institutional Animal Care and Use Committee at Texas A&M University (2018-0426).

Horses and Management

Thirteen open and dry multiparous mares (mean±SEM; 18.22 ± 0.68 years; 5.8 ± 0.22 BCS; 493.82 ± 12.74 kg BW) from an established herd were used in this study. Daily observation for signs of estrus were determined by teasing mares with a stallion and follicular development and ovulation was monitored daily using B-Mode (gray scale) ultrasonography with a rectal probe and rectal palpation of the ovaries. Following the first estrous cycle of the season, each mare was inseminated with fresh semen from one of two fertile stallions that belong to Texas A&M University. Semen was collected using a Missouri-Style artificial vagina and evaluated for concentration of sperm cells and total progressive motility by one investigator. Each insemination consisted of a dose of 500 x 10^6 fresh, progressively motile spermatozoa extended in INRA 96 (IMV Technologies, L'Aigle, France) to a total volume of 30 mL. Inseminations occurred 24 h following deslorelin acetate injection, and inseminations continued every other day until the mare ovulated. A Micromaxx ultrasound (Micromaxx®, Sonosite, Bothell, WA) with a 10–5MHz broadband, 52mm linear array was utilized to scan ovaries of mares to monitor follicular development, ovulation, and pregnancy.

Treatments and Housing

Dietary treatments began when pregnancy is confirmed on d 14 post-ovulation and ended on the day of parturition. Mares were randomly assigned to dietary treatments and balanced by age, BW, BCS, and stallion pairing. Mares (n=7) received a dietary supplement of either 50 mg/kg BW of arginine (treatment; ARG) per d, or the same diet supplemented with 100 mg/kg BW of L-alanine (n=6) to achieve isonitrogenous diets (control; CON). Arginine supplementation rate in the current study is based on positive effects without evidence of impairment to amino acid absorption, decreases in uterine fluid accumulation and large fetuses (Kelley et al., 2014; Mesa et al., 2015; and Köhne et al., 2018).

Throughout the initial two trimesters of gestation, mares were fed concentrate at 1.18% BW/d (on an as-fed basis) of concentrate (Triumph Active 12 Pellet Horse Feed, Nutrena, Minneapolis, MN) and was split into two equal meals per day using individual feeding bags (Derby Originals Breathable Canvas Feed Bag with No-Spill Design, Royal International LLC, North Canton OH), and allowed 50 min to consume concentrate. Amino acids (Ajinomoto AminoScience LLC, Raleigh, NC, USA) were top-dressed onto the concentrate and thoroughly mixed immediately prior to feeding. Mares readily consumed their respective dietary treatments, and no refusals were recorded. Once mares entered their third trimester, concentrate intake increased to 1.25%. Mares were fed throughout the study according to 2007 National Research Council's recommendations for feeding pregnant mares relative to stage of gestation. Grain intake for mares was adjusted every 28 d according to changes in BW. All mares were group-housed in dry lots (58.7 × 79.2 m) and kept under natural light at the Texas A&M University Equine Center where they had *ad libitum* access to forage in the form of round bales of bermudagrass (*Cynodon dactylon*) hay, water, and trace mineral salt. Composited hay and grain

samples were analyzed by a commercial laboratory (Equi-Analytical Laboratories, Ithaca, NY) for nutrient composition (Table 6), and in house for amino acid composition was measured by HPLC as previously described (Wu et al. 1995; Table 7).

Mare Measurements

Mare BW, BCS, and rump fat (**RF**) measurements were collected every 28 d until parturition. Body weight was obtained utilizing a calibrated platform scale (Bastrop Scale Inc., Bastrop, TX). One trained investigator determined BCS on a scale of 1 to 9 as described by (Henneke et al., 1983) with 1 = poor and 9 = extremely fat. Rump fat was measured via ultrasonic images (ECO 5, Chison) on the left hip at a point 5 cm dorsal of the halfway point between the first coccygeal vertebrae and the ischium (Westervelt et al., 1976). Body fat predicted by RF thickness was calculated by using the prediction equation developed by Westervelt et al. (1976), as follows: body fat (%) = 8.64 + 4.70 × RFT (cm).

Uterine and Fetal Ultrasonography

Color doppler ultrasonography was performed transrectally to measure blood flow to the reproductive tract beginning on d 21 and continued every 7 d until d 154 of gestation when microcotyledon development ceases (Samuel et al., 1974; Samuel et al., 1975), and prior to parturition. Transrectal examinations of blood flow to the reproductive tract of all mares took place between 0800 h and 1000 h. Times at which doppler measurements were obtained coincide with peak post-prandial concentrations of arginine in plasma (Kelley et al., 2014).

Blood flow measurements were calculated as pulsatility index (**PI**) and resistance index (**RI**) the uterine artery ipsilateral to uterine horn of established pregnancy; defined gravid uterine artery (**GUA**). The arteries were identified as described by Bollwein et al. (1998). Doppler blood flow measurements for uterine arteries were obtained using an algorithm package of the Chison

ECO (ECO 5, Chison) with a 10–5MHz broadband, transrectal R7-A transducer. The measurements include resistance index (RI) along with pulsatility index (PI) (Ginther, 2007).

Foaling Variables

Nine of the twelve original mares were observed at parturition due to horse availability (ARG, n=5 and CON, n=4). Mares were monitored for signs of impending parturition and brought into individual stalls when foaling appeared to be imminent. Bermudagrass (*Cynodon dactylon*) hay was offered to mares. All foalings were attended and foaling variables (gestation length and time from water breaking to birth, from birth to placental expulsion, from birth to standing, and from birth to nursing) were recorded. Immediately following parturition, BW of foals were taken prior to nursing. Additionally, colostrum refractometer (equine colostrum refractometer, Animal Reproduction Systems, Chino, CA) readings provided measurements of Brix percentage as an indirect quantification of IgG in pre-suckle colostrum. At 12 h after parturition, foal BW, body length, wither height, and hip height were determined. No incidences of dystocia or retained placenta were observed.

Collection and Fixation of the Placentae

At parturition, time of placental expulsion, placental weight, and placental volume were recorded. To measure volume, the allantochorion was submerged in a graduated cylinder half-filled with water. After allowing for the ingress of water (no $\geq$ 5 min) into any air spaces, the volume of water displacement was measured (Wilsher and Allen, 2003). The total displacement was summed to give the volume of the allantochorion in mL. Placental samples were harvested from the same location of the pregnant uterine horn, non-pregnant uterine horn, bifurcation of the uterine horns, and cervical star area of the placenta. Samples from each location were snap frozen in liquid nitrogen and stored at -80°C for protein analyses. Additional samples from each

region were fixed in 4% buffered paraformaldehyde and embedded in paraffin for immunohistochemical analysis of histoarchitecture and cell-specific localization of proteins of interest.

Immunohistochemistry Sample Analysis

Samples from the pregnant horn of the placenta were preserved using 4% paraformaldehyde and were analyzed using immunohistochemistry (**IHC**) imaging techniques. Sections (5 μm) of paraffin embedded placental samples were mounted to glass slides. Antigen retrieval was performed using boiling 0.01 M sodium citrate buffer (pH 6.0) for vascular endothelial growth factor using a rabbit *VEGF* polyclonal antibody (Catalog # 19003-1-AP; Thermo Fisher Scientific, Waltham, MA USA 02451) at 1:150 concentration, as well as a rabbit Anti-CAT1 antibody (Catalog # ab37588; Abcam, Cambridge, UK) at 1:150. Purified nonrelevant rabbit IgG was used as a negative control. Immunoreactive protein was visualized using Vectastain ABC Kit (Catalog no. PK 6101 for rabbit IgG; Vector Laboratories, Burlingame, CA) following manufacturer's instructions and 3,3'-diaminobenzidine tetrahydrochloride (catalog no. D5637; Sigma-Aldrich, St. Louis, MO) as the color substrate. Sections were prepared with a haemotoxylin counterstain, and a coverslip was fixed using Permount mounting medium (SP15-500; Thermo Fisher Scientific, Waltham, MA). Digital images were captured using Nikon DS-Ri1 camera with NIS-Element AR 4.30.02 software. Semiquantitative analyses were performed using 10 non-overlapping images per sample fixed in a 10X field (Fiji ImageJ v1.2).

Western Blot Sample Analysis

Protein was extracted using 100 mg of snap frozen placental tissue. Samples were homogenized in protein lysis buffer, centrifuged at max for 15 min at 4°C. Protein

concentrations for each sample were determined using the Bradford protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. For western blot analyses, 50 μg protein was separated on 12% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked using 5% nonfat dry milk in Tris-buffered-saline with 0.1% Tween (TBST) for 1 h at room temperature. Polyclonal antibodies against phosphorylated and total *VEGF*, and *SLC7A1* were incubated overnight in 2% milk-TBST at 4°C. A polyclonal tubulin (1:1000; 7291; abcam) was used as a control protein for all placental regions. Membrane blots were washed for 30 min at room temperature with TBST followed by incubation with the appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature and a final wash in TBST. Proteins were detected using chemiluminescence (SuperSignal West Pico PLUS, Thermo Fisher Scientific, Waltham, MA) using manufacturer's recommendations. The amount of protein present was quantified by measuring the intensity of bands using a ChemiDoc EQ system and Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA).

Statistical Analysis

Data for mare performance throughout early gestation (ARG, n=6 and CON, n=6) were analyzed using PROC MIXED in SAS v9.4 (SAS Inst., Inc., Cary, NC). All data were analyzed as a randomized design with effects for treatment, d, and treatment × d interaction. Data for foaling performance and placental variables (ARG, n=5 and CON, n=4) were analyzed using the GLM procedure of SAS and the model contained a main effect of treatment. Foal sex and sire were use in the statistical model but were non-significant and removed to conserve degrees of freedom. Data were tested for normality and outliers were identified using box plots of the residuals and removed if greater than two standard deviations from the mean. Main effects were

considered significant when $P \leq 0.05$ and were considered a trend toward significance when $P \leq 0.10$.

RESULTS

Feed Intake of Mares

There were no treatment differences on concentrate intake throughout gestation ($P = 0.52$), concentrate intake averaged at (6.34 ± 0.06 kg/d) for unsupplemented mares and (6.29 ± 0.06 kg/d) for arginine supplemented mares. The intake of concentrate did however have a main effect of time, as mare body weights increased throughout the entirety of gestation, so did their concentrate intake. Arginine was supplemented at 0.4% of the concentrate diet.

Mare Performance up to 154 d gestation

Mare BCS was influenced by treatment ($P < 0.01$; Table 8) in early gestation, as control mares had a higher BCS (5.94 ± 0.12) when compared to supplemented mares (5.25 ± 0.11). However, mare BW and RF thickness did not differ ($P = 0.70$) between diets. Mare % body fat was influenced by treatment ($P = 0.05$; Table 8) as arginine supplemented mares had a higher % body fat (10.52 ± 0.05 %) compared to control mares (10.37 ± 0.06 %) during the initial 154 days of gestation. Mare BW numerically increased across treatments from d 14 (502.88 ±13.52 kg) to d 154 (538.03 ±13.52 kg) with advancing gestation. A diet × day tendency ($P = 0.08$; Fig. 4) was observed the pulse index was higher at the beginning of the trial specifically day 21 and 35 in arginine supplemented mares when compared to treatment mares. A main effect of time ($P < 0.01$; Fig. 5) was observed for resistance index of the gravid horn decreased regardless of dietary treatment, as resistance decreased as gestation progressed up to day 154.

Mare Performance to Parturition

Only 9 (ARG, n=5 and CON, n=4) of the 12 mares were observed from d 154 to parturition due to horse availability. There was a diet × day interaction ($P = 0.03$; Fig. 6) on RF thickness as arginine supplementation lowered RF (3.29 ± 0.30 mm) on d 294 of gestation compared to control mares (4.55 ± 0.35 mm). Mare BCS differed by diet ($P = 0.04$, Table 9) throughout the entirety of gestation, as BCS was lower in arginine than control mares (4.83 ± 0.20 and 5.55 ± 0.24 BCS, respectively). A diet × day interaction ($P = 0.03$; Fig. 7) was observed on day 70 as arginine supplemented mares had a higher % body fat (10.61 ± 0.12 %) compared to control mares (10.26 ± 0.14 %). However, on day 182 to parturition control mares had higher % body fat (10.52 ± 0.13 %) when compared to treatment mares (10.18 ± 0.14 %). Mare BW increased ($P < 0.01$) in all mares as they approached parturition regardless of dietary treatment ($P = 0.70$). At parturition mare BCS was influenced by dietary treatment ($P < 0.01$) as control mares had a higher BCS (5.94 ± 0.11) at parturition when compared to mares in dietary treatment group (5.25 ± 0.10). Rump fat thickness at parturition was not influenced by dietary treatment ($P = 0.67$). Furthermore, there was no dietary influence on gestation length ($P = 0.84$), as well as on mare body weights pre- and/or post-partum ($P = 0.74$). Treatment mares demonstrated to have a lower RI prior to parturition ($P = 0.01$; Table 10) on the uterine artery ipsilateral to the pregnant uterine horn when compared to unsupplemented mares. This dietary difference however was not noted for PI ($P = 0.25$, Table 10).

Foaling Variables

All mares delivered normal, healthy foals without difficulty or placental retention. Foal BW at parturition and 12 h post-partum was not influenced ($P \geq 0.92$; Table 10) by maternal dietary supplementation of arginine. Foal size parameters (e.g., wither height, hip height, or body

length) or brix percentage evaluation of colostrum were also not influenced ($P \leq 0.47$; Table 11) by dietary treatment. Additionally, placental expulsion and foal standing times were not different ($P = 0.24$) between diets. Placental variables (Table 12) were not affected by the dietary supplementation of arginine throughout gestation ($P = 0.23$).

DISCUSSION

In the present study, mares on the treatment diet were offered 50 mg/kg/d BW of arginine, or 100 mg/kg BW of L-alanine per d as dietary control. Dosing of arginine was derived from previously reported data of arginine supplementation with no adverse effects (Kelley et al., 2014; Mesa et al., 2015; and Köhne et al., 2018). Due to the increased amount of nitrogen in the treatment diet, the same basal diet was supplemented with 100 mg/kg BW of L-alanine to mares as the isonitrogenous control, similar to previously reported studies (Wu 2013; Wu et al. 2004).

The supplementation of arginine (50 mg/kg BW/d) influenced BCS as control mares appeared to have a higher BCS (5.94 ± 0.12) than mares supplemented with arginine (5.25 ± 0.11) throughout the entirety of the study (d 14 - parturition). Although BCS were statistically different, both scores remain near a 5, which should be acknowledged as these mares were all of advanced age. Furthermore, limited information exists evaluating BCS of aged pregnant mares following the onset of arginine supplementation. However, the authors do acknowledge that although frequently utilized throughout the industry, the BCS scoring system (Henneke et al., 1983) is a subjective form to obtain physiological information. Furthermore, RF thickness, an objective observation, was not influenced by dietary treatment throughout the initial 154 d of gestation.

Intriguingly, it has been reported (Jobgen et al., 2009, Wu at el., 2009, Tan et al., 2012 and Boon et al., 2019) that the supplementation of arginine leads to a reduction in white adipose

tissue in different species (e.g., humans, pigs, and rats). Arginine is known to stimulate lipolysis in adipocytes, effectively mobilizing adipose tissue reserves (Wu et al., 2007). In obese rats, dietary supplementation with arginine beneficially stimulates the expression of genes for mitochondrial biogenesis and, thus, the oxidation of fatty acids in white adipose tissue (Fu et al. 2005). Similarly, partly through activating the production of carbon monoxide, arginine supplementation promotes the oxidation of both fatty acids and glucose in rat skeletal muscle (McKnight et al. 2010).

In the current study, the thickness of RF mirrored changes in BCS as a diet × day interaction was observed as rump fat thickness decreased as a response to dietary treatment. Although not analyzed in the current study, arginine supplementation has an effect on pancreas insulin production. The short-term supplementation of arginine has been demonstrated to increases insulin secretion in ponies (Holdstock et al., 2004). However, insulin secretion is reduced following the long-term supplementation of arginine (Mullooly et al., 2014), possibly due to amino acid imbalances or antagonisms. Robles et al. (2019) reported that 100 g/d increased insulin sensitivity in primiparous pregnant mares when compared to non-supplemented mares. The increased sensitivity to insulin could explain gradual decrease in rump fat thickness for arginine-treated mares into mid-gestation. Finally, there were no differences in rump fat thickness in response to dietary treatment at parturition, as arginine supplemented mares returned to above baseline levels of rump fat thickness. As arginine supplementation progressed through gestation in a "long-term" manner, we can presume that metabolic changes (e.g., lipolysis) occurred in the treatment group of mares. Further studies are warranted to test this hypothesis.

As expected, all mares, regardless of dietary treatment, increased in BW from 505.30 kg to 523.33 kg duration of this experimental phase (d 14 to d 154 of gestation). Therefore, all

mares had a 3.57% increase of bodyweight up to d 154, which is similar to previously reported data by Lawrence et al. (1992) who reported a 4.55% increase in BW up to d 150 in pregnant non-lactating mares. The bodyweight of all mares, regardless of dietary treatment, continued to increase into parturition (554.19 kg) resulting in a 9.84% body weight increase.

Ginther (2007) stated that color doppler ultrasonography indices (RI and PI) are becoming a commonly used diagnostic tool to evaluate and quantify blood flow velocity to reproductive tissues via the uterine arteries in livestock animals. In the current study, there was no dietary influence on RI or PI throughout the initial 154 days of pregnancy. There was, however, an effect of time, as all mares regardless of diet had decreased pulsatility and resistance indices, which reflects the increased flow to the uterus (pregnant horn, via the GUA) throughout early gestation (d14 – 154). This decrease was expected, as it is a normal incidence for gestating mares. Furthermore, as mares neared parturition, the arginine-supplemented mares demonstrated increased blood perfusion noted by a decreased RI possibly due to an enhanced synthesis of nitric oxide by endothelial cells, when compared to non-supplemented mares. Similarly, Mortensen et al. (2011) noted that arginine-supplemented mares (100g/d) had greater blood flow (lower resistance index in the GUA) than control mares. As previously stated by Mortensen et al. (2011), this could indicate that during pre-parturition mares exhibit maximal blood flow through the GUA. Because of a limited number of mares used in the present study, an effect of dietary arginine supplementation on their blood flow was not detected with our use of the doppler ultrasound technique.

The supplementation of arginine to gilts (first time mothers) between d 14 and 25 of gestation or between d 30 and 114 of gestation enhanced litter size and litter weight (Mateo et al., 2007; Li et al., 2014). Chavatte-Palmer et al. (2018) reported that primiparous mares

supplemented with 100 g/d of L-arginine during late gestation produced foals with heavier birthweights than unsupplemented primiparous mares. However, the current study did not have a dietary difference in pre suckle foal BW, or at 12 h post – partum, foal size (wither height, hip height, and body length), or standing time post - partum. Furthermore, Mortensen et al. (2011) reported that arginine supplemented mares (100g/d) had a significantly shorter gestation length when compared with non-supplemented controls. However, this same observation was not seen in the current study, as statistical significance was not attained in gestation length between diets, likely due to a small population size.

Furthermore, no differences were noted for placental weight, volume, or umbilical length. Robles et al. (2019) reported similar results as they observed no difference in placental weight, surface, and volume as well as foal wither height at birth. The absence of differences in foal sizes in both studies can most likely be attributed to a small sample size (*n*=8, Robles et al.,2019; *n*=5 present study). Additionally, there was no difference in placental expulsion times, as all mares expelled the entirety of the fetal membranes within a normal time (Ginther, 1992). Also, the supplementation of arginine did not affect the Brix % reading when evaluating pre-suckle colostrum.

In conclusion, these results indicate dietary arginine supplementation (50 mg/kg BW/d) is safe for gestating mares and promotes mobilization of white adipose tissue demonstrated by BCS evaluation (Henneke et al., 1983). However, a larger number of mares is required to determine effects of supplemental arginine on embryonic/fetal survival and growth in mares, as the number of mares decreased throughout the progression of the current study, ultimately leading to inconclusive results related to the placental transfer of nutrients from the mare to the foal. A small sample size, particularly in this area of research is a limiting factor of this study.

Low "*n*" may not demonstrate any differences to gross measures such as bodyweight, however more sensitive molecular changes can be observed in smaller populations. This is indicative as maternal nutrition affects the cellular and molecular mechanisms of placental function that are responsible for the phenotypical regulation of the foal *in utero* (Fowden et al., 2013).

CHAPTER IV

IMPLICATIONS AND SUMMARY

Arginine is essential for placental development, fetal survival, and optimal growth. However, a specific dietary arginine requirement has yet to be determined for the horse. Therefore, quantifying the endogenous synthesis of this amino acid by enterocytes in the small intestine is a necessary step towards determining the requirement for supplemental arginine in the equine diet. Additionally, the current studies serve as a precedent for other research to advance understanding of nutrient requirements and how to optimize the diet of aged gestating mares as well as horses in general throughout their various life stages of growth, development, performance, fertility, and reproductive health.

Classified as a conditionally essential amino acid, arginine is a precursor for the synthesis of many metabolites necessary for whole-body homeostasis, including ornithine, polyamines, proline, glutamine, creatine, agmatine, and nitric oxide (Wu et al., 2013). These biological molecules are essential for stimulating cell proliferation, cell migration, cellular remodeling, angiogenesis, and dilation of blood vessels, as well as stimulation of various cell signaling pathways. The initial study demonstrated that equine enterocytes used glutamine as a major metabolic fuel, similar to the pig and sheep. In contrast, equine enterocytes did not oxidize proline to CO_2 possibly due to the absence or limited activity of P5C dehydrogenase in their enterocytes. Additionally, regardless of age, equine enterocytes synthesized ornithine from glutamine and proline, but failed to convert ornithine into citrulline and arginine. Therefore, due to the absence of *de novo* synthesis of arginine, the provision of this amino acid in young and adult horses must depend on their diets or arginine administration.

Previous studies have reported that enterocytes of the small intestine play a pivotal role in the whole-body synthesis of citrulline, which is the immediate precursor for the endogenous synthesis of arginine. As incubated cells of other species have produced citrulline and arginine from precursor amino acids, *in vitro* and *in vivo*. Although this seems to be the first study evaluating the equine enterocyte's inability to synthesize arginine and citrulline, an implication is that enzyme expression was not evaluated. Therefore, future replicated studies are warranted to further investigate the equine enterocyte's ability to synthesize arginine *in vitro* and *in vivo* as well as to determine the expression of all enzymes involved in glutamine and proline catabolism in equine enterocytes.

In the second study, although no statistical significance was observed in mare or foaling performance, a main effect of diet was observed in pre-partum mares as supplementation of arginine (50 mg/kg BW/d) throughout pregnancy led to decreased resistance in blood flow via the GUA, leading to increased delivery of blood to the pregnant horn. Unfortunately, there were limited differences based on dietary intervention through the supplementation of arginine throughout gestation that was likely due to small sample size and measured variables.

REFERENCES

Allen, W.R., S. Wilsher, C. Turnbull, F. Stewart, J. Ousey, P.D. Rossdale, and A.L. Fowden. 2002. Influence of maternal size on placental, fetal and postnatal growth in the horse. I. Development in utero. Reprod. Camb. Engl. 123, 445–453.

Allen, W. R., S. Gower, and S. Wilsher. 2007. Immunohistochemical localization of vascular endothelial growth factor (VEGF) and its two receptors (Flt-I and KDR) in the endometrium and placenta of the mare during the oestrous cycle and pregnancy. Reprod Domest Anim 42(5):516-526. doi: 10.1111/j.1439-0531.2006.00815.x

Assaad, H., L. Zhou, R. J. Carroll, and G. Wu. 2014. Rapid publication-ready MS-Word tables for one-way ANOVA. SpringerPlus 3:474. doi: 10.1186/2193-1801-3-474

Aurich, J., M. Köhne, M. Wulf, C. Nagel, E. Beythien, C. Gautier, J. Zentek, and C. Aurich. 2019. Effects of dietary L-arginine supplementation to early pregnant mares on conceptus diameter-Preliminary findings. Reprod. Domest. Anim. 54(5):772-778. doi: 10.1111/rda.13422.

Baldwin, R. L., and K. R. McLeod. 2000. Effects of diet forage:concentrate ratio and metabolizable energy intake on isolated rumen epithelial cell metabolism in vitro. J. Anim. Sci. 78(3):771-783. doi: 10.2527/2000.783771x

Bazer, F. W., G. A. Johnson, and G. Wu. 2015. Amino acids and conceptus development during the peri-implantation period of pregnancy. Adv Exp Med Biol 843:23-52. doi: 10.1007/978-1-4939-2480-6_2

Bazer, F. W., T. E. Spencer, and W. W. Thatcher. 2012. Growth and development of the ovine conceptus. J Anim Sci 90(1):159-170. doi: 10.2527/jas.2011-4180

Bertolo, R. F. and D. G. Burrin. 2008. Comparative aspects of tissue glutamine and proline metabolism. J. Nutr. 138(10):2032s-2039s. doi: 10.1093/jn/138.10.2032S

Bollwein, H., J. Maierl, R. Mayer, and R. Stolla. 1998. Transrectal color Doppler sonography of the A. uterina in cyclic mares. Theriogenology 49(8):1483-1488. doi: 10.1016/s0093-691x(98)00094-6

Boon, M. R., M. J. W. Hanssen, B. Brans, C. J. M. Hülsman, J. Hoeks, K. J. Nahon, C. Bakker, J. B. van Klinken, B. Havekes, G. Schaart, I. M. Jazet, P. C. N. Rensen, and W. D. van Marken Lichtenbelt. 2019. Effect of l-arginine on energy metabolism, skeletal muscle and brown adipose tissue in South Asian and Europid prediabetic men: a randomised double-blinded crossover study. Diabetologia 62(1):112-122. doi: 10.1007/s00125-018-4752-6

Bracher V., S. Mathias, W.R. Allen. 1996. Influence of chronic degenerative endometritis (endometrosis) on placental development in the mare. Equine Vet J 28:180e8

Bosh,K., D. Powell, B. Shelton and W. Zent. 2009. Reproductive performance measures among Thoroughbred mares in central Kentucky, during the 2004 mating season. *Equine Vet. J.* 41:883-888.

Cetin, I. 2001. Amino Acid Interconversions in the Fetal-Placental Unit: The Animal Model and Human Studies In Vivo. Pediatric Research 49(2):148-154. doi: 10.1203/00006450-200102000-00004

Chavatte-Palmer, P., E. Derisoud, M. Robles, C. Dubois, C. Richard, M. Dahirel, L. Wimel, 2018. Effects of dietary arginine supplementation in pregnant mares on maternal metabolism and foal birthweight. *Journal of Equine Veterinary Science, 66*2018.05.112

Chung, Y., R. Sharman, R. Carlsen, S. W. Unger, D. Larson, and T. Jue. 1998. Metabolic fluctuation during a muscle contraction cycle. American Journal of Physiology-Cell Physiology 274(3):C846-C852. doi: 10.1152/ajpcell.1998.274.3.C846

Coenen, M., E. Kienzle, I. Vervuert, and A. Zeyner. 2011. Recent German Developments in the Formulation of Energy and Nutrient Requirements in Horses and the Resulting Feeding Recommendations. Journal of Equine Veterinary Science 31(5):219-229. doi: https://doi.org/10.1016/j.jevs.2011.03.204

Curthoys, N. P., and M. Watford. 1995. Regulation of glutaminase activity and glutamine metabolism. Annu Rev Nutr 15:133-159. doi: 10.1146/annurev.nu.15.070195.001025

Davis, T. A., H. V. Nguyen, R. Garcia-Bravo, M. L. Fiorotto, E. M. Jackson, and P. J. Reeds. 1994b. Amino acid composition of the milk of some mammalian species changes with stage of lactation. Br. J. Nutr. 72(6):845-853. doi: 10.1079/bjn19940089.

Davis, T.A., H.V. Nguyen, R. Garcia-Bravo, M.L. Fiorotto, E.M. Jackson, D.S. Lewis, D.R. Lee, and P.J. Reeds. 1994a. Amino acid composition of human milk is not unique. J. Nutr. 124:1126–1132.

Dillon, E.L. and G. Wu. 2021. Cortisol enhances citrulline synthesis from proline in enterocytes of suckling piglets. Amino Acids 53:1957-1966. doi: 10.1007/s00726-021-03039-y.

Dunlap, K.A., J.D. Brown, A.B. Keith, and M.C. Satterfield. 2015. Factors controlling nutrient availability to the developing fetus in ruminants. J Anim Sci Biotechnol 6 16.

Du, M., M. J. Zhu, W. J. Means, B. W. Hess, and S. P. Ford. 2005. Nutrient restriction differentially modulates the mammalian target of rapamycin signaling and the ubiquitin-proteasome system in skeletal muscle of cows and their fetuses1. Journal of Animal Science 83(1):117-123. doi: 10.2527/2005.831117x

Elmetwally, M. A., X. Li, G. A. Johnson, R. C. Burghardt, C. M. Herring, A. C. Kramer, C. J. Meininger, F. W. Bazer, and G. Wu. 2022. Dietary supplementation with l-arginine between days 14 and 25 of gestation enhances NO and polyamine syntheses and the expression of angiogenic proteins in porcine placentae. Amino Acids 54(2):193-204. doi: 10.1007/s00726-021-03097-2

Faber J.J. and K.L. Thornburg. 1983. Placental physiology. Structure and function of fetomaternal exchange. New York: Raven

Flynn, N. E. and G. Wu. 1997. Enhanced metabolism of arginine and glutamine in enterocytes of cortisol-treated pigs. Am. J. Physiol. 272(3 Pt 1): G474-480. doi: 10.1152/ajpgi.1997.

Foote, A.K., S.W Ricketts, and F.E. Whitwell. 2012. A racing start in life? The hurdles of equine feto-placental pathology. *Equine Vet. J*. 44:120-129.

Fowden A.L., J.K. Jellyman, O.A. Valenzuela, A. J. Forhead. 2013. Nutritional Programming of Intrauterine Development: A Concept Applicable to the Horse? J Equine Vet Sci, 33:295-304.

Fu, W.J., T.E. Haynes, R. Kohli, J. Hu, W. Shi, T.E. Spencer, R.J. Carroll, C.J. Meininger, and G. Wu. 2005. Dietary L-arginine supplementation reduces fat mass in Zucker diabetic fatty rats. J. Nutr. 135:714-721.

Gao, K., Z. Jiang, Y. Lin, C. Zheng, G. Zhou, F. Chen, L. Yang, and G. Wu. 2012. Dietary L-arginine supplementation enhances placental growth and reproductive performance in sows. *Amino Acids* 42:2207.

Gilbreath, K.R., F.W. Bazer, M.C. Satterfield, and G. Wu. 2021. Amino acid nutrition and reproductive performance in ruminants. Adv. Exp. Med. Biol. 1285:43-61.

Ginther, O. J., editor. 2007. Ultrasonic Imaging and Animal Reproduction: Color-Doppler Ultrasonography. Equiservices Publishing, Cross Plains, WI

Ginther O.J.1992. Parturition, puerperium, and puberty. pp. 457–473. In: Reproductive Biology of the Mare Basic and Applied Aspects, 2nd ed., Equiservices, Wisconsin.

Henneke, D. R., G. D. Potter, J. L. Kreider, and B. F. Yeates. 1983. Relationship between condition score, physical measurements and body fat percentage in mares. Equine Vet. J. 15:371–372. doi: 10.111/j.2042-3306.1983. tb01826.x

Henneke, D. R., G. D. Potter, and J. L. Kreider. 1984. Body condition during pregnancy and lactation and reproductive efficiency of mares. Theriogenology 21:897-909.

Holdstock, N., V. Allen, M. Bloomfield, C. Hales , A. Fowden. 2004. Development of insulin and proinsulin secretion in newborn pony foals. J. Endocrinol. 181:469–476. doi: 10.1677/joe.0.1810469

Hou, Y.Q., S.D. Hu, S.C. Jia, G. Nawaratna, D.S. Che, F.L. Wang, F.W. Bazer, and G. Wu. 2016. Whole-body synthesis of L-homoarginine in pigs and rats supplemented with L-arginine. Amino Acids 48:993-1001.

Hunka, M. M., E. R. R. da Silva, M. Kutschenko, E. T. Nogueira, H. E. C. da Costa Cordeiro Manso, and H. C. Manso Filho. 2016. Effects of L-arginine supplementation on lactating mares and the development of foals. Acta Sci. Vet. 44:1352.

Jobgen, W., C. J. Meininger, S. C. Jobgen, P. Li, M. J. Lee, S. B. Smith, T. E. Spencer, S. K. Fried, and G. Wu. 2009. Dietary L-arginine supplementation reduces white fat gain and enhances skeletal muscle and brown fat masses in diet-induced obese rats. J. Nutr. 139(2):230-237. doi: 10.3945/jn.108.096362

Kelley, D. E., L. K. Warren, and C. J. Mortensen. 2013. Oral L-arginine supplementation impacts several reproductive parameters during the postpartum period in mares. Anim Reprod Sci 138(3-4):233-240. doi: 10.1016/j.anireprosci.2013.03.001

Kelley, D. E., L. K. Warren, and C. J. Mortensen. 2014. Orally supplemented l-arginine impairs amino acid absorption depending on dose in horses1. Journal of Animal Science 92(12):5560-5566. doi: 10.2527/jas.2014-7690

Krause, B. J., M. A. Hanson, and P. Casanello. 2011. Role of nitric oxide in placental vascular development and function. Placenta 32(11):797-805. doi: 10.1016/j.placenta.2011.06.025

Köhne, M., M. Wulf, E. Beythien, C.Gauthier, K. Männer, J. Zentek, and C. Aurich. 2018. Effects of L-arginine supplementation to early pregnant mares on conceptus development and growth. J. Equine Vet. Sci. 66;201. doi: 10.1016/j.jevs.2018.05.090

Lawrence, L. M., J. DiPietro, K. Ewert, D. Parrett, L. Moser, and D. Powell. 1992. Changes in body weight and condition of gestating mares. Journal of Equine Veterinary Science 12(6):355-358. doi: https://doi.org/10.1016/S0737-0806(06)81361-4

Lee, U., T. P. Garcia, R. J. Carroll, K. R. Gilbreath, and G. Wu. 2019. Analysis of repeated measures data in nutrition research. Front. Biosci. 24:1378-1390.

Li, X.L., F.W. Bazer, G.A. Johnson, R.C. Burghardt, J.W. Frank, Z.L. Dai, J.J. Wang, Z.L. Wu, I. Shinzato, and G. Wu. 2014. Dietary supplementation with L-arginine between days 14 and 25 of gestation enhances embryonic development and survival in gilts. *Amino Acids* 46:375-384.

Mateo, R. D., G. Wu, F. W. Bazer, J. C. Park, I. Shinzato, and S. W. Kim. 2007. Dietary L-arginine supplementation enhances the reproductive performance of gilts. J. Nutr. 137(3):652-656. doi: 10.1093/jn/137.3.652

Martinez, R. E., J. L. Leatherwood, A. N. Bradbery, B. L. Silvers, J. Fridley, C. E. Arnold, E. A. Posey, W. He, F. W. Bazer, and G. Wu. 2022. Equine enterocytes actively oxidize l-glutamine, but do not synthesize l-citrulline or l-arginine from l-glutamine or l-proline in vitro. Journal of Animal Science 100(4)doi: 10.1093/jas/skac077

McKnight, J.R., M.C. Satterfield, W.S. Jobgen, S.B. Smith, T.E. Spencer, C.J. Meininger, C.J. McNeal, and G. Wu. 2010. Beneficial effects of L-arginine on reducing obesity: Potential mechanisms and important implications for human health. Amino Acids 39:349-357.

Mesa, A. M., L. K. Warren, J. M. Sheehan, D. E. Kelley, and C. J. Mortensen. 2015. L-Arginine supplementation 0.5% of diet during the last 90 days of gestation and 14 days postpartum reduced uterine fluid accumulation in the broodmare. Anim Reprod Sci 159:46-51. doi: 10.1016/j.anireprosci.2015.05.011

Mortensen, C. J., D. E. Kelley, and L. K. Warren. 2011. Supplemental l-Arginine Shortens Gestation Length and Increases Mare Uterine Blood Flow before and after Parturition. Journal of Equine Veterinary Science 31(9):514-520. doi: https://doi.org/10.1016/j.jevs.2011.01.004

Mullooly, N., W. Vernon, D.M. Smith, and P. Newsholme. 2014. Elevated levels of branched-chain amino acids have little effect on pancreatic islet cells, but l-arginine impairs function through activation of the endoplasmic reticulum stress response. Exp. Physiol. 2014;99:538–551. doi: 10.1113/expphysiol.2013.077495.

Nagy, R. 2006. Effects of Management on Reproductive Efficiency in Thoroughbreds. Masters Theses. University of Kentucky., Lexington.

Newsholme, P., M. M. Lima, J. Procopio, T. C. Pithon-Curi, S. Q. Doi, R. B. Bazotte, and R. Curi. 2003. Glutamine and glutamate as vital metabolites. Braz J Med Biol Res 36(2):153-163. doi: 10.1590/s0100-879x2003000200002

Niekerk, F. E., and C. H. Niekerk. 1997. The effect of dietary protein on reproduction in the mare. II. growth of foals, body mass of mares and serum protein concentration of mares during the anovulatory, transitional and pregnant periods. *Journal of the South African Veterinary Association, 68*: 81-85.

NRC. 2007. Nutrient requirements of horses. 6th rev. ed. Natl. Acad. Press, Washington, DC.

Oba, M., R. L. Baldwin, and B. J. Bequette. 2004. Oxidation of glucose, glutamate, and glutamine by isolated ovine enterocytes in vitro is decreased by the presence of other metabolic fuels. J. Anim. Sci. 82(2):479-486. doi: 10.2527/2004.822479x

Parimi, P. S., M. M. Kadrofske, L. L. Gruca, R. W. Hanson, and S. C. Kalhan. 2005. Amino Acids, Glutamine, and Protein Metabolism in Very Low Birth Weight Infants. Pediatric Research 58(6):1259-1264. doi: 10.1203/01.pdr.0000185130.90205.1f

Peugnet, P., M. Robles, L. Mendoza, L. Wimel, C. Dubois, M. Dahirel, D. Guillaume, S. Camous, V. Berthelot, and M. P. Toquet. 2015. Effects of moderate amounts of barley in late pregnancy on growth, glucose metabolism and osteoarticular status of pre-weaning horses.

Reynolds, L. P., P. P. Borowicz, J. S. Caton, K. A. Vonnahme, J. S. Luther, D. S. Buchanan, S. A. Hafez, A. T. Grazul-Bilska, and D. A. Redmer. 2010. Uteroplacental vascular development and placental function: an update. Int J Dev Biol 54(2-3):355-366. doi: 10.1387/ijdb.082799lr

Ricketts, S. W., and S. Alonso. 1991. The effect of age and parity on the development of equine chronic endometrial disease. Equine Vet J 23(3):189-192. doi: 10.1111/j.2042-3306.1991.tb02752.x

Robles, M., P. Peugnet, C. Dubois, F. Piumi, L. Jouneau, O. Bouchez, M. C. Aubrière, M. Dahirel, J. Aioun, and L. Wimel. 2018. Placental function and structure at term is altered in broodmares fed with cereals from mid-gestation. Placenta 64:44-52.

Robles, M., A. Couturier-Tarrade, E. Derisoud, A. Geeverding, C. Dubois, M. Dahirel, J. Aioun, A. Prezelin, J. Calvez, C. Richard, L. Wimel, P. Chavatte-Palmer. 2019. Effects of dietary arginine supplementation in pregnant mares on maternal metabolism, placental structure and function and foal growth. *Sci Rep* 9**,** 6461

Robles, M., S. Loux, A. M. de Mestre, and P. Chavatte-Palmer. 2022. Environmental constraints and pathologies that modulate equine placental genes and development. Reproduction 163(3):R25-R38. doi: 10.1530/REP-21-0116

Rogers, Q. R. and J. M. Phang. 1985. Deficiency of pyrroline-5-carboxylate synthase in the intestinal mucosa of the cat. J. Nutr. 115:146-150

Silver, M., Fowden, A.L., Taylor, P.M., Knox, J. and Hill, C.M., 1994. Blood amino acids in the pregnant mare and fetus: the effects of maternal fasting and intrafetal insulin. Experimental Physiology, 79,4230433

Samuel, C. A., W. R. Allen, and D. H. Steven. 1974. STUDIES ON THE EQUINE PLACENTA. Reproduction 41(2):441-445. doi: 10.1530/jrf.0.0410441

Samuel, C. A., W. R. Allen, and D. H. Steven. 1975. Ultrastructural development of the equine placenta. J Reprod Fertil Suppl (23):575-578.

Samuel, C. A., W. R. Allen, and D. H. Steven. 1976. Studies on the equine placenta. II. Ultrastructure of the placental barrier. J. Reprod. Fertil. 48, 257–64

Shashar, M., T. Chernichovski, O. Pasvolsky, S. Levi, A. Grupper, R. Hershkovitz, T. Weinstein, and I. F. Schwartz. 2017. Vascular Endothelial Growth Factor Augments Arginine Transport and Nitric Oxide Generation via a KDR Receptor Signaling Pathway. Kidney and Blood Pressure Research 42(2):201-208. doi: 10.1159/000476016

Tan, B. e., X. Li, Y. Yin, Z. Wu, C. Liu, C. D. Tekwe, and G. Wu. 2012. Regulatory roles for L-arginine in reducing white adipose tissue. Front Biosci (Landmark Ed) 17(6):2237-2246. doi: 10.2741/4047

Vonnahme, K. A., M. E. Wilson, and S. P. Ford. 2001. Relationship between placental vascular endothelial growth factor expression and placental/endometrial vascularity in the pig. Biol Reprod 64(6):1821-1825. doi: 10.1095/biolreprod64.6.1821

Vosatka, R. J., P. M. Hassoun, and K. B. Harvey-Wilkes. 1998. Dietary L-arginine prevents fetal growth restriction in rats. Am. J. Obstet. Gynecol. 178(2):242-246. doi: 10.1016/s0002-9378(98)80007-0

Wakabayashi, Y. 1995. The glutamate crossway. In: Amino Acid Metabolism and Therapy in Health and Nutritional Disease (Cynober, L.A. ed.) pp. 88-98. CRC Press, New York, NY.

Westervelt, R. G., J. R. Stouffer, H. F. Hintz, and H. F. Schryver. 1976. Estimating Fatness in Horses and Ponies. Journal of Animal Science 43(4):781-785. doi: 10.2527/jas1976.434781x

Wilsher, S. and W. R. Allen. 2003. The effects of maternal age and parity on placental and fetal development in the mare. *Equine Vet. J.* 35:476-483.

Winther, H., A. Ahmed, and V. Dantzer. 1999. Immunohistochemical localization of vascular endothelial growth factor (VEGF) and its two specific receptors, Flt-1 and KDR, in the porcine placenta and non-pregnant uterus. Placenta 20(1):35-43. doi: 10.1053/plac.1998.0350

Wu, G. 1993. Determination of proline by reversed-phase high-performance liquid chromatography with automated pre-column o-phthaldialdehyde derivatization. J. Chromatogr. A 641:168-175. doi: https://doi.org/10.1016/0021-9673(93)83471-4.

Wu, G. 1997. Synthesis of citrulline and arginine from proline in enterocytes of postnatal pigs. Am. J. Physiol. 272:G1382-G1390. doi: 10.1152/ajpgi.1997.272.6.G1382.

Wu, G. 2022. Amino Acids: Biochemistry and Nutrition, 2nd edition. CRC Press, Boca Raton, Florida

Wu, G., D. A. Knabe, and S. W. Kim. 2004. Arginine Nutrition in Neonatal Pigs. The Journal of Nutrition 134(10):2783S-2790S. doi: 10.1093/jn/134.10.2783S

Wu, G. and D.A. Knabe. 1994. Free and protein-bound amino acids in sow's colostrum and milk. J. Nutr. 124:415-424.

Wu, G. and S. M. Morris, Jr. 1998. Arginine metabolism: nitric oxide and beyond. Biochem. J. 336:1-17. doi: 10.1042/bj3360001

Wu, G., and C.J. Meininger. 2008. Analysis of citrulline, arginine, and methylargininines using high-performance liquid chromatography. Methods Enzymol. 440:177-189. doi: 10.1016/S0076-6879(07)00810-5.

Wu, G., C. J. Meininger, C. J. McNeal, F. W. Bazer, and J. M. Rhoads. 2021. Role of L-arginine in nitric oxide synthesis and health in humans. Adv. Exp. Med. Biol. 1332:167-187.

Wu, G., D. A. Knabe, and N. E. Flynn. 1994. Synthesis of citrulline from glutamine in pig enterocytes. Biochem. J. 299:115-121. doi: 10.1042/bj2990115

Wu, G., D.A. Knabe, W. Yan, and N.E. Flynn. 1995. Glutamine and glucose metabolism in enterocytes of the neonatal pig. Am. J. Physiol. 268:R334-R342. doi: 10.1152/ajpregu.1995.268.2.R334.

Wu, G., F. W. Bazer, G. A. Johnson, and Y. Q. Hou. 2018. Arginine nutrition and metabolism in growing, gestating and lactating swine. J. Anim. Sci. 96:5035-5051. doi: 10.1093/jas/sky377.

Wu, G., F. W. Bazer, M. C. Satterfield, K. R. Gilbreath, E. A. Posey, and Y. X. Sun. 2022. L-Arginine nutrition and metabolism in ruminants. Adv. Exp. Med. Biol. 1354:177-206.

Wu, G., F. W. Bazer, M. C. Satterfield, X. Li, X. Wang, G. A. Johnson, R. C. Burghardt, Z. Dai, J. Wang, and Z. Wu. 2013. Impacts of arginine nutrition on embryonic and fetal development in mammals. Amino Acids 45(2):241-256. doi: 10.1007/s00726-013-1515-z

Wu, G., F. W. Bazer, T. A. Davis, S. W. Kim, P. Li, J. Marc Rhoads, M. C. Satterfield, S. B. Smith, T. E. Spencer, and Y. Yin. 2009. Arginine metabolism and nutrition in growth, health and disease. Amino Acids 37(1):153-168. doi: 10.1007/s00726-008-0210-y

Wu, G., F.W. Bazer, J.M. Wallace, and T.E. Spencer. 2006. Board invited review. Intrauterine growth retardation: implications for the animal sciences. *J. Anim. Sci*. 84:2316- 2337.

Wu, G., F.W. Bazer, T. Davis, S. Kim, P. Li, J. Marc Rhoads, Y. Yin. 2009. Arginine metabolism and nutrition in growth, health and disease. *Amino Acids*, 37(1), 153-168.

Wu, G., M.-J. Lee, and S. K. Fried. 2007. The arginine-NO pathway modulates lipolysis in adipose tissues of obese human subjects. The FASEB Journal 21(6):A1052-A1052. doi: https://doi.org/10.1096/fasebj.21.6.A1052-b

Wu, G., P. K. Davis, N. E. Flynn, D. A. Knabe, and J. T. Davidson. 1997. Endogenous synthesis of arginine plays an important role in maintaining arginine homeostasis in postweaning growing pigs. J. Nutr. 127(12):2342-2349. doi: 10.1093/jn/127.12.2342.

Wu, X., Y.L. Yin, Y.Q. Liu, X.D. Liu, Z.Q. Liu, T.J. Li, R.L. Huang, Z. Ruan, Z.Y. Deng. 2012. Effect of dietary arginine and N-carbamoylglutamate supplementation on reproduction and gene expression of *eNOS, VEGFA* and *PlGF1* in placenta in late pregnancy of sows, *Animal Reproduction Science*, 132:187-192.

Young, M., W. R. Allen, and N. E. P. Deutz. 2003. Free amino-acid concentrations in the equine placenta: relationship to maternal and fetal plasma concentrations. Research in Veterinary Science 74(3):279-281. doi: https://doi.org/10.1016/S0034-5288(02)00193-5

Zhu, C., X. L. Li, F. W. Bazer, G. A. Johnson, R. C. Burghardt, Z. Y. Jiang, and G. Wu. 2021. Dietary L-arginine supplementation during days 14-25 of gestation enhances aquaporin expression in the placentae and endometria of gestating gilts. Amino Acids 53:1287-1295. doi: 10.1007/s00726-021-03038-z.

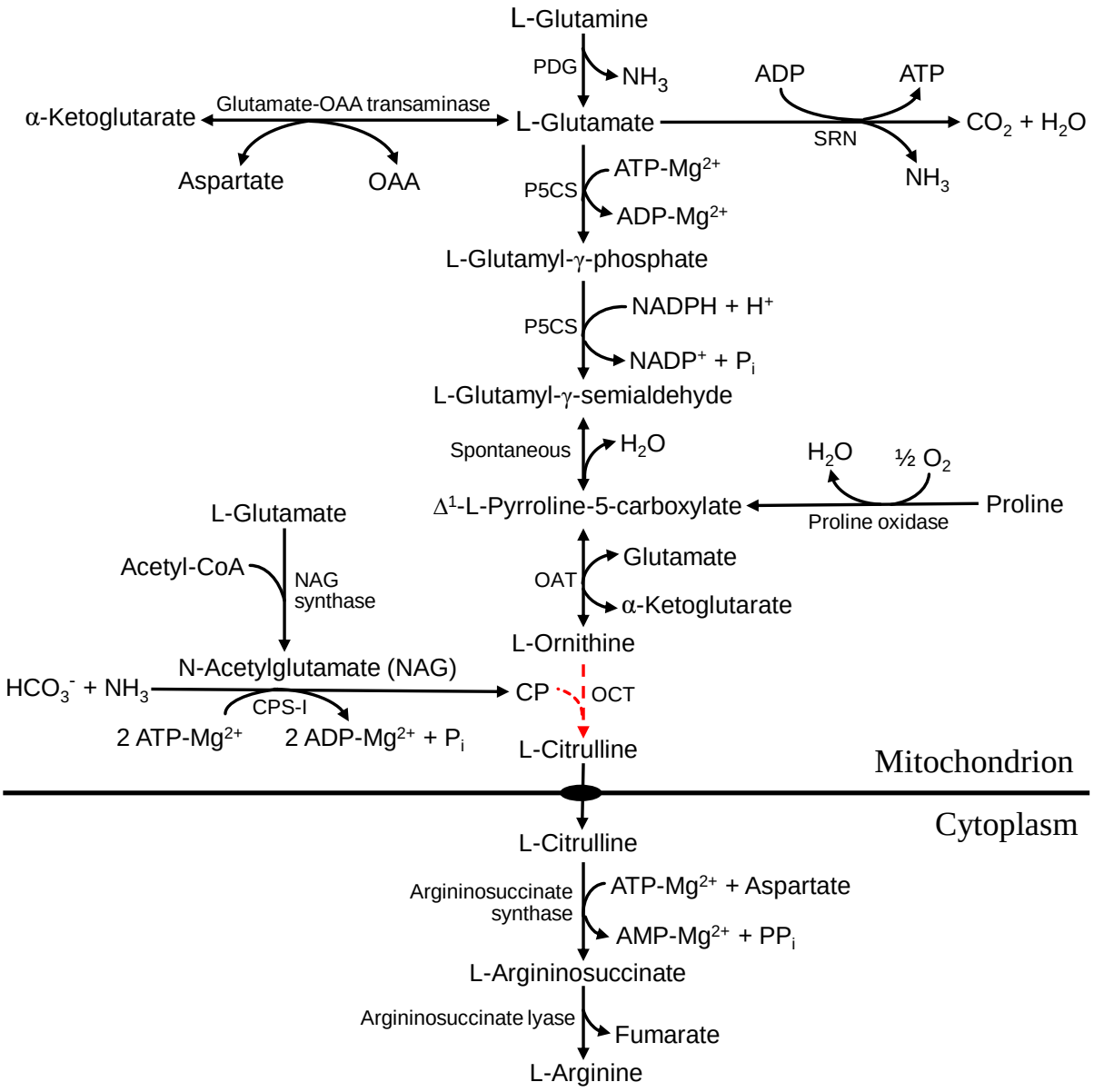

Figure 1. Synthesis of ornithine from glutamine and ornithine in enterocytes of young and adult horses. The conversion of glutamine, glutamate, and proline into ornithine occurs in the mitochondria of enterocytes. However, equine enterocytes do not form citrulline from ornithine. In the cytosol of all mammalian cells, citrulline can be converted into arginine. CP, carbamoyl phosphate; CPS-I, carbamoylphosphate synthetase I; OAA, oxaloacetate; OAT, ornithine aminotransferase; OCT, ornithine carbamoyltransferase; PDG, phosphate-activated glutaminase; P5CS, Δ^1-pyrroline-5-carboxylate synthase (a bifunctional enzyme); and SRN, a series of enzyme-catalyzed reactions. N-Acetylglutamate is the allosteric activator of CPS-I.

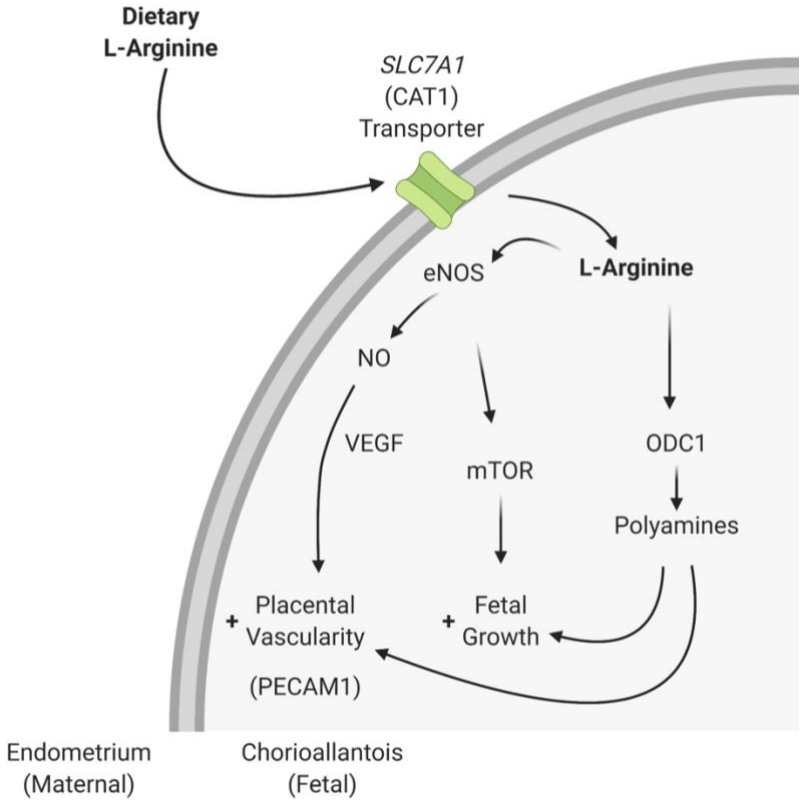

Figure 2. High affinity cationic amino acid transporter 1 (*SLC7A1* also known as CAT1) demonstrates the intracellular transport of L-arginine of maternal origin to fetal membranes. Providing the necessary precursor for the biological products necessary for placental growth and vascularity (PECAM1), fetal development, and growth *(*NO, VEGF, ODC1 and mTOR).

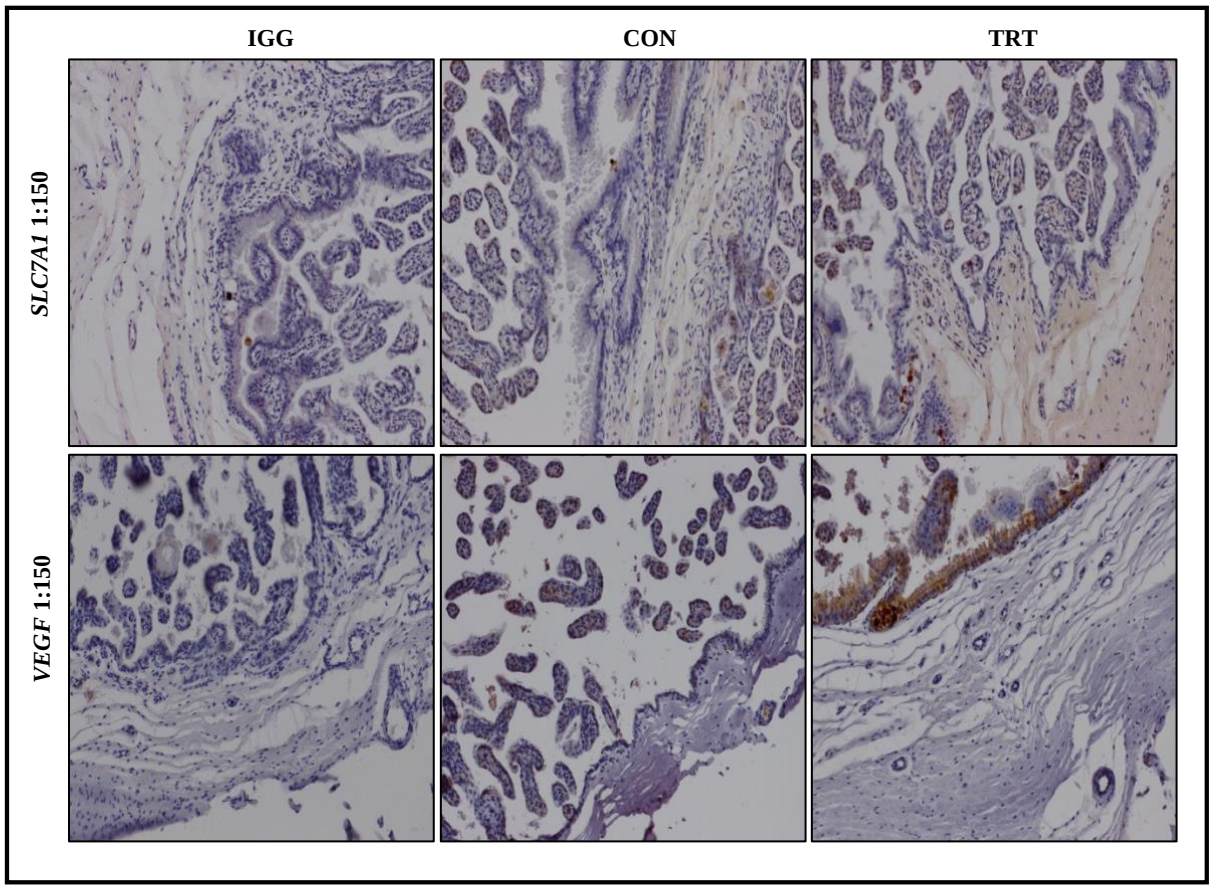

Figure 3. Histological sections (10x) of the pregnant horn stained with antibodies of interest *SLC7A1* & *VEGF* and a haemotoxylin counter stain. *SLC7A1* staining intensity can be localized to the columnar trophoblast cells, microcotyledons, of the chorion as well as the fetal connective tissue that surrounds the fetal vascular network. The *VEGF* staining intensity can be localized to the columnar trophoblast cells, microcotyledons of the chorion. IgG negative control images are provided on the left side

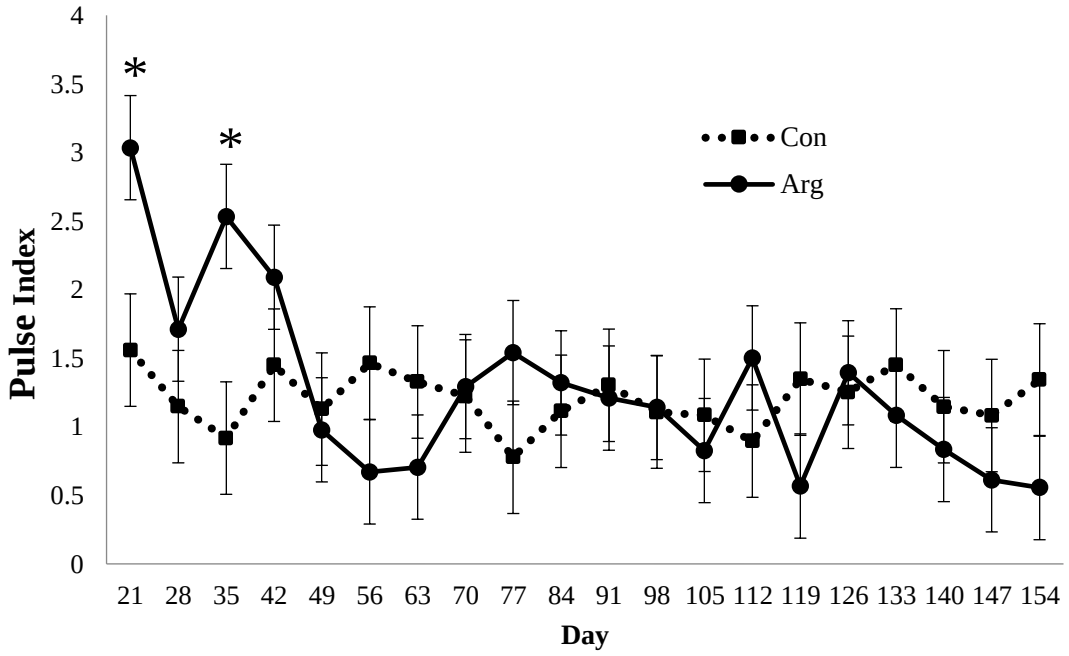

Figure 4. Pulse Index (PI) of gravid uterine artery (GUA) in aged mares supplemented with either 50 mg/kg BW of arginine (treatment; Arg) per d, or the same diet supplemented with 100 mg/kg BW of L-alanine to achieve isonitrogenous diets (control; Con). Main effects included diet ($P = 0.82$), day ($P \leq 0.01$), and diet × day ($P = 0.08$). * Within time point, RI tends to differ between diets.

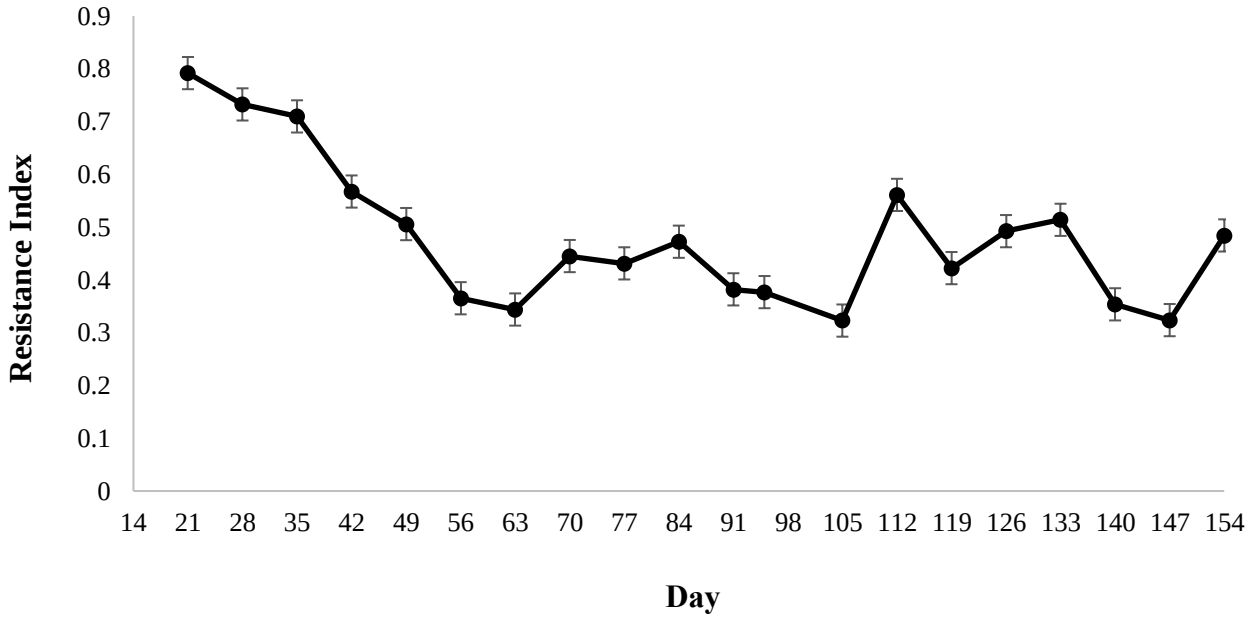

Figure 5. Resistance Index (RI) of gravid uterine artery (GUA) in aged mares supplemented with either 50 mg/kg BW of arginine (treatment; ARG) per d, or the same diet supplemented with 100 mg/kg BW of L-alanine to achieve isonitrogenous diets (control; CON) for the initial 154 of gestation. Main effects included diet ($P = 0.29$), day ($P \leq 0.01$), and diet × day ($P = 0.44$).

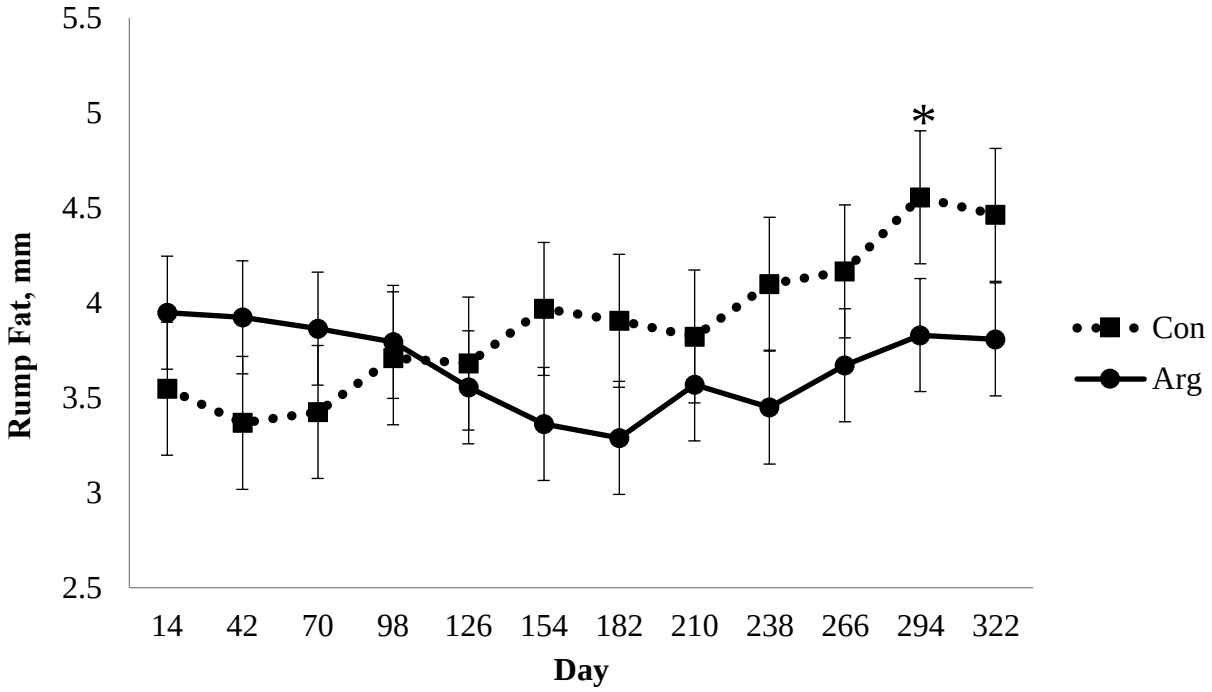

Figure 6. Rump fat thickness of aged mares supplemented with either 50 mg/kg BW of arginine (treatment; ARG) per d, or the same diet supplemented with 100 mg/kg BW of L-alanine (n=6) to achieve isonitrogenous diets (control; CON) throughout gestation. Main effects included diet ($P = 0.52$), day ($P = 0.34$), and diet × day ($P = 0.03$). * Within time point, refers to a dietary difference ($P < 0.05$).

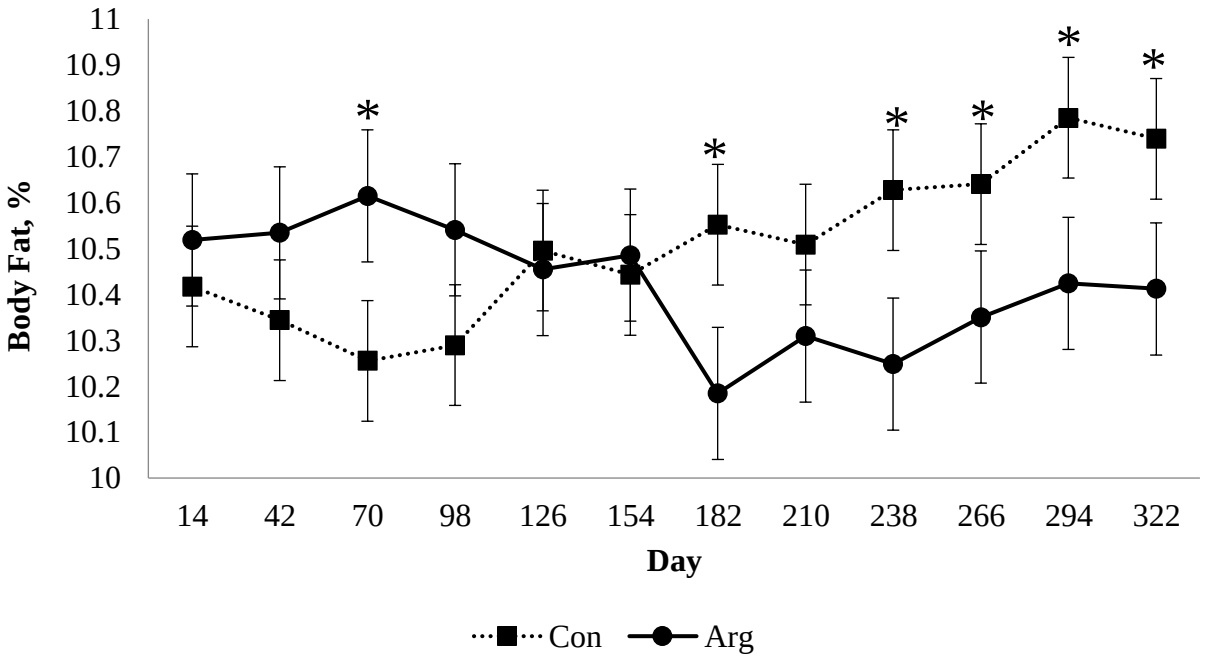

Figure 7. Body fat % of aged mares supplemented with either 50 mg/kg BW of arginine (treatment; ARG) per d, or the same diet supplemented with 100 mg/kg BW of L-alanine to achieve isonitrogenous diets (control; CON) throughout gestation. Main effects included diet ($P = 0.13$), day ($P = 0.93$), and diet × day ($P = 0.03$). * Within time point, refers to a dietary difference ($P < 0.05$).

Study	% CP basal diet	% Arginine basal diet	Arginine supplementation rate
Chavatte-Palmer et al., 2018	*	*	100 g/d
Köhne et al., 2018	*	42 g/meal	0.0125% BW
Mesa et al., 2015	16%	*	0.50% of diet
Kelley et al., 2014	16%	*	0.025% BW
Kelley et al., 2014	16%	*	0.0125% of BW
Mortensen et al., 2011	16%	*	100 g/d

Table 1. Reported levels of arginine previously supplemented to horses
*Values not reported

Table 2. Age, sex, bodyweight (kg), and body condition score (BCS) of horses

Group	**Age**[1]	**Sex**	**BW**[1]	**BCS**[3]
Neonates	7.54 ± 2.36 d	3 colts, 1 filly	ND[2]	ND[2]
Adults	6.2 ± 0.67 yr	3 geldings, 6 mares	502.2 ± 22.3	6.0 ± 0.53
Geriatrics	22.9 ± 3.0 yr	1 gelding, 5 mares	424.0 ± 71.5	4.58 ± 0.36

[1]Values (mean ± SEM)
[2]ND = data not obtained
[3]Obtained using the 1 to 9 scale (Henneke et al., 1983)

Table 3. Production of $^{14}CO_2$ by equine enterocytes incubated for 30-min with 2 mM L-[U-^{14}C]glutamine or 2 mM L-[U-^{14}C]proline plus 2 mM L-glutamine

	Age Groups[1]			
Incubation Medium	Neonates (*n*=4)	Adults (*n*=9)	Aged (*n*=6)	*P*-value
2 mM L-[U-^{14}C]Gln	81.1 ± 25.6[a]	2.47 ± 0.67[c]	7.07 ± 0.86[b]	0.01
2 mM L-[U-^{14}C]Pro + Gln	ND[2]	ND	ND	---

[1]Values (mean ± SEM) expressed as nmol $CO_2/10^6$ cells/30 min

[2]ND = not detected

[a-c]Within a row, means not sharing the same superscript letters are different ($P < 0.05$), as analyzed by one-way ANOVA for baseline-normalized data, followed by the Student-Newman-Keuls multiple comparison test

Table 4. Concentrations of arginine, citrulline, and ornithine in incubation medium plus cell extracts of equine enterocytes before and after 30-min incubation period

		30-min incubation			
Age Groups[1]	No Incubation	0 mM	2 mM Gln	2 mM Pro + 2 mM Gln	*P*-value
			Arginine		
Neonates (n=4)	6.44 ± 0.55[b]	13.91 ± 2.55[a]	14.17 ± 3.11[a]	14.56 ± 3.05[a]	< 0.01
Adults (n=9)	7.64 ± 1.42[b]	13.10 ± 2.50[a]	12.71 ± 2.35[a]	13.17 ± 2.56[a]	< 0.01
Geriatrics (n=6)	5.51 ± 0.86[b]	9.78 ± 2.44[a]	10.23± 2.90[a]	9.91 ± 2.53[a]	< 0.01
			Citrulline		
Neonates (n=4)	0.72 ± 0.19	0.84 ± 0.19	0.76 ± 0.22	0.90 ± 0.13	0.88
Adults (n=9)	0.86 ± 0.26	0.92 ± 0.22	0.90 ± 0.19	0.95 ± 0.23	0.85
Geriatrics (n=6)	0.67 ± 0.06	0.77 ± 0.12	0.87 ± 0.07	0.81 ± 0.10	0.91
			Ornithine		
Neonates (n=4)	5.24 ± 1.10[d]	7.75 ± 1.64[c]	11.84 ± 2.08[b]	15.1 ± 0.98[a]	< 0.01
Adults (n=9)	0.23 ± 0.04[d]	0.47 ± 0.09[c]	0.87 ± 0.13[b]	1.34 ± 0.12[a]	< 0.01
Geriatrics (n=6)	1.88 ± 0.62[d]	2.69 ± 0.93[c]	4.38± 0.91[b]	6.78 ± 1.65[a]	< 0.01

[1]Values (mean ± SEM) expressed as nmol/10^6 cells

[a-d] Within a row, means not sharing the same superscript letters are different ($P < 0.05$), as analyzed by one-way ANOVA for baseline-normalized data, followed by the Student-Newman-Keuls multiple comparison test

Table 5. Concentrations of arginine, citrulline, and ornithine in incubation medium plus cell extracts of enterocytes from 7-day-old pigs (*n*=6) before and after 30-min incubation with 2 mM L-[U-^{14}C]glutamine, and production of $^{14}CO_2$ from 2 mM L-[U-^{14}C]glutamine

Variable	No incubation	30-min incubation	
		0 mM Gln	2 mM Gln
Amino acid in incubation medium plus cell extracts (mean ± SEM; nmol/10^6 cells)			
Arginine	1.05 ± 0.05^{c}	2.19 ± 0.10^{b}	3.47 ± 0.15^{a}
Citrulline	0.42 ± 0.02^{c}	0.61 ± 0.03^{b}	1.85 ± 0.08^{a}
Ornithine	0.15 ± 0.01^{c}	0.22 ± 0.02^{b}	0.36 ± 0.01^{a}
Production of $^{14}CO_2$ (nmol/10^6 cells/30 min)	---	---	113.3 ± 6.9

[a-c]Within a row, means not sharing the same superscript letters are different ($P < 0.05$), as analyzed by one-way ANOVA for baseline-normalized data, followed by the Student-Newman-Keuls multiple comparison test

Table 6. Analysis of nutrient composition of commercial concentrate and Coastal bermudagrass (*Cynodon dactylon*) hay offered to pregnant mares

Nutrient[1]	**Concentrate**[2]	**Coastal bermudagrass hay**[3]
DE, Mcal/kg	2.91	1.66
CP, %	15.90	9.30
Crude Fat, %	7.40	0.90
NDF, %	33.30	69.00
ADF, %	19.30	42.50
Starch, %	23.80	3.60
Ca, %	1.32	0.49
P, %	0.83	0.14
K, %	1.16	0.97
Mg, %	0.39	0.12
Na, %	0.425	0.01
Cl, %	0.73	0.19
S, %	0.25	0.24
Co, ppm	1.77	2.32
Fe, ppm	268.00	598.00
Zn, ppm	208.00	38.00
Cu, ppm	45.00	13.00
Mn, ppm	163.00	44.00

[1] Values presented on a 100% DM basis
[2] Concentrate = basal grain diet fed to all horses at 1.18% - 1.25% BW (as-fed basis) per d
[3] Coastal bermudagrass (*Cynodon dactylon*) hay was offered *ad libitum* to all pregnant mares

Table 7. Amino Acid composition of commercial concentrate and Coastal bermudagrass (*Cynodon dactylon*) hay offered to pregnant mares as determined by HPLC analysis

Amino Acid[1]	**Concentrate**[2]	**Coastal bermudagrass hay**[3]
Aspartate + Asparagine	7.92	5.89
Glutamate + Glutamine	18.21	5.68
Serine	5.11	2.27
Histidine	2.63	0.61
Glycine	4.73	2.97
Threonine	3.72	2.30
Arginine	5.50	2.33
Alanine	6.20	3.08
Tyrosine	3.85	2.28
Methionine	1.59	0.31
Valine	5.16	2.94
Phenylalanine	4.47	2.48
Isoleucine	3.79	2.25
Leucine	8.42	4.02
Lysine	8.42	7.82

[1] Values presented on a g/kg of diet (as-fed basis)
[2] Concentrate = basal grain diet fed to all horses at 1.18% - 1.25% BW (as-fed basis) per d
[3] Coastal bermudagrass (*Cynodon dactylon*) hay was offered *ad libitum* to all horses

Table 8. Effect of arginine[1] supplementation to aged pregnant mares on mare performance d 14 to 154 d of gestation

	Diet		*P*-values		
Mare Performance[3]	Con[2] (*n*=6)	Arg[1] (*n*=7)	Diet	Day	Diet × Day
Body Weight, kg	516.10 ± 8.10	514.03 ± 7.5	0.80	0.30	0.97
BCS[4]	5.94 ± 0.12[a]	5.25 ± 0.11[b]	<0.01	0.76	0.96
Rump Fat, mm	3.84 ± 0.12	3.88 ± 0.11	0.81	0.99	0.87
Body Fat, %[6]	10.37 ± 0.06	10.52 ± 0.05	0.05	0.99	0.70
Resistance Index[5]	0.47 ± 0.03	0.51 ± 0.03	0.29	<0.01	0.44
Pulse Index[5]	1.29 ± 0.09	1.32 ± 0.08	0.82	<0.01	0.08

[1]Arginine supplementation at 50 mg/kg BW/d
[2]Alanine supplementation at 100 mg/kg BW/d
[3]Values (mean ± SEM)
[4]Obtained using the 1 to 9 scale (Henneke et al., 1983)
[5]Blood flow measurements obtained as pulsatility index (PI) and resistance index (RI) from uterine artery ipsilateral to pregnant uterine horn; defined gravid uterine artery
[6]% Body fat predicted by rump fat thickness
[a,b] indicate differences ($P \leq 0.05$) in dietary treatment

Table 9. Effect of arginine[1] supplementation to aged pregnant mares on mare performance from d 14 to parturition

	Diet		*P*-values		
Mare Performance[3]	Con[2] (*n*=6)	Arg[1] (*n*=7)	Diet	Day	Diet × Day
Body Weight, kg	539.10 ± 18.31	529.72 ± 15.84	0.7	<0.01	0.30
BCS[4]	5.55 ± 0.24	4.83 ± 0.20	0.04	<0.01	0.07
Rump Fat, mm	3.89 ± 0.26	3.67 ± 0.21	0.52	0.34	0.03
Body Fat, %[5]	10.51 ± 0.04	10.42 ± 0.03	0.13	0.93	0.03

[1]Arginine supplementation at 50 mg/kg BW/d
[2]Alanine supplementation at 100 mg/kg BW/d
[3]Values (mean ± SEM)
[4]Obtained using the 1 to 9 scale (Henneke et al., 1983)
[5]% Body fat predicted by rump fat thickness

Table 10. Effect of arginine[1] supplementation to aged pregnant mares on mare performance at parturition

	Diet		
Foaling Performance[3]	Con[2] (*n*=4)	Arg[1] (*n*=5)	*P*-values
Gestating Days	337.24 ± 5.67	338.80 ± 5.08	0.84
Pre-partum BW, kg	554.73 ± 19.57	547.40 ± 17.79	0.79
Post-partum BW, kg	490.73 ± 19.76	499.13 ± 17.68	0.74
BCS[4]	5.94 ± 0.11[a]	5.25 ± 0.10[b]	<0.01
Rump Fat, mm	4.23 ± 0.30	4.05 ± 0.27	0.67
Body Fat, %[6]	10.63 ± 0.13	10.56 ± 0.11	0.70
Pulse Index[4]	2.77 ± 0.33	2.2 ± 0.29	0.25
Resistance Index[4]	1.22 ± 0.09[a]	0.89 ± 0.08[b]	0.01

[1]Arginine supplementation at 50 mg/kg BW/d
[2]Alanine supplementation at 100 mg/kg BW/d
[3]Values (mean ± SEM)
[4]Obtained using the 1 to 9 scale (Henneke et al., 1983)
[5]Blood flow measurements obtained as pulsatility index (PI) and resistance index (RI) from uterine artery ipsilateral to pregnant uterine horn; defined gravid uterine artery (GUA) prior to parturition
[6] % Body fat predicted by rump fat thickness
[a,b] indicate differences ($P \leq 0.05$) in dietary treatment.

Table 11. Effect of arginine[1] supplementation to aged pregnant mares on foal performance at parturition and at 12 h post-parturition

	Diet		
Foaling Variables[3]	Con[2] (*n*=4)	Arg[1] (*n*=5)	*P*-values
Pre-Suckle BW kg	40.59 ± 3.35	40.55 ± 2.99	0.99
12 h BW kg	42.86 ± 3.62	42.37 ± 3.24	0.92
Wither Height cm	94.77 ± 2.07	97.00 ± 2.07	0.47
Hip Height cm	94.53 ± 2.72	97.3 ± 2.72	0.52
Body Length cm	71.05 ± 2.96	70.13 ± 2.96	0.76
Colostrum[4]	23.00 ± 1.66	25.2 ± 1.48	0.35
Standing Time, min	37.00 ± 3.67	32.40 ± 3.28	0.38
Placental Expulsion, min	66.50 ± 13.04	44.2 ± 11.6	0.24

[1]Arginine supplementation at 50 mg/kg BW/d
[2]Alanine supplementation at 100 mg/kg BW/d
[3]Values (mean ± SEM)
[4]Brix Refractometer Scale (0-32%)

Table 12 Effect of arginine[1] supplementation to aged pregnant mares on placental performance at term

	Diet		
Placental Variables[3]	Con[2] (*n*=4)	Arg[1] (*n*=5)	*P*-values
Placental Weight, kg	4.13 ± 0.32	4.39 ± 0.28	0.56
Placental Volume, mL	4000.00 ± 342.06	4125.00 ± 305.95	0.40
Umbilical Length, cm	62.55 ± 5.07	69.58 ± 4.55	0.34
VEGF[4]	7.57 ± 2.01	10.06 ± 1.74	0.39
SLC7A1[4]	1.37 ± 1.25	3.63 ± 1.08	0.23

[1]Arginine supplementation at 50 mg/kg BW/d
[2]Alanine supplementation at 100 mg/kg BW/d
[3]Values (mean ± SEM)
[4]Data obtained following semiquantitative analyses were performed using 10 non-overlapping images per sample fixed in a 10X field (Fiji ImageJ v1.2) SLC7A1 - High affinity cationic amino acid transporter 1 of lysine and arginine , VEGF - Vascular Endothelial Growth Factor

APPENDIX A

Responses to an intra-articular lipopolysaccharide challenge following dietary supplementation of *Saccharomyces cerevisiae* fermentation product in young horses[*]

R. E. Martinez*[1], J. L. Leatherwood*, C. E. Arnold†, K. G. Glass†, K. W. Walter[¶], H. C. Valigura*, S. A. Norton[#], and S. H. White-Springer*

*Department of Animal Science, Texas A&M University and Texas A&M AgriLife Research, College Station, TX USA 77843; †Department of Large Animal Clinical Sciences, Texas A&M University, College Station, TX USA 77843; ¶Department of Agricultural Science, Truman State University, Kirksville, MO USA 63501; [#]Diamond V Mills, Inc. Cedar Rapids, IA USA 52404

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ABSTRACT: Dietary intervention may be a valuable strategy to optimize the intra-articular environment in young horses to prolong their performance career. To test the hypothesis that dietary supplementation of a *Saccharomyces cerevisiae* fermentation product would reduce markers of joint inflammation and increase markers of cartilage metabolism following a single inflammatory insult, Quarter Horse yearlings (mean ± SD; 9 ± 1.0 mo) were balanced by age, sex, body weight (BW), and farm of origin and randomly assigned to: 1.25% BW/d (dry matter basis) custom-formulated concentrate only (CON; n = 9) or concentrate top dressed with 21 g/d *Saccharomyces cerevisiae* fermentation product (SCFP; n = 10) for 98 d. Horses had *ad libitum* access to Coastal bermudagrass hay. On d 84, one randomly selected radial carpal joint from each horse was injected with 0.5 ng lipopolysaccharide solution (LPS). The remaining carpal joint was injected with sterile lactated Ringer's solution as a contralateral control. Synovial fluid obtained before supplementation (d 0) and on d 84 at pre-injection h 0, and 6, 12, 24, 168, and 336 h post-injection was analyzed for prostaglandin E_2 (PGE_2), carboxypeptide of type II collagen (CPII), and collagenase cleavage neopeptide (C2C) by commercial assays. Rectal temperature, heart rate, respiration rate, carpal surface temperature, and carpal circumference (CC) were recorded prior to each sample collection and for 24 h post-injection. Data were analyzed using linear models with repeated measures. From d 0 to 84, synovial C2C declined ($P \leq 0.01$) and the CPII:C2C ratio increased ($P \leq 0.01$) in all horses with no effect of diet. In response to intra-articular LPS, synovial PGE_2 increased by h 6 ($P \leq 0.01$) and returned to baseline by h 336, CPII increased by h 12, remained elevated through h 168 ($P \leq 0.01$), and returned to baseline by h 336, and C2C increased by h 6 ($P \leq 0.01$) but did not return to baseline through h 336 ($P \leq 0.01$). Post-intra-articular injection, PGE_2 levels were lower in SCFP than CON horses ($P = 0.01$) regardless of injection type. Synovial CPII and the CPII:C2C ratio

demonstrated stability during the LPS challenge in SCFP compared to CON horses ($P \leq 0.01$). Clinical parameters were not influenced by diet but increased in response to repeated arthrocentesis ($P \leq 0.01$). Dietary SCFP may favorably modulate intra-articular inflammation following an acute stressor and influence cartilage turnover in young horses.

Key words: cartilage, equine, lipopolysaccharide, *saccharomyces cerevisiae*, synovial

ABBREVIATIONS

BCS – body condition score

BW – body weight

CC – carpal circumference

CT – carpal surface temperature

C2C – collagenase cleavage neopeptide

CPII – carboxypropeptide of type II collagen

DM – dry matter

ELISA – enzyme linked immunosorbent assay

HR – heart rate

LPS – lipopolysaccharide

LRS – lactated Ringer's solution

OA – osteoarthritis

PGE_2 - prostaglandin E_2

PIH - pre-injection h

RR – respiratory rate

RT – rectal temperature

INTRODUCTION

Inflammatory joint disorders are a common cause of lameness and a leading cause for early retirement and loss of athletic performance in young horses (Brama et al., 2000b). Once initial injury or degradation of cartilage occurs, inflammation results as an attempt to stop the damage. In the short term, this process is designed to limit the use of the joint and promote healing. However, with continuous exercise and damage, excessive cytokines and eicosanoids may be released that could result in chronic inflammation and pain, and elicit degradation of articular cartilage (Palmer and Bertone, 1994).

Inflammation of the synovial membrane, or synovitis, is a common consequence of repeated trauma and stress to the joint. Recently, interest has been placed on dietary mitigation of systemic inflammation through gut modulation (Hernández et al., 2017). It is suggested that an alteration to the microbiome's microenvironment through dietary manipulation may improve the host's overall immunological response. Furthermore, the microbial composition of the gut has an intrinsic role in the development of the immune system, nutritional efficiency, storage of adipose tissue, and behavior (Sekirov et al., 2010; Cryan and O'Mahony, 2011). An imbalance in the microbial population, or dysbiosis, is now recognized to have an extensive influence on the development of several metabolic diseases and systemic inflammation (Hand et al., 2016), which may be associated with joint disease (Szychlinska et al., 2019). Therefore, a modification to the diet, and consequently, to the microbiome's microenvironment, has the potential mitigate the immunological response of extended joint inflammation (Collins et al., 2015).

An example of such a dietary component is *Saccharomyces cerevisiae* fermentation product. Dietary supplementation of *Saccharomyces cerevisiae* fermentation product decreased plasma haptoglobin concentrations, a biological marker that increases following an inflammatory

response, in lactating Holstein dairy cows following a subacute ruminal acidosis challenge (Guo et al., 2017). Similarly, the paws of *Saccharomyces cerevisiae* fermentation product supplemented rats produced lesser prostaglandin E_2 (**PGE_2**), following an inflammatory insult compared to un-supplemented controls (Evans et al., 2012). Finally, adult horses supplemented with *Saccharomyces cerevisiae* fermentation product had lower white blood cell counts compared to non-supplemented controls following transport (Faubladier et al., 2013). The reduction of biological markers related to inflammation demonstrates the potential for *Saccharomyces cerevisiae* fermentation product supplementation to attenuate an intra-articular inflammatory response.

Measuring the effectiveness of dietary interventions related to joint health is challenging in young horses, as cartilage responds to both growth and exercise (Firth, 2006). Intra-articular lipopolysaccharide (**LPS**) injection is an established model for inducing acute localized inflammation in the young horse (de Grauw et al., 2009; Lucia et al., 2013). Following LPS injection, synovial concentrations of PGE_2, an eicosanoid indicative of naturally occurring arthritis (Bertone et al., 2001), increases in young and mature horses (Kahn et al., 2017). Similarly, biomarkers of cartilage metabolism, including anabolic carboxypropeptide of type II collagen (**CPII**) and catabolic collagenase cleavage neopeptide (**C2C**), also increase as a result of localized inflammation from LPS (de Grauw et al., 2006). The objective of the current study was to evaluate the effect of *Saccharomyces cerevisiae* fermentation product supplementation on markers of cartilage metabolism and joint inflammation in response to an intra-articular LPS challenge in young horses. Authors hypothesized that horses receiving *Saccharomyces cerevisiae* fermentation product would have reduced intra-articular inflammation and increased cartilage

metabolism markers compared to non-supplemented horses following the intra-articular LPS challenge.

MATERIALS AND METHODS

All care, handling, and sampling of horses was reviewed and approved by the Institutional Animal Care and Use Committee at Texas A&M University (2016-0294).

Horses and Management

Nineteen Quarter Horses (11 fillies and 8 colts) entering their yearling year (mean ± SD; 9 ± 1 mo of age; initial body weight (**BW**) 280 ± 31 kg) were utilized in a randomized complete design for a 98-d study. Yearlings originated from two sources: Texas A&M University (College Station, TX; n = 8) and Birdsong Farms (Hearne, TX; n = 11). Horses arrived 30 d prior to the start of the study in order to centralize housing and standardize diet. Horses were randomly assigned to treatment groups that were balanced by age, sex, BW, and farm of origin. Treatments consisted of: control (**CON**; basal diet with no supplementation, n = 9) or *Saccharomyces cerevisiae* fermentation product supplementation (**SCFP**; basal diet + 21 g/d Original XPC, Diamond V Mills, Inc.; Cedar Rapids, IA; n = 10); the SCFP was top-dressed onto the concentrate immediately prior to each feeding (10.5 g/feeding).

The basal diet was formulated to meet or exceed the requirements of growing horses (NRC, 2007). Horses were offered 1.25% BW/d on a dry matter (DM) basis of a custom formulated pelleted concentrate that was free of *Saccharomyces cerevisiae* fermentation product and was split into two equal meals per day. Horses were placed into individual stalls (3.2 x 3.2 m) and allowed 1 h to consume their respective dietary treatments at 12 h intervals. Horses were group-housed in dry lots (58.7 × 79.2 m) and had free-choice access to Coastal bermudagrass hay (*Cynodon dactylon*) in the form of round bales.

Every 14 d, BW was obtained utilizing a calibrated platform scale (Bastrop Scale Inc., Bastrop, TX) and concentrate intake was adjusted accordingly. Body condition scores (**BCS**) were also obtained by three independent observers using the 1 to 9 scale described by Henneke et al. (1983). Composited hay and grain samples were analyzed by a commercial laboratory (Equi-Analytical Laboratories, Ithaca, NY) for nutrient composition (Table 1). On d 56, all horses performed a 2-h submaximal exercise test for a related study (Valigura et al., 2021) to evaluate the effects of SCFP supplementation on markers of exercise-induced stress and inflammation. This exercise test represented the only forced exercise that horses received for the duration of the study.

Intra-articular LPS Challenge

On d 84, all horses underwent an intra-articular LPS challenge as previously described (Lucia et al., 2013). Briefly, carpal joints for each horse were randomly assigned one of two colors. A pharmacist at the Texas A&M Veterinary Hospital prepared sterile lactated Ringer's solution (**LRS**) and 0.5 ng LPS derived from *Escherichia coli* O55:B5 (Sigma-Aldrich, St. Louis, MO) diluted in LRS using aseptic techniques. The pharmacist assigned each solution a color, which corresponded to the color-coded carpal joints, allowing investigators to remain blinded to treatments until completion of data analysis. Horses were first sedated with xylazine HCL (Bimeda-MC Animal Health Inc., Cambridge, Ontario, Canada) that was administered intravenously at recommended doses (0.5 mg/kg BW) then carpal arthrocentesis was hygienically performed by a veterinarian from the Texas A&M University Large Animal Clinic.

A volume of 0.8 mL of either LRS or LPS was injected into each carpal joint (following color coding). The LRS-injected joint served as a contralateral control. For sampling consistency, ease of collection, and to obtain the volume of fluid required, carpal joints were

aseptically prepared and then aspirated utilizing a location medial to the extensor carpi radialis tendon in the palpable depression between the radial carpal bone and the third carpal bone, to a depth of approximately 12.7 mm to avoid unnecessary contact with articular cartilage (McIlwraith and Trotter, 1996). Throughout the first 24 h post-injection, all horses were closely monitored in individual 3.2 × 3.2 m stalls. During that time, rectal temperature (**RT**), heart rate (**HR**), and respiratory rate (**RR**) were recorded at pre-injection h (**PIH**) 0, and 6, 12, and 24 h post-injection. Carpal circumference (**CC**) was also determined (cm) at these time points at the level of the accessory carpal bone utilizing a soft tape measure, and surface temperature (**CT**) of the dorsal surface of each carpal joint was obtained at the same location from a distance of 1.2 m by the use of an infrared camera (FLIR E60 Series, Flir Systems Inc., Wilsonville, OR). Immediately prior to imaging, the dorsal surface of the carpus was cleaned using a dry brush to remove any particulate from the joint surface.

Images were analyzed in duplicate by a certified thermographer (Infrared Training Center, Nashua, NH; certificate no. 81467) using the FLIR Tools software (Flir Tools 2.0). For each image, emissivity was set to 0.95, and ambient temperature and humidity were entered. Additionally, reflected apparent temperature was accounted for on each image using a diffuse reflector (International Organization for Standardization ISO 18434-1:2006 and ASTM E1862-97(2002)e1, Standard Test Methods for Measuring and Compensating for Reflected Temperature Using Infrared Imaging Radiometers, ASTM International, West Conshohocken, PA, 1997). The ellipse measurement tool was then used to evaluate the average temperature of the entire carpal surface. Average ambient temperature and humidy were 22°C and 70%, 27°C and 64%, 29°C and 53%, and 23°C and 66% at PIH 0, and 6, 12, and 24 h post-injection, respectively.

Synovial fluid (1 to 4 mL) was obtained at the beginning of the study (d 0), as well as surrounding the LPS challenge on d 84 at PIH 0 and 6, 12, 24, 168 (7 d) and 336 h (14 d) post-injection. Collected synovial fluid was immediately transferred into sterile non-additive tubes (serum blood collection tubes, BD Vacutainer, Kendall Co., Mansfield, MA) then placed on ice for transfer back to the laboratory. Within 2 h of collection, samples were aliquoted and stored at -80°C for later analysis of PGE_2, CPII and C2C.

Sample Analysis

Synovial fluid samples were analyzed in duplicate for concentrations of PGE_2 utilizing a commercially available enzyme linked immunosorbent assay (**ELISA**; R&D Systems, Minneapolis, MN) previously utilized in horses (Bertone et al., 2001; de Grauw et al., 2006) using a plate reader (Synergy H1 Hybrid Multi-Mode Reader, BioTek Instruments, Inc. Winooski, VT). Synovial fluid samples were diluted with provided calibrator diluents 1:1 to 1:10 depending on time post-injection to remain within detectable limits of the assay. Intra-assay precision for PGE_2 ranged between 2.06% and 9.07% and inter-assay precision between 8.27% and 9.80%.

Concentrations of CPII and C2C were determined using commercially available ELISA kits (IBEX Pharmaceuticals Inc., Quebec, Montreal, Canada) previously reported for use in equine synovial fluid (Billinghurst et al., 2001; Frisbie et al., 2008). Synovial fluid samples were diluted 1:4 with assay buffer provided by the manufacturer. Samples were analyzed in duplicate with an intra-assay range CV of 0.10% to 9.84% and an inter-assay CV of 1.38% to 7.96% for CPII and intra-assay range CV of 0.33% to 9.56% and an inter-assay CV of 2.28% to 6.76% for C2C. All markers were read (Synergy H1 Hybrid Multi-Mode Reader) at an optical density of 450 nm.

Statistical Analysis

Data were analyzed using PROC MIXED in SAS v9.4 (SAS Inst., Inc., Cary, NC) with repeated measures (time). The responses to diet (d 0 to PIH 0 on d 84) were analyzed separately from responses to the intra-articular LPS challenge at d 84. Data were tested for normality and outliers were identified using box plots of the residuals and removed if greater than two standard deviations from the mean.

For responses to the pre-LPS dietary adaptation period, the model contained fixed effects of diet, time, and the diet × time interaction, and a random effect of horse(diet). Where d 0 values differed by treatment (PGE_2), d 0 was included in the model as a covariate. Sex and knee were also included in the model as fixed effects but were removed when $P > 0.15$ to conserve degrees of freedom. For responses to the intra-articular LPS challenge at d 84, the model contained fixed effects of diet, injection type, time, and all interactions, and a random statement of horse(diet × injection type). All data are presented as least squares means ± SEM. Significance was declared at $P \leq 0.05$, and $P \leq 0.10$ was considered a trend toward significance.

RESULTS

Responses to Dietary Treatment

There was no influence of dietary treatment on BW or BCS ($P \geq 0.9$); however, all horses, regardless of treatment, gained BW (280 ± 8 to 344 ± 8 kg) and increased BCS (5.3 ± 0.1 to 5.7 ± 0.1) over the 98-d trial ($P \leq 0.01$). During the pre-LPS period, synovial fluid C2C concentrations decreased ($P \leq 0.01$) and the ratio of CPII to C2C increased ($P \leq 0.01$) in all horses from d 0 to 84 but PGE_2 and CPII concentrations remained stable (Table 2). Synovial concentrations of PGE_2, CPII, C2C, and the ratio of CPII to C2C were unaffected by diet or the interaction of diet and time from d 0 to 84 pre-LPS challenge (Table 2).

Responses to the Intra-articular LPS Challenge

Clinical parameters. Throughout the intra-articular LPS challenge, dietary treatment did not influence HR, RR, or RT, and all values remained within normal physiological ranges over the 24-h period. Regardless of diet, HR and RR increased from 0 to 6 h post-injection (P 0.01; Suppl. Fig. 1A and B) and remained elevated at 12 h post-injection (P 0.01). Heart rate returned to pre-injection values by 24 h post-injection (Suppl. Fig. 1A), while RR decreased from 12 to 24 h post-injection ($P \leq 0.01$) but remained greater than pre-injection values at 24 h post-injection ($P = 0.05$; Suppl. Fig. 1B). Conversely, RT was greater at 12 and 24 h compared to 0 and 6 h post-injection (P 0.01; Suppl. Fig. 1C). Neither dietary treatment nor intra-articular injection of LPS affected CC or CT (Suppl. Fig. 2). Following injection, CC increased at 6 h ($P \leq 0.01$) and remained above pre-injection values through 336 h in response to repeated arthrocentesis ($P \leq 0.01$; Suppl. Fig. 2A). At 6 h post LPS injection, CT increased at in all horses and peaked at 12 h post-LPS injection ($P \leq 0.01$) but decreased to levels similar to pre-injection temperatures by 24 h post-injection (Suppl. Fig. 2B).

Acute synovial inflammation. Across dietary treatments, synovial PGE_2 concentrations increased at 6 h post-injection ($P \leq 0.01$; Fig. 1). Synovial PGE_2 then returned to baseline by 12 h post-injection in LRS (contralateral control) knees and by 24 h post-injection in LPS knees. At 6 and 12 h after injection, intra-articular LPS resulted in greater synovial PGE_2 concentrations compared to the contralateral control (LRS) regardless of dietary treatment ($P \leq 0.01$; Fig. 1). However, throughout the challenge, horses receiving SCFP had lesser synovial PGE_2 concentrations compared to CON horses ($P \leq 0.01$), which was particularly evident at 6 h post-injection ($P \leq 0.01$; Fig. 1).

Markers of cartilage metabolism. In response to the LPS injection, synovial concentrations of the cartilage synthesis marker, CPII, increased from 0 to 6 h ($P \leq 0.01$), remained elevated through 168 h post-injection ($P \leq 0.01$) and returned to baseline at 336 h (Fig. 2A). Conversely, in knees that received the LRS injection (contralateral control), CPII concentrations remained unchanged through 24 h, increased at 168 h ($P \leq 0.01$) but returned to baseline by 336 h. At 6, 12, 24, and 168 h, LPS knees had greater CPII concentrations than LRS knees ($P \leq 0.01$). At 24 h post-injection, CPII concentrations in LPS knees of horses supplemented with SCFP were greater than LPS knees of CON horses ($P \leq 0.01$), as well as LRS injected knees regardless of diet ($P \leq 0.01$; Fig. 2A).

Across dietary treatments, the cartilage degradation marker, C2C, increased at 6 h in LPS knees ($P \leq 0.01$) and at 12 h in LRS knees ($P = 0.03$) then remained elevated through 336 h post-injection in both knees of all horses ($P \leq 0.01$; Fig. 2B). Similar to CPII, C2C concentrations were greater in LPS-injected knees compared to the contralateral control knees (LRS) at 6, 12, 24, and 168 h regardless of dietary treatment ($P \leq 0.01$; Fig. 2B).

Throughout the challenge, there was a 3-way interaction of diet, time, and injection type for the ratio of CPII:C2C ($P \leq 0.01$; Fig. 2C). There was no difference in the CPII:C2C ratio between groups at 0 h. In contralateral control knees of horses receiving the CON diet (CON-LRS), the CPII:C2C ratio did not change through 168 h; however, the ratio decreased from 168 to 336 h ($P = 0.01$) to be lower at 336 h than 0 ($P = 0.02$). In LPS knees of CON horses (CON-LPS) and the control knees of SCFP horses (SCFP-LRS), the CPII:C2C ratio increased from 0 to 6 h ($P \leq 0.02$), remained similar from 6 to 12 h, decreased from 12 to 24 h ($P \leq 0.01$), increased from 24 to 168 h ($P \leq 0.01$), then decreased from 168 to 336 h ($P \leq 0.01$) to be lower at 336 than 0 h ($P \leq 0.02$). Finally, the CPII:C2C ratio in LPS knees of SCFP horses (SCFP-LPS)

increased from 0 to 6 h ($P \leq 0.01$), remained stable from 6 through 168 h, then decreased from 168 to 336 h ($P \leq 0.01$) to be the similar at 336 h compared to h 0 (Fig. 2C). These changes over time resulted in the following differences at each time point. At 6, 12, 24, and 168 h, CON-LRS had lesser CPII:C2C than all other groups ($P \leq 0.01$). At 12 and 168 h, CON-LPS had greater CPII:C2C than SCFP-LRS ($P \leq 0.03$) and at 24 h, SCFP-LPS had greater CPII:C2C than all other groups ($P \leq 0.01$). At 336 h, the CPII:C2C ratio tended to be greater in LPS knees regardless of dietary treatment (CON-LPS and SCFP-LPS) than LRS knees of horses receiving the CON diet ($P = 0.06$; Fig. 3C).

DISCUSSION

The current study investigated the effects of dietary *Saccharomyces cerevisiae* fermentation product supplementation on synovial biomarkers related to joint inflammation and cartilage metabolism in young horses challenged with an acute intra-articular inflammatory insult. Acute joint inflammation altered synovial PGE_2, CPII, C2C, as well as CPII:C2C, and SCFP supplementation impacted the degree of response. Specifically, SCFP horses responded more favorably to the acute inflammatory challenge evidenced by reduced PGE_2 concentrations in combination with sustained elevated CPII concentrations as well as the CPII:C2C ratio compared to non-supplemented horses. Therefore, these data provide foundational knowledge related to the ability of dietary provision of SCFP to result in stabilization of cartilage synthesis and mitigation of acute synovial inflammation in young growing horses.

During the 84 d pre-LPS supplementation period, horses readily consumed their concentrate with no significant feed refusals across treatment groups. Yearlings consumed energy and associated nutrients to meet or exceed recommended requirements (NRC, 2007) as

BW and BCS increased in all horses as expected with growth but did not differ by dietary treatment.

Prior to the LPS challenge, C2C concentrations decreased and the ratio of type II collagen synthesis to degradation (CPII:C2C) increased over time. This was expected, as intra-articular collagen synthesis occurs with growth as horses adapt to increases in BW (Brama et al., 2000a). Concentrations of PGE_2 also increased numerically over the 84 d of supplementation, but this change was not statistically significant, nor did it differ by dietary treatment. Therefore, this change may be related to growth of the yearlings. Similar to PGE_2, CPII, C2C, and the ratio of CPII to C2C were not affected by diet during the pre-LPS period. The horses in the current study received no forced exercise outside of the previously described submaximal exercise test, which occurred 4 wk prior to the LPS challenge (Valigura et al., 2021). As opposed to horses at rest, forced exercise in young horses is more commonly associated with alterations within the joint space due to repetitive hyperextensions and concussions that are generated during early training and exercise (Palmer and Bertone, 1994). Since horses were allowed turnout but were not enrolled in a regular exercise training program, a lack of dietary effect during the pre-LPS period was expected in these clinically healthy yearlings.

While diet did not affect cartilage inflammation or turnover while horses were at rest, the LPS challenge at d 84 allowed determination of dietary impacts on synovial markers following an acute inflammatory insult. Clinical measures during the LPS challenge, including HR, RR, and RT remained within physiologically normal ranges through 24 h post-injection, which confirmed a localized inflammatory response. Carpal circumference was measured in an attempt to quantify the degree of joint distention, which might be expected to increase more in joints injected with LPS compared to LRS. However, both carpal circumference and CT increased over

time without an effect of injection type. This indicates increased joint effusion from the initiation of an inflammatory response in both carpal joints due to repeated arthrocentesis (Bliss, 1998; van den Boom et al., 2005). These responses were expected and are similar to previous reports (Hawkins et al., 1993; de Grauw et al., 2009; Lucia et al., 2013). While the external physical characteristics that reflect joint distension/effusion were similar between injection types, cellular markers of inflammation and cartilage turnover within synovial fluid are likely more indicative of changes within the joint space. As further discussed below, inflammatory PGE_2 was greater in LPS compared to LRS joints, indicating a greater induction of inflammation in LPS over LRS knees even though this differential response was not captured by carpal circumference and CT measures.

The dietary supplementation of postbiotics such as *Saccharomyces cerevisiae* fermentation product may elicit positive effects for treating acute stress related gut dysmobility (West et al., 2016). Postbiotics are by-products, or metabolic waste released from a metabolic activity carried out by probiotics following the digestion of prebiotics in the gut (Żółkiewicz et al., 2020). Interestingly, postbiotics demonstrate beneficial health effects much like probiotics, strengthening the intestinal microbiome (Klemashevich et al., 2014). However, postbiotics aren't made up of any live organisms like probiotics, decreasing risks associated with intake (Żółkiewicz et al., 2020). These organisms allow for the maintenance of tight junctions by increasing the production of mucin along the intestinal lining and improving the life of intestinal epithelial cells to increase the resistance of colonization from harmful bacteria (Oelschlaeger, 2010). Systemic and local inflammatory affects, such as intra-articular inflammation, are achieved by the modification of cytokine production by the intestinal epithelial cells and their

effect on the innate immune system cells such as macrophages and dendritic cells (Watanabe et al., 2009).

In obese mice, supplementation of oligofructose allowed for the re-establishment of beneficial *Bifidobacteria* (Schott et al., 2018). *Bifidobacteria* are associated with supporting intestinal epithelial proliferation, barrier function (Arboleya et al., 2016), and reducing the circulation of endotoxins and inflammatory cytokines, as well as protecting the infiltration of macrophages into the joint capsule (Schott et al., 2018). Therefore, synovial cells may be affected by a variety of mediators that directly impact cartilage structure and maintenance through the diffusion from systemic circulation into synovial fluid. However, there is limited information regarding the effects on the dietary inclusion of *Saccharomyces cerevisiae* fermentation product on joint health in a young, growing equine model. This gap in knowledge allows for the potential that gut microbial manipulation from postbiotic supplementation could be utilized as a dietary intervention strategy to address systemic and intra-articular inflammation in young growing horses.

In the current study, intra-articular inflammation was assessed by quantification of synovial PGE_2 concentrations. Prostaglandin E_2 has both pro- and anti-inflammatory effects, but its pro-inflammatory effects cause the articular structural changes that characterize arthritic disease (Martel-Pelletier et al., 2003); therefore, PGE_2 is used as a marker of synovial inflammation (Sokolove and Lepus, 2013). In the present study, synovial PGE_2 peaked at 6 h and began to decline by 12 h post-injection regardless of injection type. However, knees that were challenged with LPS had greater concentrations of PGE_2 compared to contralateral control knees, indicating elevated inflammation in the presence of LPS. Furthermore, the PGE_2 peak at 6 h did not parallel the CT peak that occurred at 12 h but did mirror the increase in CC noted at 6 h

post-injection of both LPS- and LRS-injected knees. The increased synthesis of PGE_2 likely enhanced carpal edema as well as the extravasion of plasma (Higgs et al., 1984) causing an increase in CC. Currently, the time frame of physiological occurrences that lead to increased swelling of the joint, including increases of synovial PGE_2 in growing horses, are minimally understood and warrant further investigation.

In previous work investigating the impacts of *Saccharomyces cerevisiae* fermentation product supplementation on PGE_2, rats that had received 14 d of supplementation of 7 mg/kg BW *Saccharomyces cerevisiae* fermentation product (EpiCor, Embria Health Sciences, Ankeny, IA) had decreased PGE_2 in their paw tissue following an inflammatory insult (Evans et al., 2012). In the current study, regardless of injection type, SCFP horses exhibited lower synovial PGE_2 than CON horses. In humans, PGE_2 has suppressive effects on collagen cleavage, allowing for the maintenance of a normal chondrocyte phenotype for appropriate collagen matrix maintenance of type II collagen (Tchetina et al., 2007). Although the present study did not evaluate the systemic immune response or associated changes in the gut microbiota, it can be hypothesized that the supplementation of SCFP modified the intestinal microbial population in a favorable manner, leading to a muted pro-inflammatory response following the acute LPS stressor. The mechanism of action whereby dietary SCFP impacted synovial PGE_2 warrants further investigation. While PGE_2 was the only biomarker evaluated to understand intra-articular inflammation in the current study, it would be of benefit to quantify and determine any dilutional effects of other synovial biomarkers related to inflammation, including cytokines, to obtain a more elaborate understanding of intra-articular inflammation. It was reported that horses supplemented with SCFP had lower serum concentrations of inflammatory cytokines, IL-1β, IL-6, and tumor necrosis factor alpha (TNFα), when compared to CON horses throughout the

recovery period following the submaximal exercise test at d 56 (Valigura et al., 2021). However, possible cytokine levels in serum and their potential relationship with joint cytokines at the time of the LPS challenge were not evaluated, as serum cytokines were only analyzed surrounding the exercise test at d 56 and the evaluation of synovial cytokines was outside of the scope of the current study.

Any inflammatory insult to the joint, including LPS or an exercise-induced inflammatory event (Frisbie et al., 2008), may lead to degradation of the type II collagen fibril within articulating joints. Degradation is initiated by unwinding of the collagen, exposing the normally hidden epitope, catabolic C2C, and may be utilized as a marker of collagen degradation (Trumble et al., 2009). Regardless of dietary treatment, synovial C2C concentrations were greater in LPS joints compared to LRS joints. The increased inflammatory response to LPS compared to LRS joints, as evidenced by greater levels of PGE_2, likely lead to cartilage degradation, which would explain the elevation of catabolic C2C concentrations in LPS joints (Garvican et al., 2010). Interestingly, dietary SCFP did not impact synovial C2C concentrations in the current study.

During the regeneration process, CPII is released from the type II collagen precursor, procollagen (Nelson et al., 1998). In arthritic joints, the synovial concentration of CPII has been correlated to the direct upregulation of collagen production (Nelson et al., 1998; Sugiyama et al., 2003). Therefore, with a half-life of approximately 16 h in humans, CPII within synovial fluid can be utilized as an indicator of new collagen synthesis (Nelson et al., 1998). Although the half-life of CPII is unknown in the horse, type II collagen synthesis and degradation pathways appear to be homologous between mammalian species (Kadler, 1995) so it can be inferred that CPII serves as an appropriate indicator of new collagen synthesis in the horse. In the current study,

synovial CPII concentrations increased 6 h post-injection, and remained elevated through 168 h post-injection, but returned to near baseline levels by 336 h.

The dietary treatment of SCFP appeared to modulate the CPII response to the LPS challenge. Previous investigations of *Saccharomyces cerevisiae* fermentation product on joint health have shown improved lameness and arthritic scores in dogs and rats (Beynen and Legerstee, 2010; Evans et al., 2012). Although these studies demonstrated the potential for dietary supplementation of *Saccharomyces cerevisiae* fermentation product to improve joint function, they did not evaluate synovial concentrations of cartilage metabolism. In the current study, supplementation of SCFP allowed for CPII concentrations to plateau through 168 h post-LPS injection while LPS knees in CON horses exhibited a drop in concentrations from 12 to 24 h post-injection, only to increase once more at h 168. Additionally, CPII concentrations remained greater in LRS knees of SCFP horses compared to LRS knees of CON horses throughout the post-injection period. The supplementation of SCFP demonstrated the ability to stabilize collagen synthesis throughout the 336 h following the intra-articular inclusion of LPS, as well as favoring increases in synovial concentrations of CPII regardless of injection type. However, additional biological markers related to collagen metabolism would be necessary to definitively determine a potential improvement in cartilage repair.

In addition to elevated CPII concentrations, LPS also induced an increase in the ratio of CPII:C2C, and further, the CPII:C2C ratio remained stable in LPS knees of SCFP horses throughout the post-injection period before returning to pre-injection levels at h 336, similar to CPII concentrations. This is in contrast to control knees in SCFP horses and LPS knees in control horses, which both demonstrated fluctuations in CPII:C2C throughout the post-injection period. The CPII:C2C ratio is an important variable to consider to gain a more holistic understanding of

cartilage health within the equine joint as it provides insight into net increases or decreases in cartilage synthesis and/or degradation. Given that SCFP horses maintained a sustained net positive cartilage synthesis:degradation ratio post-LPS injection, specifically at 24 h, it can be inferred that SCFP may help to positively regulate the cartilage metabolic response to an acute intra-articular inflammatory insult.

In conclusion, the dietary supplementation of SCFP to young horses for 84 d demonstrated potential to improve joint health following an acute stressor by reducing markers of intra-articular inflammation and improving markers of cartilage metabolism by stabilizing collagen formation. The dietary supplementation of SCFP may potentially reduce the susceptibility of intra-articular inflammation in growing horses. However, further studies are recommended to investigate the effects of SCFP supplementation on marker responses reflective of prolonged intra-articular inflammation and cartilage metabolism in horses.

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CONFLICT OF INTEREST

The authors affirmatively acknowledge that they were free from influence by any funding sources or their employees that would result in any conflict of interest.

LITERATURE CITED

Arboleya, S., C. Watkins, C. Stanton, and R. P. Ross. 2016. Gut *bifidobacteria* populations in human health and aging. Front Microbiol. 7(1204):1-9. doi:10.3389/fmicb.2016.01204

Bertone, A. L., J. L. Palmer, and J. Jones. 2001. Synovial fluid cytokines and eicosanoids as markers of joint disease in horses. Vet. Surg. 30:528–531. doi:10.1053/ jvet.2001.28430

Beynen, A. C. and E. Legerstee. 2010. Influence of dietary beta-1,3/1,6-glucans on clinical signs of canine osteoarthritis in a double-blind, placebo-controlled trial. Amer. J. Anim. Vet. Sci. 5(2):97-101. doi:10.3844/ajavsp.2010.97.101

Billinghurst, R. C., E. M. Buxton, M. G. Edwards, M. S. McGraw, and C. W. McIlwraith. 2001. Use of an anti-neoepitope antibody for identification of type-II collagen degradation in equine articular cartilage. Amer. J. Vet. Res. 62:1031-1039. doi: 10.2460/ajvr.2001.62.1031

Bliss, M. R. 1998. Hyperaemia. J. Tissue Viability. 8:4–13. doi:10.1016/S0965-206(98)80028-4

Brama, P. A. J., J. M. Tekoppele, A. Bank, R. A. Barneveld, and P. R. van Weeren, 2000a. Functional adaptation of equine articular cartilage: The formation of regional biochemical characteristics up to age one year. Equine Vet J. 32(3):217-221. doi: 10.2746/042516400776563626

Brama, P. A. J., J. M. Tekeoppele, R. A. Bank, A. Barneveld, E. C. Firth, and P. R.van Weeren. 2000b. The influence of strenuous exercise on collagen characteristics of articular cartilage in Thoroughbreds age 2 years. Equine Vet. J. 32:551-554. doi:10.2746/042516400777584613

Collins, K. H., H. A. Paul, R. A. Reimer, R. A. Seerattan, D. A. Hart, and W. Herzog. 2015. Relationship between inflammation, the gut microbiota, and metabolic osteoarthritis

development: Studies in a rat model. Osteoarthr. Cartil. 23(11):1989-1998. doi:10.1016/j.joca.2015.03.014

Cryan, J. F., and S. M. O'Mahony. 2011. The microbiome-gut-brain axis: From bowel to behavior. J. European Gastro. Motility Soc. 23(3):187–192. doi:10.1111/j.1365-2982.2010.01664.x

de Grauw, J. C., C. H. A. van de Lest, R. van Weeren, H. Brommer, and P. A. J. Brama. 2006. Arthrogenic lameness of the fetlock: synovial fluid markers of inflammation and cartilage turnover in relation to clinical joint pain. Equine Vet. J. 38:305–311. doi:10.2746/042516406777749236

de Grauw, J. C., C. H. van de Lest, P. A. J. Brama, B. P. Rambags, and P. R. van Weeren. 2009. In vivo effects of meloxicam on inflammatory mediators, MMP activity and cartilage biomarkers in equine joints with acute synovitis. Equine Vet. J. 41:693–699. doi:10.1186/ar2640

Evans, M., S. Reeves, and L. E. Robinson. 2012. A dried yeast fermentate prevents and reduces inflammation in two separate experimental immune models. Evidence-Based Complementary and Alternative Med. 2012:973041. doi: 10.1155/2012/973041

Faubladier, C., F. Chaucheyras-Durand, L. da Veiga, and V. Julliand. 2013. Effect of transportation on fecal bacterial communities and fermentative activities in horses: impact of *Saccharomyces cerevisiae* CNCM I-1077 supplementation. J. Anim. Sci. 91(4):1736–1744. doi:10.2527/jas.2012-5720

Firth, E. C. 2006. The response of bone, articular cartilage and tendon to exercise in the horse. J. Ana. 208(4):513-526. doi:10.1111/j.1469-7580.2006.00547.x

Frisbie, D. D., F. Al-Sobayil, R. C. Billinghurst, C. E. Kawcak, and C. W. McIlwraith. 2008. Changes in synovial fluid and serum biomarkers with exercise and early osteoarthritis in horses. Osteoarthr. Cartil. 16:1196–1204. doi:10.1016/j. joca.2008.03.008

Garvican, E. R., A. Vaughn-Thomas, J. F. Innes, and P. D. Clegg. 2010. Biomarkers of cartilage turnover. Part 1: markers of collagen degradation and synthesis. Vet. J. 185:36-42. doi:10.1016/j.tvjl.2010.04.011

Guo, J., H. Khalouei, F. Kelsey, V. Senaratne, Z. Zhang, H. Derakhshani, M. Scott, G. Crow, I. Yoon, E. Khafipour, and J. Plaizier. 2017. Effects of *Saccharomyces cerevisiae* fermentation products on endotoxins and acute phase proteins in lactating dairy cows. Pages 161-162 in Proc. Amer. Dairy Sci. Assoc. Annu. Meet. Pittsburgh, PA.

Hand, T. W., I. Vujkovic-Cvijin, V. K. Ridaura, and Y. Belkaid. 2016. Linking the microbiota, chronic disease, and the immune system. Trends Endocrinol. Metab. 27(12):831-843. doi: 10.1016/j.tem.2016.08.003

Hawkins D. L., R. J. MacKay, G. G. Gum, P. T. Colahan, and J. C. Meyer. 1993. Effects of intra-articularly administered endotoxin on clinical signs of disease and synovial fluid tumor necrosis factor, interleukin 6 and prostaglandin E_2 values in horses. Am. J. Vet. Res. 54:379–386.

Henneke, D. R., G. D. Potter, J. L. Kreider, and B. F. Yeates. 1983. Relationship between condition score, physical measurements and body fat percentage in mares. Equine Vet. J. 15:371–372. doi: 10.111/j.2042-3306.1983. tb01826.x

Hernández-Alonso, P., D. Cañueto, S. Giardina, J. Salas-Salvadó, N. Cañellas, X. Correig, and M. Bulló. 2017. Effect of pistachio consumption on the modulation of urinary gut

microbiota-related metabolites in prediabetic subjects. J. Nutr. Biochem. 45:48-53. doi:10.1016/j.jnutbio.2017.04.002

Higgs, G. A., S. Moncada, and J. R. Vane. 1984. Eicosanoids in inflammation. Ann Clin Res 16(5-6):287-299.

Kadler, K. 1995. Extracellular matrix 1: Fibril-forming collagens. Protein Profile 2(5):491-619.

Kahn, M. K., J. A. Coverdale, J. L. Leatherwood, C. E. Arnold, R. A. Dabareiner, A. N. Bradbery, A. A. Millican, and T. H. Welsh, Jr. 2017. Age-related effects on markers of inflammation and cartilage metabolism in response to an intra-articular lipopolysaccharide challenge in horses. J. Anim. Sci. 95:657–670. doi:10.2527/jas2016.1078

Klemashevich, C., C. Wu, D. Howsmon, R. C. Alaniz, K. Lee, and A. Jayaraman. 2014. Rational identification of diet-derived postbiotics for improving intestinal microbiota function. Curr Opin Biotechnol 26:85-90. doi: 10.1016/j.copbio.2013.10.006

Lucia, J. L., J. A. Coverdale, C. E. Arnold, and K. N. Winsco. 2013. Influence of an intra-articular lipopolysaccharide challenge on markers of inflammation and cartilage metabolism in young horses. J. Anim. Sci. 91:2693–2699. doi:10.2527/jas.2012-5981

Martel-Pelletier, J., J. P. Pelletier, and H. Fahmi. 2003. Cyclooxygenase-2 and prostaglandins in articular tissues. Semin Arthritis Rheum 33(3):155-167. doi: 10.1016/s0049-0172(03)00134-3

McIlwraith, C. W. and G. W. Trotter. 1996. Joint Disease in the Horse. W.B. Saunders Co., Philadelphia, PA.

Nelson F., L. Dahlberg, S. Laverty, A. Reiner, I. Pidoux, M. Ionescu, G. L. Fraser, E. Brooks, M. Tanzer, L. C. Rosenberg, P. Dieppe, and A. R. Poole. 1998. Evidence for altered

synthesis of type II collagen in patients with osteoarthritis. J. Clin. Invest. 102:2115–25. doi: 10.1172/JCI4853.

NRC. 2007. Nutrient Requirements of Horses. 6th rev. ed. National Academies Press, Washington, DC.

Oelschlaeger, T. A. 2010. Mechanisms of probiotic actions – A review. Intl. J. Medical Micro. 300(1):57-62. doi:10.1016/j.ijmm.2009.08.005

Palmer, J. L., and A. L. Bertone. 1994. Experimentally-induced synovitis as a model for acute synovitis in the horse. Equine Vet. J. 26: 492-495. doi:10.1111/j.2042-3306.1994.tb04056.x

Schott, E. M., C. W. Farnsworth, A. Grier, J. A. Lillis, S. Soniwala, G. H. Dadourian, R. D. Bell, M. L. Doolittle, D. A.Villani, H. Awad, J. P. Ketz, F. Kamal, C. Ackert-Bicknell, J. M. Ashton, S. R. Gill, R. A. Mooney, and M. J. Zuscik. 2018. Targeting the gut microbiome to treat the osteoarthritis of obesity. Amer. Soc. Clin. Invest. 3(8):e95997 doi:10.1172/jci.insight.95997

Sekirov, I., S. L. Russell, L. C. Antunes, and B. B. Finlay. 2010. Gut microbiota in health and disease. Physiol. Rev. 90(3):859–904. doi:10.1152/physrev.00045.2009

Sokolove, J., and C. M. Lepus. 2013. Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations. Therapeutic advances in musculoskeletal disease. 5(2):77–94. doi:10.1177/1759720X12467868

Sugiyama, S., M. Itokazu, Y. Suzuki, and K. Shimizu. 2003. Procollagen II C propeptide level in the synovial fluid as a predictor of radiographic progression in early knee osteoarthritis. Ann. Rheum. Dis. 62:27-32. doi:10.1136/ard.62.1.27

Szychlinska, M. A., M. Di Rosa, A. Castorina, A. Mobasheri, and G. Musumeci. 2019. A correlation between intestinal microbiota dysbiosis and osteoarthritis. Heliyon. 5:1.e01134. doi:10.1016/j.heliyon.2019.e01134

Tchetina, E. V., J. A. Di Battista, D. J. Zukor, J. Antoniou, and A. R. Poole. 2007. Prostaglandin PGE_2 at very low concentrations suppresses collagen cleavage in cultured human osteoarthritic articular cartilage: this involves a decrease in expression of proinflammatory genes, collagenases and *COL10A1*, a gene linked to chondrocyte hypertrophy. Arthritis Res. Ther. 9:R75. doi:10.1186/ar2273

Trumble, T. N., A. B. Scarbrough, and M. P. Brown. 2009. Osteochondral injury increases type II collagen degradation products (C2C) in synovial fluid of Thoroughbred racehorses. Osteoarth. and Cart. 17(3):371-374. doi: 10.1016/j.joca.2008.07.014

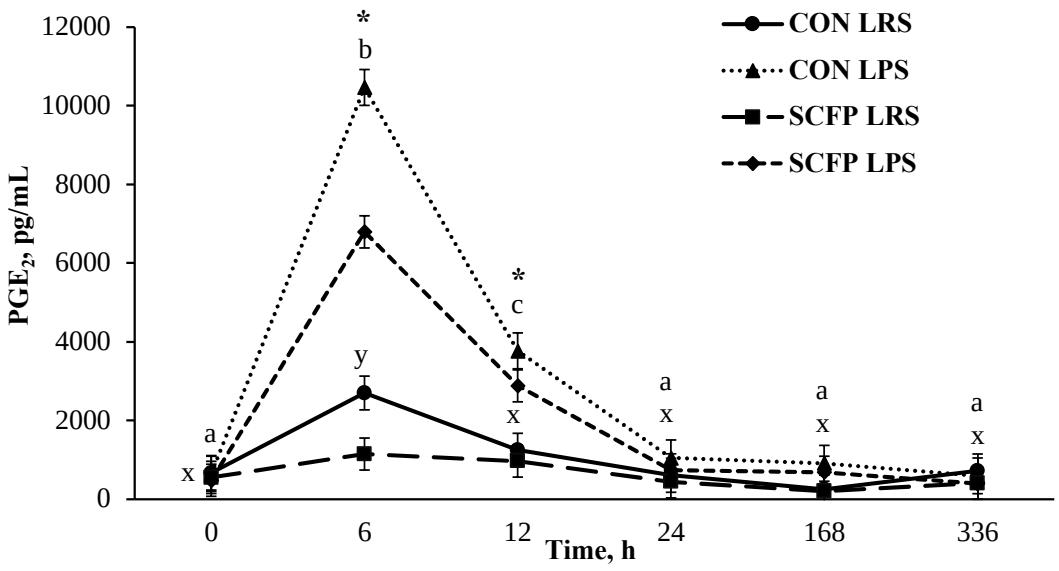
Valigura, H. C., J. L. Leatherwood, R. E. Martinez, S. Norton, and S. H. White-Springer. 2021. Dietary supplementation of a Saccharomyces cerevisiae fermentation product attenuates exercise-induced stress markers in young horses. Journal of Animal Science doi: 10.1093/jas/skab199

van den Boom, R., C. H. van de Lest, S. Bull, R. A. Brama, P. R. van Weeren, and A. Barneveld. 2005. Influence of repeated arthrocentesis and exercise on synovial fluid concentrations of nitric oxide, prostaglandin E_2 and glycosaminoglycans in healthy equine joints. Equine Vet. J. 37(3):250-256. doi: 10.2746/0425164054530740

Watanabe, T., H. Nishio, T. Tanigawa, H. Yamagami, H. Okazaki, K. Watanabe, and T. Arakawa. 2009. Probiotic *lactobacillus casei* strain shirota prevents indomethacin-induced small intestinal injury: Involvement of lactic acid. Amer. J. of Physiology-Gastrointestinal and Liver Phys. 297(3):G506-G513. doi:10.1152/ajpgi.90553.2008

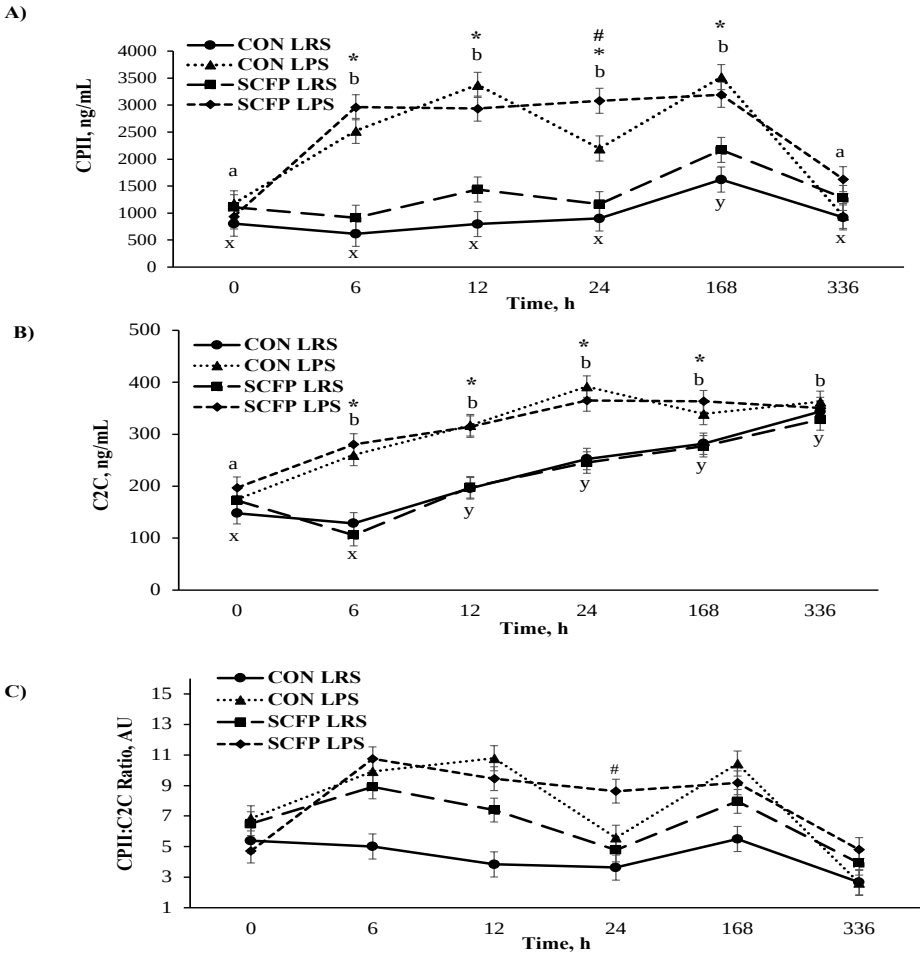
West, C., A. M. Stanisz, A. Wong, and W. A. Kunze. 2016. Effects of *Saccharomyces cerevisiae* or boulardii yeasts on acute stress induced intestinal dysmotility. World J. Gastroenterol. 22(48):10532-10544. doi: 10.3748/wjg.v22.i48.10532

Żółkiewicz, J., A. Marzec, M. Ruszczyński, and W. Feleszko. 2020. Postbiotics—a step beyond pre- and probiotics. Nutrients 12(8):2189. doi: 10.3390/nu12082189



Appendix Figure 1. Synovial prostaglandin E_2 (PGE_2) concentrations of yearling horses in response to an intra-articular injection of either 0.5 ng lipopolysaccharide derived from *Escherichia coli* O55:B5 (LPS) or sterile lactated Ringer's solution (LRS) after receiving either no supplement (CON; n = 9) or 21 g/d of *Saccharomyces cerevisiae* fermentation product (SCFP; n = 10) for 84 d. Main effects included diet ($P \leq 0.01$), time ($P \leq 0.01$), injection type ($P \leq 0.01$), diet × time ($P \leq 0.01$), diet × injection type ($P = 0.17$), time × injection type ($P \leq 0.01$), and diet × time × injection type ($P = 0.4$). [a,b,c,x,y] Across dietary groups but within injection type, time points with different letters differ ($P < 0.05$). * Within time point, LPS differs from LRS ($P < 0.05$).

Figure 2.



Appendix Figure 2. Synovial concentrations of A) CPII, B) C2C, and C) the ratio of CPII:C2C of yearling horses in response to an intra-articular injection of either 0.5 ng lipopolysaccharide derived from *Escherichia coli* O55:B5 (LPS) or sterile lactated Ringer's solution (LRS) after receiving either no supplement (CON; n = 9) or 21 g/d of *Saccharomyces cerevisiae* fermentation product (SCFP; n = 10) for 84 d. Main effects included diet ($P = 0.02$, $P = 0.99$, $P \leq 0.01$), time ($P \leq 0.01$, $P \leq 0.01$, $P \leq 0.01$), injection type ($P \leq 0.01$, $P \leq 0.01$, $P \leq 0.01$), diet × time ($P = 0.3$, $P = 0.6$, $P = 0.09$), diet × injection type ($P = 0.3$, $P = 0.7$, $P = 0.01$), time × injection type ($P \leq 0.01$, $P \leq 0.01$, $P \leq 0.01$), and diet × time × injection type ($P = 0.04$, $P = 0.8$, $P \leq 0.01$) for panels A, B, and C, respectively. [a,b,x,y] Across dietary groups but within injection type, time points with different letters differ ($P < 0.05$). * Within time point, LPS differs from LRS ($P < 0.05$). [#] Within time point, CON LPS differs from SCFP LPS ($P < 0.05$).

Appendix Table 1. Nutrient composition of custom-formulated concentrate and Coastal bermudagrass (*Cynodon dactylon*) hay offered to yearling horses

Nutrient[1]	**Concentrate**[2]	**Coastal bermudagrass hay**[3]
DE, Mcal/kg	0.61	0.39
CP, %	18.00	13.00
CF, %	8.80	1.70
NDF, %	30.40	71.60
ADF, %	15.20	40.30
Starch, %	18.00	1.00
Crude Fat, %	8.40	1.70
Ca, %	1.40	0.44
P, %	1.06	0.22
K, %	1.40	0.88
Mg, %	0.57	0.17
Na, %	0.62	0.30
Cl, %	1.08	0.52
S, %	0.30	0.24
Co, ppm	2.00	0.50
Fe, ppm	813.00	184.00
Zn, ppm	217.00	34.00
Cu, ppm	56.00	7.00
Mn, ppm	189.00	241.00

[1] Values presented on a 100% DM basis
[2] Concentrate = basal grain diet fed to all horses at 1.25% BW (DM basis) per d
[3] Coastal bermudagrass (*Cynodon dactylon*) hay was offered *ad libitum* to all horses

Appendix Table 2. Mean synovial inflammatory and cartilage biomarkers of yearling horses before (d 0) and after 84 d of receiving either no supplementation (CON; n = 9) or 21 g *Saccharomyces cerevisiae* fermentation product (SCFP; n = 10) per day

					P-values		
Synovial Biomarker[1]	Diet	d 0	d 84	SEM	Diet	Time	Diet Time
PGE_2, pg/mL[2]	CON	454.07	460.59	49.94	0. 79	0.19	0.25
	SCFP	392.02	496.16				
CPII, ng/mL	CON	955.38	993.20	114.24	0.46	0.76	0.83
	SCFP	866.60	873.43				
C2C, ng/mL	CON	228.86	161.31*	9.96	0.41	< 0.01	0.13
	SCFP	226.70	182.52*				
CPII:C2C, AU	CON	4.07	5.76*	0.51	0.37	< 0.01	0.44
	SCFP	3.84	4.94*				

[1] PGE_2 = prostaglandin E_2; CPII = carboxypropeptide of type II collagen; C2C = collagenase cleavage neopeptide

[2] Synovial concentrations differed between CON and SCFP at d 0 so d 0 was included as a covariate in statistical analyses

[*] Within row, d 0 differs from d 84 ($P < 0.05$)