EFFECTS OF GLYCINE SUPPLEMENTATION ON CREATINE SYNTHESIS AND GROWTH OF INTRAUTERINE GROWTH RESTRICTED PIGS (*SUS SCROFA DOMESTICUS*) POST-WEANING

A Dissertation

by

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ABSTRACT

This study was designed to test the hypothesis that dietary glycine (Gly) supplementation would improve the growth rate and survivability of post-weaning growing pigs through improved creatine synthesis. Pigs were obtained at weaning (21 d) and divided according to weight as either intrauterine growth restricted (IUGR) or normal, and then assigned randomly to treatment with a dietary supplement of either1% Gly or 1.19% alanine (Ala, isonitrogenous control). Pigs were slaughtered at market weight (188 d) and tissue samples were obtained to determine activities of creatine-synthetic enzymes, and expression of their respective mRNAs. Carcass quality was assessed at the time of slaughter.

Normal pigs receiving dietary Gly and Ala had similar growth rates, final live body weights, and carcass quality. However, dietary Gly supplementation increased growth rate and final body weight ($P < 0.05$), and improved carcass quality of IUGR pigs. Further, metabolic analyses of tissues from all groups revealed that Gly supplementation increased creatine and creatine-phosphate concentrations in semimembranosus muscle, pancreas, and kidneys of IUGR and normal pigs ($P < 0.05$). Concentrations of creatine were greater ($P < 0.05$) in livers of IUGR pigs receiving Gly supplementation, but were similar in plasma from all pigs.

Enzymatic activities and expression of mRNAs for creatine-synthetic enzymes also increased in response to Gly supplementation. Arginine:glycine amidinotransferase (AGAT) activity was greater ($P < 0.05$) in kidneys of Gly-treated IUGR pigs, and

expression of AGAT mRNA followed a similar pattern. Guanidinoacetate N-methyltransferase (GAMT) activity was greater ($P < 0.05$) in the pancreas of Gly-treated IUGR and normal pigs, and greater in pancreases than in all other tissues analyzed ($P < 0.05$). While GAMT activity was unchanged in the liver ($P > 0.05$), GAMT mRNA expression was greater ($P < 0.01$) for Gly-treated IUGR pigs. The activities and expression of mRNAs for both AGAT and GAMT were lowest in all tissues analyzed from IUGR control pigs. Expression of creatine kinase mRNA was not different due to treatment group or tissue analyzed. Dietary Gly increased ($P < 0.05$) expression of mTOR mRNA in the kidney and skeletal muscle.

Results of this study indicate that: (1) dietary Gly supplementation can improve the growth and carcass quality of IUGR pigs; (2) IUGR pigs are less able to synthesize creatine beginning at the pre-translational level, but have similar abilities to express creatine kinase an store creatine as creatine-phosphate in tissues analyzed; (3) the rate of creatine synthesis can be enhanced through dietary Gly supplementation; (4) Gly stimulates mTOR expression in kidney and skeletal muscle of post-weaning IUGR and normal pigs. Collectively, these results indicate that dietary Gly supplementation is vital for IUGR pigs to synthesize sufficient creatine and it provides a novel means to manage IUGR pigs.

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This work was supervised by a dissertation committee consisting of Dr. Guoyao Wu, Dr. Fuller W. Bazer, and Dr. Stephen B. Smith, Department of Animal Science, College of Agriculture and Life Sciences, and Dr. Gregory A. Johnson, Department of Veterinary Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences.

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NOMENCLATURE

1C	One-carbon
AA	Amino acid
AGAT	Arginine:glycine amidinotransferase
AKB	2-amino-3-ketobutyrate
CEAA	Conditionally essential amino acid
CK	Creatine kinase
CR	Creatine
CRN	Creatinine
CRP	Creatine phosphate (also known as phosphocreatine)
EAA	Essential amino acid
GA	Guanidinoacetate
GAMT	Guanidinoacetate N-methyltransferase
Gly	Glycine
Hyp	Hydroxyproline
IACUC	Institutional Animal Care and Use Committee
IUGR	Intrauterine growth restricted
ME	Metabolizable energy
meTHF	N^5, N^{10}-methylenetetrahydrofolate
mTOR	Mechanistic target of rapamycin
NAD/NADH	Nicotinamide adenine dinucleotide
NEAA	Nonessential amino acid

SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SHMT	Serine hydroxymethyl transferase
TDH	Threonine dehydrogenase
THF	Tetrahydrofolate

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Swine production represents a large portion of animal agriculture worldwide, providing high quality animal protein for human consumption. Domestic swine (*Sus scrofa domesticus*) are unique within the animal agriculture sector for their prolific nature, with high farrowing rates and high numbers of offspring per litter. However, not all offspring that are produced will make it to market for human consumption. This is due to the phenomenon of intrauterine growth restriction (IUGR) that occurs naturally in swine production enterprises and represents 20-25% of all pigs born in the swine industry(Wu 2010). This growth restriction can also be attributed to inadequate uterine capacity in the sow (commonly seen in sows in commercial swine production) or inadequate maternal nutrition (Wu et al. 2006). The IUGR pigs have birthweights that are only one-half or even one-third of the weight of their normal littermates, with considerably underdeveloped and undersized skeletal muscles and small intestine tissues (Wang et al. 2008). Due to this, IUGR pigs are usually culled by producers at birth as most (76%) of them will die before weaning and they will not reach market weight at the same pace as their normal birthweight litter mates (Ji et al. 2017). This represents a huge profit lost for producers, as well as wasted animals, neither of which are beneficial to the industry as a whole. There is currently no intervention protocol for IUGR pigs to allow them to be rescued and raised to mature to market weight at the same time as their normal littermates. However, recent work has revealed that some biochemical pathways related to amino acid (AA) synthesis are severely underdeveloped in IUGR pigs.

Specifically, those pathways that synthesize arginine (Arg) and glycine (Gly) endogenously were found to be inadequate for meeting daily requirements for growth and metabolism (Hu et al. 2017). Amino acids are vital for all aspects of life from growth to survivability, so a deficit in synthesis, especially of AAs that are not commonly supplemented in swine diets (such as Gly and Arg) can have dramatic impacts on the growth and survivability of IUGR pigs, which begin life at a deficit. Despite this, this finding also highlights a potential area for intervention. However, it is first important to establish the importance of all AAs, and especially Gly, to overall health and as promoters of growth and survivability in IUGR pigs.

Amino acids are important compounds for all humans and animals, including pigs. These amino- and carboxyl-containing compounds have numerous roles in maintaining normal physiological functions each day (Wu et al. 2014), one of which is protein synthesis. The primary function of the 20 "proteinogenic" AAs is to serve as building block for hundreds of cellular and tissue proteins. The 20 proteinogenic amino acids are: arginine, alanine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, tyrosine, threonine, tryptophan, and valine. While all of these AAs are similar in base structure (an amino group and a carboxyl group), their differences in sidechains confers variability in the functional roles of each of them. Some of these roles include substrate for synthesis of nitric oxide and polyamines, antioxidants, enzymatic regulation, maintenance of normal pH through ammonia metabolism and detoxification via the urea cycle and providing links for various metabolic pathways and cycles, as well as whole body inter-organ metabolism (Broer and Broer 2017; Wu 2013a). Overall, there is a need for continuing research to establish a

complete understanding of the many functional roles of amino acids to advancing scientific understanding of those roles, especially as it relates to health and development.

The first step in establishing this understanding is determining daily requirements for AAs and how deficits or excesses of AAs can impact overall animal and human health. Original classifications for AA requirements were based on the idea of nitrogen balance or observations of the effects of AA restrictions on growth of animals. In this way, the original classifications of "essential" versus "nonessential" AAs were used initially in 1912 and subsequently established. Nutritionally essential amino acids (EAAs) were those that had no identified pathway for synthesis in animals, e.g., sulfur-containing AAs such as methionine (Osborne et al. 1917). While nutritionally nonessential amino acids (NEAAs) were those that had established synthesis pathways identified, with the idea being that if the synthesis pathway existed, all requirements for those AAs could be met through endogenous provision. These classifications were further clarified and confirmed decades later by W.C. Rose (1968) based on nitrogen balance in young adults consuming diets lacking a specific AA. As nutritional science progressed, so did that of animal dietary development, and requirements for specific AAs became of greater importance for optimizing swine production. Accordingly, in the 1990's, Chung and Baker (1992) developed the ratios of AA requirements for swine diets based on lysine, considered the "ideal AA pattern" for swine diets, which would later inform the AA requirements listed in the 1998 NRC Swine Nutrition Requirements. However, no one seemed to consider the potential importance of the so-called "NEAAs" (Kim et al. 2001) on growth and overall health of swine. Since the early 1990s, there has been active research that has highlighted the importance of providing dietary sources of NEAAs (such as arginine, cysteine, glycine, proline, and tyrosine) for

many different species (Kim and Wu 2004; Wang et al. 2014a; Wang et al. 2013a; Wu 2013b; Wu et al. 2011; Wu et al. 2014; Wu et al. 2008; Wu et al. 1994; Zhang et al. 2021). While these AAs were previously classified as "nonessential" due to the existence of endogenous pathways for synthesis, it's now known that these pathways do not always meet the metabolic demands of the animal, especially during times of growth, reproduction, lactation, or disease. It was recently suggested by Hou and Wu (2017, 2018) that EAAs be defined as AAs whose carbon skeletons cannot be synthesized de novo via endogenous pathways, or those AAs that are not synthesized in adequate amounts to meet maintenance requirements as well as the increased requirements for optimum growth and health. This would indicate a need for dietary supplementation of many AAs for all classifications of animals. Additionally, those AAs that would be synthesized de novo can be affected by a variety of factors, including life stage, age, production status, and overall health. Those AA-synthetic pathways affected by external factors should, therefore, be further classified as conditionally essential AAs (CEAAs). For example, glycine must be supplemented in diets of sow-reared piglets due to the insufficient supply of glycine in sow's milk (Wang et al. 2013a). This also true for arginine, glutamine, and proline in gestating and lactating mammals, whose demands for all AAs are greatly increased (Kim and Wu et al. 2004; Li et al. 2007; Rhoads and Wu 2009; Wang et al. 2014a; Wu 2013a; Wu et al. 2008).

As mentioned previously, glycine is one of the AAs that should be classified as a CEAA due to the high demand for it during growth of preweaning piglets (Wang et al. 2014a). Glycine is the simplest of all proteinogenic AAs, having a single hydrogen as its side chain group. Because of this, Gly lacks an asymmetrical carbon and, therefore, does not undergo isomerization and has no D- or L- structure (Hall 1998; Jackson 1991; Wang et al.

2013a). Glycine has been studied for over two centuries. It was first obtained in 1820 from gelatin (acid hydrolysates of whole animal protein) by H. Braconnot. Glycine was then isolated from gelatin utilizing alkaline hydrolysis. Its name is derived from the Greek word "glykys," meaning sweet, owing to its sweet taste (Meister 1992).

Due to the simple nature of its side chain, Gly confers a great deal of flexibility to proteins. Therefore, it is no surprise that Gly is especially abundant (making up one-third of total AA content) in some of the most flexible tissues, collagen and elastin. Further, as collagen and elastin make up one-third of all body protein, this makes Gly the most abundant AA in all animals on a molar basis (Wang et al. 2013a; Wu et al. 1999). Interestingly, Gly is also the most abundant AA in the plasma of piglets during the preweaning stage of life (Wu 2013b). However, it is very deficient in plant protein sources, such as corn and soybean meal (Hou et al. 2019; Li and Wu 2020).

Glycine can be synthesized from multiple precursors such as serine, threonine, choline, and (recently confirmed in pigs) 4-hydroxyproline (Chao et al. 1953; Hu et al. 2017; Shemin et al. 1948; Soloway and Stetten 1953; Tressel et al. 1986). Due to the existence of multiple pathways for synthesis, Gly has traditionally been classified as a NEAA. However, previous work has shown that the daily needs for Gly are much greater than can be provided by endogenous synthesis alone (Wang et al. 2013a). Specifically, it has been shown that the endogenous sources of Gly can only provide, at most, 50% of the requirements for protein synthesis alone, and represents < 30% of the total Gly needed by mammals as a functional AA (Gersovitz et al. 1980; Jackson 1991; Wang et al. 2013a; Wu 2020; Yu et al. 1985). Additional studies have confirmed that endogenous synthesis of Gly cannot meet the demands for supporting optimal metabolic health or growth in poultry, rats, and young pigs

(He et al. 2021; Jackson 1991; Melendez-Hevia and De Paz-Lugo 2008; Wang et al. 2014a; Wu et al. 2013).

Additionally, Gly is a precursor for the metabolically important compound, creatine. An entire molecule of Gly is fully incorporated into 1 mol of creatine, making creatine synthesis a considerable drain on available Gly (estimated that 14.5% of all Gly [dietary + endogenous] is utilized for creatine synthesis each day), with 87% of dietary Gly going to creatine synthesis (Melendez-Hevia et al. 2009; Wu and Morris 1998; Wu et al. 2018). Creatine plays many important roles in growth and skeletal muscle accretion, as well as sharing many of the important functional roles of its precursor AA (Gly, arginine, and methionine) such as being an antioxidant, providing membrane stability, and regulating enzymatic activities (Almeida et al. 2006; Wu 2013; Wu 2020). Additionally, creatine is heavily involved in cellular energy metabolism, especially in skeletal muscle, where the majority of creatine is stored as creatine phosphate (Brosnan and Brosnan 2007; Brosnan et al. 2011). Creatine is synthesized via the renal-hepatic axis and, therefore, involves interorgan metabolism and varying degrees of regulation (Edison et al. 2007; Van Pilsum et al. 1972; Wyss and Kaddurah-Daouk, 2000). As Gly is essential for the first step in creatine synthesis, and direct dietary creatine supplementation is ineffective at promoting growth or skeletal muscle accretion for animals, an adequate supply of Gly for young pigs during high growth phases is vital (Dolan et al. 2019; Marzuca-Nassr et al. 2019; Wu 2010). Such evidence provides one of the convincing bases for the new nutritional concept of functional AAs (Wu 2010). This is especially true for IUGR pigs in which pathways for synthesis of Gly are insufficient, as mentioned previously (Hu et al. 2017). Because of the many biologically important roles of Gly, including contributions to creatine synthesis, during

growth and other high-demand phases of production, and the overall limitations in endogenous synthesis and dietary supply, Gly should be considered a conditionally essential amino acid (Wu 2013b).

Glycine

Synthesis from serine

Glycine synthesis from serine was discovered in 1946 and was the first of the Gly synthesis pathways discovered. D. Shemin utilized labeled serine, ^{15}N and ^{13}C, and found that radiolabeled Gly was produced at a rapid rate (Shemin 1946). This endogenous synthesis pathway is also one of the simplest, only requiring one step, as shown in Figure 1. 1. Tetrahydrofolate (THF) is required as a cofactor for serine hydroxymethyl transferase (SHMT) to successfully convert serine into Gly. In this process, serine loses a one-carbon (1C) unit to be converted to Gly, and the 1C is transferred to THF, forming N^5-N^{10}-methylene-tetrahydrofoalte (meTHF) (Wu 2013a). Despite the long-held belief that serine was the primary substrate for Gly synthesis in animals, this is now known to be untrue at least in pigs (Wang et al. 2013a). The most important role of this conversion is its contribution to one-carbon metabolism, as was later determined following the development of better analytical techniques, which allowed for a more nuanced understanding of amino acid biochemistry.

Serine hydroxymethyl transferase (SHMT) relies on both THF, as mentioned previously, and pyridoxal phosphate for enzymatic activity (Wang et al. 2013a). Two isoforms of SHMT are present in most animal cells, either within the cytoplasm (cSHMT or SHMT1) or mitochondria (mSHMT or SHMT2). Overall, mSHMT exhibits much greater activity and is the primary isoform of the mammalian enzyme for Gly synthesis (Schirch and

Peterson 1980). In 1996, Narkewicz et al. confirmed this by eliminating the mSHMT isoform in ovarian cells of the Chinese hamster, which decreased Gly production and the accumulation of serine. SHMT is also heavily regulated by a variety of factors that appear to act in a cell- and tissue-dependent manner (Burns and Jackson 1982; Sanborn et al. 1975; Snell 1984; Snell et al. 1988). Hormones such as estradiol and testosterone increase the metabolism of serine in the uterus and prostate, respectively by upregulating SHMT activity (Sanborn et al. 1975). Tumor cells also appear to have a higher requirement for Gly than normal cells, as tumor cells have much higher SHMT activity (Snell et al. 1988). It is worth noting, however, that none of these studies utilized other substrates to determine if serine was the preferred substrate for Gly synthesis, which is now known to be untrue, at least in pigs, as noted previously.

Synthesis from threonine

One of the primary hurdles in identifying Gly synthesis from threonine for many years was the misidentification of the enzyme threonine aldolase. Schirch and Gross (1968) claimed for many years that the SHMT enzyme also converted threonine into Gly. While SHMT does have some ability to convert threonine to Gly in some tissues (e.g., the rabbit liver) under in vitro conditions with exceedingly high concentrations of threonine, subsequent work has proven that SHMT differs from threonine aldolase. The two enzymes differ in both function and AA sequences (Bird and Nunn 1983; Dale 1978; Ogawa et al. 2000; Yeung 1986).

The synthesis of Gly from threonine can occur through two pathways. One pathway requires threonine dehydrogenase (TDH). This is a two-step pathway in the liver of animals that begins with the conversion of threonine into 2-amino-3-ketobutyrate (AKB) by TDH

and the concurrent reduction of NAD^+ to NADH + H^+. The subsequent reaction is the conversion of AKB into Gly by AKB-lyase. This two-step pathway also requires CoA as a cofactor and generates acetyl-CoA (Darling et al. 2000; House et al. 2001; Wu 2013a). The net reaction for this pathway for Gly synthesis is:

$$\text{Threonine} + NAD^+ + \text{CoA} \Rightarrow$$

$$\text{Glycine} + \text{Acetyl-CoA} + \text{NADH} + H^+ \text{ (TDH \& AKB lyase)}$$

This is the pathway responsible for the majority of threonine degradation in mammalian livers (Bird and Nunn 1983; Davis and Austic 1994; Wu 2013a). However, there are conflicting reports, based on isotopic studies, that this pathway may have only a minor role in threonine degradation in adult humans, while also being responsible for one-half of threonine degradation in newborn humans (Darling et al. 2000; Parimi et al. 2005). These conflicting results may indicate a temporal effect on expression of threonine degradation enzymes in the human liver or differences in the experimental methods. Additionally, the second pathway that converts threonine into Gly involves threonine aldolase that converts threonine directly into Gly; however, most researchers consider this the minor pathway for threonine degradation.

Synthesis from choline

Choline is a metabolite found in many human and animal foods and feed ingredients. Choline plays a wide variety of roles in overall health, especially maintaining normal cellular functions. The important cellular functions affected by choline include: one carbon metabolism, maintenance of cell membrane integrity, neurotransmission, and cell signaling

(Zeisel 1981; Zeisel and Blusztajn 1994). Some of these functions are shared by its metabolite, Gly (Wu 2013a). Choline is converted into Gly via oxidative degradation into the metabolite betaine in a very tissue-specific pathway (Soloway and Stetten 1953; Zhang et al. 1992). Choline is first oxidized into betaine by choline dehydrogenase and betaine dehydrogenase in mitochondria of liver and kidney cells. Betaine is then methylated to form dimethylbetaine (DMB), in a reaction catalyzed by betaine transmethylase and further oxidized to sarcosine (N-methylglycine) by dimethylglycine oxidase. Sarcosine is then converted to Gly as the final step in the reaction. However, the sarcosine to Gly pathway exists in relatively few tissues, so this is one limitation of this pathway for Gly synthesis (Bergeron et al. 1998; Yeo and Wagner 1994; Zhang et al. 1992). Overall, the net reaction for Gly synthesis from choline is:

$$\text{Choline} + NAD^+ + 4O_2 + 4H_2O \Rightarrow \text{Gly} + 4H_2O_2 + 3HCHO + NADH + H^+ \text{ (enzymes)}$$

However, as mentioned previously, Gly synthesis from choline is very limited. Instead, choline is primarily involved in one-carbon metabolism, cell membrane maintenance, and cholinergic neurotransmission (Bergeron et al. 1998; Wang et al. 2013a; Yeo and Wagner 1994; Zeisel 1981). There are two main barriers to choline playing a substantial role in Gly synthesis. First, concentrations of choline is, overall, very low in physiological fluids, as well as in the diet, especially compared to the concentrations of other Gly precursors (serine, threonine, and 4-hydroxyproline; Donovan et al. 1997; Wu 2020). Additionally, the choline-to-sarcosine and sarcosine-to-Gly pathways are both highly cell- and tissue-specific, meaning that the former pathway exists primarily to degrade choline and

not to synthesize Gly. Instead, choline is utilized to synthesize phosphatidylcholine for maintenance of cell membrane stability, and sphingomyelin synthesis in the nervous system, and for synthesis of the neurotransmitter, acetylcholine (Wang et al. 2013a; Zeisel 1981).

Synthesis from hydroxyproline

4-Hydroxyproline (Hyp), a metabolite of proline, is abundant in collagen and elastin proteins. It has been known since the 1980's that Hyp is a precursor for Gly synthesis in the liver and kidneys of rodents (Lowry et al. 1985a; Ribaya and Gershoff 1979; Takayama et al. 2003). 4-hydroxyproline was later also shown to be metabolized in the liver and other organs in humans (Baker et al. 2004; Knight et al. 2006; Melendez-Hevia et al. 2009). The initial pathway proposed for synthesis of Gly from Hyp was based on work from Ruiz-Torres and Kürten (1976) showing that rats given radiolabeled 4-hydroxy-L-proline metabolized 80% of the labeled substrate into labeled Gly within 1 hour. This led to the later discoveries of an active hydroxyproline-glycine pathway in rodent liver and kidneys and in humans. Later, Wu et al. (2013a) proposed that the hydroxyproline-glycine pathway also exists in young pigs. This hypothesis was based on the high requirement for endogenous Gly synthesis in young pigs, rather than Gly being synthesized from other precursors such as serine, threonine, or choline, which are present in much lower concentrations in sow's milk than Hyp (an abundant AA in sow's milk; Wang et al. 2013a). This hypothesis was later confirmed by Hu et al. (2017).

The pathway for Gly synthesis from Hyp is metabolically preferred to the other Gly synthesis pathways as it is exergonic and results in the production of reducing equivalents and pyruvate that represent a net gain of 29 mols of ATP. The initial step is the rate limiting step, catalyzed by hydroxyproline oxidase (OH-POX) that oxidizes Hyp to Δ^1-pyrroline-3-

hydroxy-5-carboxylate (3-OH-P5C). 3-OH-P5C is then degraded and P5C is synthesized. This two-step process is carried out by one enzyme, 3-OH-P5C dehydrogenase (Valle et al. 1979; Li and Wu 2017). The additional pathway for Gly synthesis diverges at the synthesis of 3-OH-P5C. This metabolite can instead be converted to gamma-hydroxyglutamate and further to glyoxylate by glutamate-oxaloacetate transaminase (GOT) and 4-hydroxy-2-oxoglutarate aldolase (HOA). The final step in the synthesis of Gly is the conversion of glyoxylate into Gly by alanine:glyoxylate transaminase (AGT) (Holmes and Assimos 1998).

Hu et al. (2017) recently discovered that this pathway is also active in many tissues from 14- to 21-day-old piglets reared by sows, including the small intestine, liver, skeletal muscle, kidneys, and pancreas. This adds to the list of species that can successfully utilize this pathway for the endogenous synthesis of Gly. 4-Hydroxyproline is the most suitable candidate as a major contributor to Gly synthesis in preweaning piglets as it is abundant in sow's milk and pig tissues as the AA repeat Gly-Pro-Hyp (Hu et al. 2017; Li and Wu 2017; Wang et al. 2013b; Wu et al. 2019). This is helpful for improving the growth and survivability of preweaning IUGR pigs; however, it means that postweaning IUGR piglets, which are traditionally fed corn- and soybean meal-based diets (low in Gly and Hyp), have a low supply of substrates for endogenous synthesis of Gly (Hou et al. 2019). While Hyp is much more abundant in animal-sourced feed ingredients (e.g., hydrolyzed feather meal) than plant-sourced feedstuffs, use of the former in the diets of postweaning swine is limited. There is still the possibility of Gly deficiency in growing-finishing IUGR pigs, which have limited pathways for Hyp-Gly synthesis prior to weaning (Hu et al. 2017).

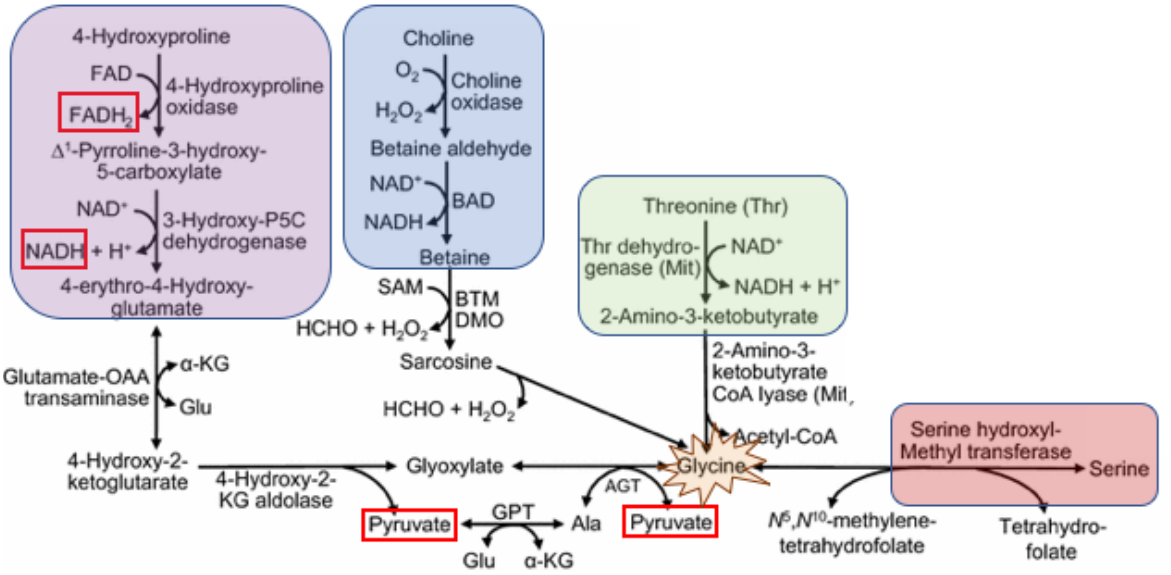


Figure 1.1 Glycine synthesis pathways in animal cells include synthesis from serine (red), threonine (green), choline (blue), and hydroxyproline (purple). Note the presence of reducing equivalents and pyruvate generated by the Hyp-Gly pathway, making this pathway more biochemically favorable than the others. Adapted from Wu 2013a.

Gly synthesis is important for generating the endogenous supply of Gly for meeting daily requirements; however, the pathways that exist are most important for producing Gly for participation in interorgan metabolism. Glycine links THF and meTHF in folate metabolism, as well as linking vital methylation pathways in the liver and contributing significantly to one-carbon metabolism homeostasis (Finkelstein and Martin 2000; Wu 2009; Wu 2013a, Wu 2013b.). Through its activities linking conversion of homocysteine to methionine via S-adenosylmethionine (SAM) methylation in the liver, Gly contributes to creatine synthesis and energy metabolism in cells (Finkelstein and Martin 2000; Wu 2013a).

Catabolism of Gly through the Gly cleavage system (GCS)

The glycine cleavage system (GCS) is the major enzymatic system responsible for Gly catabolism in animals. The overall pathway converts Gly into one-carbon units. It is widely distributed across prokaryotes and vertebrates and localized to the inner

mitochondrial membrane in animals (Kikuchi et al. 2008; Lowry et al. 1985b). Therefore, this system plays many critical roles in Gly homeostasis (Jois et al. 1992; Wang et al. 2013a). In mammals, the GCS is most active in liver and kidneys.

The GCS consists of four separate proteins with three enzymes that carryout the necessary reactions. This system is very complex for the relatively simple process of simply deaminating Gly to one-carbon units. This indicates that it is vital that minimal errors are committed regarding Gly degradation and that no excess catabolism of Gly occurs; that makes Gly a biochemically valuable metabolite. The four components of the GCS and the enzymes present within them are: Protein P (pyridoxal phosphate-dependent protein; glycine dehydrogenase), Protein T (amino-methyltransferase), Protein L (dihydrolipoamide dehydrogenase), and Protein H (structural carrier protein) (Kikuchi 1973; Kikuchi et al. 2008; Motokawa and Kikuchi 1969). These protein components and their constituent enzymes carry out the three reactions required for degradation of Gly: decarboxylation, amino-methyl transfer, and final reoxidation of the reduced lipoate group. This complex pathway is shown in Figure 1.2 and the overall reaction is:

$$\text{Gly} + NAD^{+} + H_4\text{-Folate} \rightarrow$$

$$NH_3 + CO_2 + \text{NADH} + H^{+} + \text{5,10-methylene-H4Folate (GCS)}$$

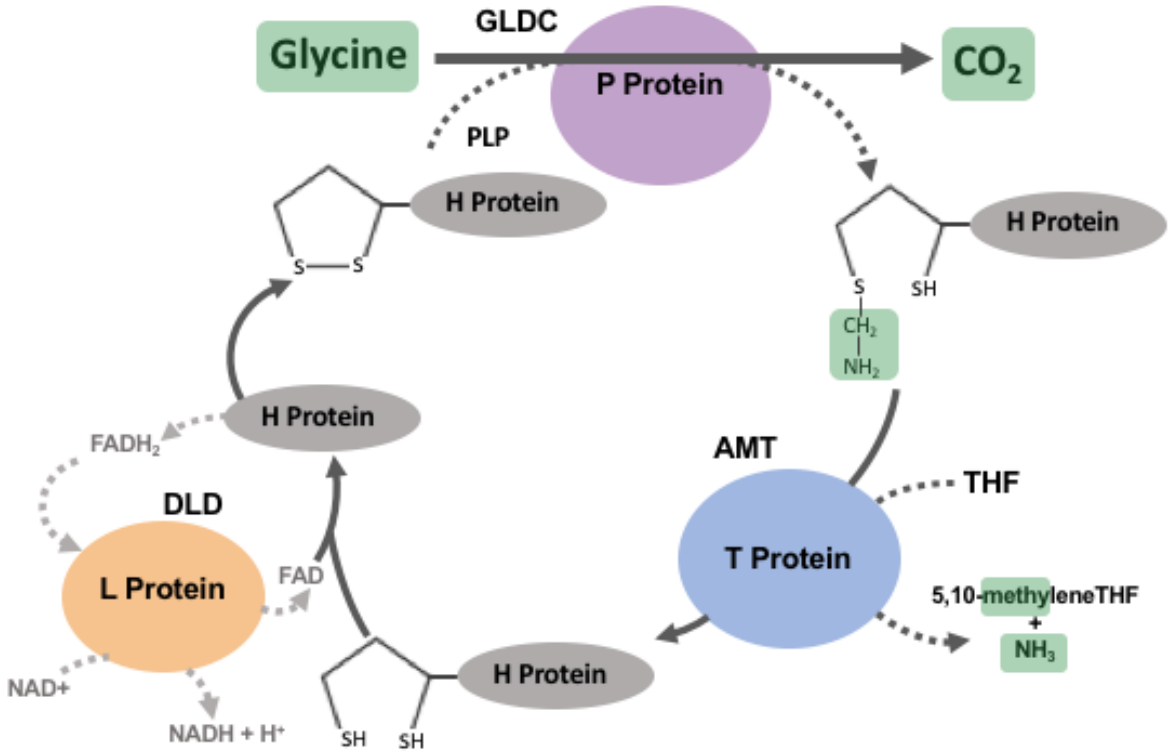


Figure 1.2 The glycine cleavage system (GCS) for glycine catabolism consists of pyridoxal phosphate-dependent (PLP) P-Protein: glycine dehydrogenase (GLDC); T-Protein: ammino-methyltransferase (AMT); L-Protein: dihydrolipoamide dehydrogenase (DLD); and H-Protein that exists as a carrier protein within the complex. Adapted from Tan et al. 2020

While it is highly unlikely that this complex system of enzymes is reversible under physiological conditions, it is subject to regulation, primarily by hormones and nutritional status, similar to the types of regulation affecting synthesis of Gly. Hormones such as glucagon enhance GCS activity by upregulating the decarboxylase complex in liver of rodents, while increases in concentrations of protein, fatty acids, and ions in plasma increase activity of GCS and Gly degradation via multiple pathways in various tissues, such as the liver and kidneys (Jois et al. 1992; Lowy et al. 1985b; Mudd et al. 2007; Wang et al. 2013a; Wu et al. 2014).

Catabolism via serine hydroxymethyl transferase (SHMT)

The reversible conversion of serine into Gly cements the role of Gly as a link to one-carbon metabolism. The SHMT degradation pathway connects Gly catabolism to nucleotide and protein biosynthesis by providing serine and one-carbon units from N-^{5},^{10}-methTHF and Gly. As this reaction is reversible, the Gly catabolic activity of SHMT is highly dependent on age and development. For example, the activity of SHMT is high in the ovary, placenta, fetal hepatocytes, and adult kidneys, locations where one-carbon units are in high demand and nucleotide biosynthesis is very active. This is crucial for reproduction, fetal development, and overall survival (Bazer et al. 2021; Lewis et al. 2004; Lowry et al. 1985b; Narkewicz et al. 1996; Thureen et al. 1995).

The SHMT reaction is also related to the role of Gly in linking various metabolic pathways through interorgan metabolism. In rats and pigs supplemented with dietary Gly, there is a nonlinear relationship with concentrations of serine in plasma (Lowry et al. 1985b; Shoham et al. 2001; Wang et al. 2013a). This indicates that the formation of serine from Gly is highly compartmentalized within cells, and that serine derived from Gly catabolism is not likely to be released into the circulation, especially from muscle cells or enterocytes, where protein biosynthesis is paramount and cell turnover is rapid, respectively (Sun et al. 2016; Wang et al. 2014).

Requirements for endogenous synthesis and metabolic functions of Gly in pigs

While results from some earlier studies indicated that diet and endogenous synthesis of Gly are adequate in meeting daily demands for Gly for optimal growth (Sanborn et al. 1975; Schirch and Gross 1968), this is not always the case, especially for sow-reared piglets (Kim and Wu 2004; Wu 2010). Previous studies with sow reared piglets revealed suboptimal

growth of piglets compared to piglets fed a milk replacer diet (Kim and Wu 2004; Wu 2010). Further, quantitative analyses performed by Wang et al. (2013a) on the demand for endogenous synthesis of Gly in sow-reared piglets as well as growing pigs was found to exceed the amount of Gly actually synthesized each day. The evidence indicated that only about 20% of Gly requirements in preweaning pigs are satisfied by sow's milk alone. (Hu et al. 2017; Wang et al. 2013a). Thus, about 80% of Gly must be synthesized endogenously each day from available substrates such as serine, choline, threonine, and hydroxyproline.

While serine was initially considered the primary substrate for Gly synthesis, it is now known that serine conversion to Gly is very limited, and that only 19% of serine present in sow's milk is available for Gly synthesis, as the rest supports rapid growth and protein synthesis of preweaning pigs (Wu 2010). This also holds true for serine present in traditional corn- and soybean-meal based diets for growing pigs. The high demand for growth and protein accretion means that serine catabolism to Gly is downregulated in favor of serine production. Gly synthesis from choline is also very limited, as the concentration of choline in sow milk and traditional growing-finishing diets of pigs is very low, and available dietary choline will be used for specific cell functions such as synthesis of phosphatidylcholine and acetylcholine (Hou et al. 2019; Li and Wu 2017). Therefore, in sow-reared piglets, approximately 36 mg/kg body weight/day of Gly will be synthesized from choline and choline esters, compared to the estimated requirement for 1.05 g Gly/kg body weight/day to meet demands of growing piglets to support gains in body weight (Wang et al. 2013a). Additionally, the amount of Gly synthesized from threonine each day is also very limited and accounts for 33 mg/kg body weight/day at most (Wang et al. 2013a). 4-Hydroxyproline accounts for the majority of Gly synthesis is preweaning and growing-finishing pigs;

however, IUGR pigs have very underdeveloped pathways for Gly synthesis from 4-hydroxyproline (Hu et al. 2017). This indicates a potential for prolonged Gly deficiencies in postweaning and growing IUGR pigs, leading to reduced growth and survivability of these animals.

The Gly present in the diet will be used primarily to support protein accretion during the rapid growth of preweaning and postweaning pigs. About 80% of all dietary Gly will be utilized for protein synthesis as Gly represents 11.5% of total amino acids in body protein of animals (Wang et al. 2013a; Wu et al. 2013; Yan and Sun 1997). Thus, 20% of the remaining requirements for Gly will have to be met by that in dietary sources and endogenous synthesis. Glycine is the primary substrate for many important metabolites such as purines, bile acid, heme, hepatic glutathione, and especially creatine (Brosnan et al. 2009; Hall 1998; Hellwing et al. 2007; Reeds et al. 1997; Wang et al. 2013a). To support all metabolic functions in animals, there is a requirement for a total of 189 mg/kg bodyweight/day Gly and, of that, 96 mg/kg bodyweight/day of Gly is oxidized to ammonia and CO_2 each day (Hu et al. 2017). These functions make up the required 1,204 mg/kg bodyweight/day requirement for optimal growth and health in growing pigs (Li and Wu 2017; Hu et al. 2017). Sow's milk and traditional diets for growing pigs are very low in Gly content (Hou et al. 2019); therefore, the majority of the Gly must come from endogenous sources, and hydroxyproline will be the major substrate to supply the Gly, but this option is limited in IUGR pigs that have underdeveloped pathways for synthesis of Gly (Hu et al. 2017). This will be a limiting factor for the growth and development of IUGR pigs for many reasons, but especially for creatine synthesis.

For each mol of creatine synthesized de novo, an entire molecule of Gly is used. The entire Gly molecule is incorporated into one mol of creatine (Wu 2013a). Creatine is only degraded via the irreversible and spontaneous conversion to creatinine which is filtered into the urine as a waste product. There is no metabolic pathway for dietary or endogenous creatine to serve as a substrate for Gly synthesis. Additionally, traditional diets for postweaning pigs are very low in creatine content, and direct dietary supplementation of creatine to non-exercising and metabolically normal animals is ineffective in improving muscle mass (Dolan et al. 2019; Hou et al. 2019; Marzuca-Nassr et al. 2019). Overall, creatine synthesis accounts for 14.5% of daily total Gly (diet + endogenous synthesis) usage, and 87% of dietary Gly usage that is a considerable drain on non-proteinogenic Gly demands each day (Melendez-Hevia 2009; Wu and Morris 1998). An adequate supply of creatine is important, however, as creatine plays many unique and vital roles in promoting both growth and health in young growing animals.

Creatine

Creatine was originally isolated from bovine skeletal muscle in 1832 by Michel Eugène Chevreul. He named the compound after the Greek word for meat, “kreas” owning to the tissue from which it was originally isolated. Therefore, to no surprise, creatine (CR) is found in highest concentrations in animal tissues and absent from all plant-sourced foods (Hou et al. 2019). Creatine is synthesized from three amino acids (Gly, arginine, and methionine) and plays many important functional roles in human health, especially in skeletal muscle growth and health (Brosnan and Brosnan, 2007). CR is not an AA. Based on CR synthesis, Gly should be considered a CEAA in growing pigs, and arginine is already considered a CEAA in many species and physiological conditions (Wu 2013a). Therefore,

while pigs do possess the necessary enzymes for synthesizing CR de novo, it is essential that, as a necessary precursor AA for synthesis of CR, Gly must be provided in adequate amounts in the diet to avoid deficiencies in CR (Li et al. 2011; Watford & Wu 2011).

Synthesis of CR via the renal-hepatic axis

In terrestrial mammals and birds, CR is synthesized via the renal-hepatic axis, as shown in Figure 1.3 (Wyss and Kaddurah-Daouk 2000; Edison et al. 2007). The pathway for synthesis of CR requires only two primary enzymes that are highly tissue-specific to allow for multiple levels of regulation. Synthesis begins in the kidneys where a two-step reaction is catalyzed by arginine:glycine amidinotransferase (AGAT, a mitochondrial enzyme). AGAT removes the amidino group from arginine, resulting in the formation of ornithine. The second step of this reaction is the addition of the amidino group to the alpha carbon of the Gly molecule, resulting in formation of guanidinoacetate (GA). While AGAT activity is highest in the kidneys, it is also active in the pancreas where it does not appear to be regulated by the same mechanisms as it is in the kidneys (Da Silva et al. 2009; Da Silva et al. 2014; Van Pilsum et al. 1972). Once GA is generated from Gly and arginine, it is transported through the blood circulation and enters the liver through the hepatic artery. In the liver, GA is methylated to form CR in a reaction catalyzed by guanidinoacetate N-methyltransferase (GAMT, a cytosolic enzyme). This reaction is dependent on the availability of methionine as S-adenosylmethionine (SAM) for methylation (Wu 2013a). SAM is a common methylating compound and participates in many methylation reactions throughout the body. The demethylated cofactor released from the reaction is S-adenosylhomocysteine (SAH), which will be remethylated in a transmethylation step of the folate and one-carbon metabolism pathways that also rely on an adequate supply of Gly for

functionality (Amelio et al. 2014). There are other tissues with GAMT activity such as the testes, the brain, the spleen, and the pancreas; however, due to organ mass, the liver is the primary site for GAMT production of CR in mammals, including swine (Brosnan 2011; Da Silva et al. 2014; Van Pilsum et al. 1972). However, isolated rat pancreatic islets in culture are able to produce CR without the participation of other organs, and synthesis of CR is not likely regulated at the same levels as that for renal AGAT (Da Silva et al. 2014). However, again, due to tissue mass, the overall contribution of the pancreas to total CR synthesis is likely minor, compared to that of the renal-hepatic axis.

Regulation for the pathway for synthesis of CR is at the AGAT step of synthesis, with feedback inhibition being the strongest (Da Silva et al. 2008). Increased dietary intake of CR decreases overall AGAT activity in rats. This mechanism likely conserves arginine and methionine when CR is in ready supply, and regulation occurs at the pre-translation level for AGAT mRNA in the kidneys, but not the pancreas (Da Silva et al. 2014; Nasrallah et al. 2010). Ornithine, one of the direct products of AGAT activity very strongly downregulates AGAT activity in the kidneys (Walker 1979), but this may not be relevant in vivo because ornithine is actively metabolized in many tissues via multiple pathways (Wu and Morris 1998). Additionally, downregulation of AGAT occurs following thyroidectomy or hypophysectomy in rats, and activity was restored with T4 and growth hormone injections, indicating hormonal and dietary influences on AGAT activity (Van Pilsum et al. 1982). Growth hormone also has a regulatory effect on AGAT expression and activity in rats (Wu and Morris 1992). There are not many factors that activate AGAT besides substrate availability (Nasrallah et al. 2010). Folic acid deficiency is one example of non-substrate activation of the AGAT enzyme; however, this is more likely due to the severe decrease in

CR production. During folic acid deficiency, the AGAT enzyme becomes overexpressed as rates of CR synthesis are reduced due to low intracellular concentrations of SAM, the necessary cofactor for the second step in synthesis of CR. Additionally, SAM and methionine levels in the liver are especially low during folic acid deficiency, which is the major site of CR synthesis (Duncan et al. 2013). There are no confirmed regulatory mechanisms influencing GAMT activity, further indicating that AGAT is the rate limiting step for CR synthesis. Thus, an adequate supply of Gly is necessary for CR synthesis (Da Silva et al. 2008; Nasrallah et al. 2010)

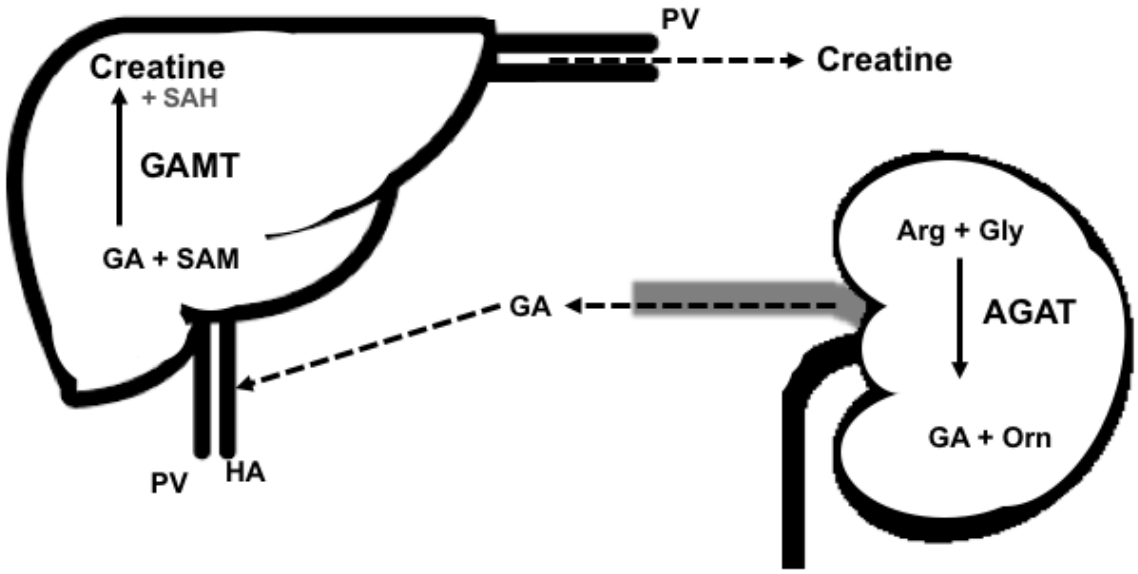


Figure 1.3. The primary pathway for synthesis of creatine involves inter-organ cooperation of the renal-hepatic axis. HA: hepatic artery; PV: portal vein; HV: hepatic vein; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; GAMT: guanidinoacetate N-methyltransferase; AGAT: L-arginine:glycine amidinotransferase; GA: guanidinoacetate. Adapted from da Silva et al. 2008.

The synthesis of CR may impose a particularly large metabolic burden on rapidly growing young animals, such as growing-finishing pigs (Brosnan et al. 2009; Riedijk et al. 2007). It is estimated that 14.5% of all Gly (dietary + endogenous) each day is utilized for

synthesis of CR, with 87% of strictly dietary Gly being used for CR synthesis (Wu and Morris 1998). The calculations for the total contribution of Gly (dietary + endogenous) to CR synthesis suggest that 2,902 mg Gly/day is the total sum of Gly from all dietary and endogenous sources in humans, and that 420 mg of this is required for CR synthesis each day (Melendez-Hevia et al. 2009). As IUGR pigs have diminished capabilities for endogenous synthesis of Gly, (Hu et al. 2017), it is likely that CR synthesis places an even greater burden on IUGR growing pigs.

Catabolism of CR via spontaneous conversion to creatinine

The catabolism of CR is very simple. It is either converted to creatine phosphate (CRP) (the storage form of CR with 95% stored in skeletal muscle) or it undergoes spontaneous cyclization to creatinine (CRN) (Brosnan and Brosnan 2007). Both CR and CRP can spontaneously and irreversibly convert to CRN at a rate of approximately 1.7% of total CR (CR + CRP) per day under physiological conditions. Creatinine is then filtered by the kidneys and is excreted in the urine. Therefore, conversion of CR/CRP to CRN represents an irreversible loss of total CR each day (Brosnan and Brosnan 2007; Cockcroft and Gault 1976; Kan et al. 2006; Wyss and Kaddurah-Daouk 2000). The conversion of CR into CRP is reversible and catalyzed by creatine kinase (CK), which exists in cytoplasmic form as creatine kinase-muscle type (CKM) and creatine kinase-brain type (CKB), and as two forms of mitochondrial enzyme, (MtCK), sarcomeric (sMtCK) and ubiquitous (uMtCK), (McGilvery and Murray 1974; Payne and Strauss 1994) The CKM enzyme is incredibly active within skeletal muscle tissue, the primary site of CRP storage, and in brain and other tissues as CKB transcript (Keller and Gordon 1991; Trask and Billadello 1990).

Creatine plays many roles in human health but is essential for proper energy metabolism in the brain and skeletal muscle, as well as other tissues such as placentae and testes (Wyss and Kaddurah-Daouk 2000). Creatine aids in energy metabolism via (1) conversion to CRP that is an easily accessible "energy pool" for rapid regeneration of ATP from ADP within tissues that contain CK, especially skeletal muscle; and (2) stability of cell membranes as CR decreases concentrations of calcium within the cell by stimulating calcium uptake by the sarcoplasmic reticulum, helping to stabilize membrane potential (Bender et al. 2005; Fortalezas et al. 2018; Wu 2013a). Creatine is also an antioxidant that helps to decrease damage due to oxidative stress associated with cardiac disease and to decrease overall systemic inflammation (Almeida et al. 2006; Wu 2020). A summary of the physiological functions of CR are summarized in Table 1.1 (adapted from Wu 2020).

Table 1.1 Summary of the physiological functions of creatine in the body

Physiological Functions and Advantages of Creatine Intake	
Location	Function
Brain	Energy metabolism; mental development; decreases intracellular calcium concentrations/increases intracellular glutamate to prevent occurrences of random membrane depolarization; free radical scavenger (antioxidant); anti-apoptotic; prevention of mitochondrial disorders/damage; reduction of neurological damage from ischemia/anoxia following a stroke; treatment of neurological degradation disorders such as: Alzheimer's, Huntington's, and Parkinson's diseases; improve cognitive function; decrease traumatic brain injury effects
Skeletal Muscle	Energy metabolism; free radical scavenger (antioxidant); anti-apoptotic; prevention of mitochondrial disorders/damage; mitigates skeletal muscle dysfunction; prevent skeletal muscle atrophy; aids in rehabilitation for tendon injury

Cardiac Muscle	Decrease in damage from ischemia/anoxia following a heart attack, treatment of congestive heart failure
Immune System	Protection from infectious diseases (provides for increased availability of arginine which allows for increased nitric oxide production); therapeutic for cancer treatment; decrease systemic inflammation

Adapted from Wu 2020.

Summary and objectives

Intrauterine growth restriction represents huge losses in profit and animals each year in the swine industry. While this is a naturally occurring phenomenon (affecting 20-25% of all pigs born), the effect on the swine industry is indelible, as IUGR pigs are born at one-half to one-third of their normal birthweight siblings (< 1.1 kg). IUGR pigs are usually culled at birth as they are unlikely to survive until weaning and, if they do, they will not reach market weight at the same rate as their normal birthweight siblings. This is due to underdeveloped and undersized skeletal muscle tissue and small intestines that likely contribute to issues with the utilization of nutrients, especially AAs such as Gly (Wu et al. 2010). While Gly can be synthesized endogenously from serine, threonine, choline, and hydroxyproline, it has been incorrectly classified as an NEAA. Glycine is a functional AA with many roles in optimal growth and overall health, including linking interorgan metabolism, one-carbon metabolism, antioxidant activity, gastrointestinal protectant, and regulator of neurotransmission as a neuroprotective (Wang et al. 2013a; Wu 2010). Therefore, Gly requirements must be met each day to ensure proper growth and metabolism. However, conventional diets of growing swine fail to provide adequate amounts of dietary Gly, placing the burden of Gly synthesis on the endogenous pathways for synthesis that is

not sufficient to meet the metabolic requirements for post-weaning piglets. Additionally, IUGR pigs have an underdeveloped capacity for the endogenous synthesis of Gly (Hu et al. 2017), resulting in an even greater deficit in available Gly (Powell et al. 2011).

One of the many effects of Gly deficiency is a failure to synthesize adequate amounts of creatine, a metabolite formed from three amino acids, including an entire molecule of Gly for each mol of creatine – using 87% of total dietary Gly intake each day. Creatine synthesis places a heavy drain on dietary Gly that is provided in inadequate amounts in diets. Creatine, primarily as CrP, is actively involved in cellular energy metabolism and. growth through skeletal muscle accretion essential for the high growth rates in modern swine production. Cr is also an antioxidant and cell protectant, as it helps to stabilize cellular membranes in excitable tissues such as skeletal muscle and brain. Creatine is lost at an irreversible rate of 1.7% through spontaneous conversion to creatinine each day (Brosnan and Brosnan 2007). However, a direct dietary supply of creatine is not only ineffective in promoting skeletal muscle accretion, but can exert a strong negative effect on AGAT, the rate-limiting enzyme for creatine synthesis, further decreasing the rate of de novo synthesis of creatine. Interestingly, AGAT activity is upregulated by substrate availability, so providing substrates for AGAT (arginine and glycine) improved overall rates of creatine synthesis (Edison et al. 2007). Dietary supplementation of arginine improves overall growth and carcass quality (Wu et al. 2013; 2018), and dietary Gly supplementation to preweaning pigs improves growth prior to weaning (Wang et al. 2014). However, the effect of dietary Gly supplementation on growing and finishing pigs postweaning and through finishing to market weight is not known. Furthermore, effects of dietary Gly on on growth, survivability, and carcass quality of IUGR pigs that are raised to market weight is not known.

The central hypothesis of the research for this dissertation is that glycine deficiency due to inadequate dietary provision and insufficient endogenous glycine synthesis pathways in IUGR pigs causes a deficiency in creatine. Further, this creatine deficiency contributes greatly to the failure of IUGR pigs to thrive postweaning and experience suboptimal growth compared to normal birthweight pigs during the postweaning and growing-finishing periods. The overall objectives of this dissertation research were to: (1) identify the effects of dietary glycine supplementation on creatine synthesis and postweaning growth of IUGR pigs compared to pigs with a normal birthweight; (2) determine the extent of the effects of increased creatine availability on growth, survivability, and carcass quality of IUGR pigs compared to normal birthweight pigs; and (3) determine the validity of standardizing dietary glycine supplementation as an intervention protocol for rescuing IUGR pigs following weaning.

Results from this research are expected to indicate direct actionable interventions for swine producers to use in order to save IUGR animals from being wasted, as well as increasing annual profits for producers each year. Additionally, these findings will allow for understanding of Gly as a nutritional intervention to promote the growth of IUGR pigs and elucidate the potential biochemical mechanisms that are responsible for regulating growth and development of neonates. Finally, this research will identify how IUGR may impact creatine synthesis in pigs. Novel findings from this dietary supplementation of glycine study—helping animals achieve optimal growth and survival despite low birthweights—will have far-reaching implications for the swine industry and animal agriculture, as well as provide evidence for a biochemical basis for new interventions that benefit the growth and survival of growth-restricted and premature infants.

CHAPTER II

ANALYSIS OF EFFECTS OF DIETARY GLYCINE SUPPLEMENTATION ON BODY WEIGHT AND MEAT QUALITY OF IUGR AND NORMAL PIGS

Introduction

Glycine is a functional amino acid that plays a wide variety of roles in maintaining homeostasis, but has especially vital roles in both muscle protein synthesis and nucleotide synthesis, as well as antioxidative effects (Fan et al. 2019; Wang et al. 2013a). These are vital factors for promotion of growth and development in neonatal and growing pigs. However, despite the importance of this amino acid, it is present in low concentrations in both sow's milk and traditional corn- and soybean meal-based diets for pigs (Flynn et al. 2000; Hou et al. 2019; Li at el. 2011; Li and Wu 2018). Inadequate provision of glycine during early neonatal life and throughout growing and finishing phases places the burden of glycine provision on endogenous pathways for its synthesis in animals (Wu 2010). Additionally, endogenous synthesis of Gly does not meet the metabolic requirements for post-weaning piglets (Powell et al. 2011). This drain on endogenous resources, such as Gly, provides a challenge to achieving maximal growth for neonatal pigs, especially neonatal IUGR pigs with below average body weights and reduced antioxidative capacity (Wang et al. 2008). Even more concerning is that IUGR pigs represent 20-25% of all pigs born in the swine industry (Wu et al. 2010). IUGR pigs are usually culled by producers at birth as most (76%) of them will die before weaning, not reach market weight at the same time as their normal birthweight litter mates or require an additional week of feeding to reach market weight (Ji et al. 2017). This represents a huge profit lost for producers, as well as a waste of

piglets that could be beneficial to a productive and efficient swine enterprise. Additionally, when IUGR pigs are raised to market weight, the carcass yields are traditionally less and meat quality is reduced, with smaller total areas and lower weights of desired cuts of meat, and higher percentages of undesirable fat depots (Nissen and Oksbjerg 2011). However, there is currently no intervention strategy for rescuing IUGR piglets with respect to survival and growth to market weight. Of note, Hu et al. (2017) reported that IUGR pigs have severely underdeveloped pathways for glycine synthesis. But, dietary glycine supplementation increases muscle protein deposition in both mouse myoblast cell lines (C2CC12) and weanling pigs, and decreases muscle protein degradation, leading to a net increase in overall protein accretion in skeletal muscle (Sun et al., 2016, Wang et al. 2014, Wu et al. 2013., Hu et al., 2017). The increases in skeletal muscle mass and body weight, desirable proportions of carcass lean tissues and less fat in carcasses of pigs. This may also result in improved carcass yields for IUGR pigs as dietary Gly supplementation may prove to be a novel interventional strategy for rescuing IUGR animals by enhancing their growth potential to meet or exceed rates of body weight gain relative to their normal birth weight siblings, and also improve overall carcass yields at market weight. To determine the validity of this intervention strategy, a feeding experiment including both normal birth weight pigs and IUGR pigs was designed to determine effects on body weight gain, feed efficiency, and overall carcass quality following dietary glycine supplementation.

Materials and methods

Animals and diets

The experimental protocol for this study was approved prior to beginning the study by the Texas A&M University Animal Care and Use Committee (IACUC).

Piglets from Yorkshire x Landrace sows bred to Duroc x Hampshire boars were obtained at 21 days of age (n=34), following weaning. Pigs were maintained in a climate-controlled room between 21 and 64 days of age at the Swine Center of the O.D. Butler Jr Animal Science Complex, and in outside pens of the Swine Center between 64 and 188 days of age. It should be noted that during Days 120-148 the type of feeder had to be changed to accommodate the growing pigs. This impacted feed intake and growth for a short time while pigs acclimated.

Pigs had free access to a corn-and-soybean-meal based diet from Producer's Co-Operative Feed Mill in Bryan, TX (Table 2.1). The diet fed was a 21% crude protein diet from d 21 to d 64 of age (Wu et al. 1996) and then transitioned to an 18% crude protein diet from d 65 to d 108 of age. At d 108 of age, pigs were transitioned to a 16% crude protein diet, and then transitioned to a final diet of 14% crude protein from d 147 to d 188 of age. The pigs remained on the 14% crude protein diet for the remainder of the experiment until they reached market weight.

Table 2.1. Composition of Diets Fed to the Pigs

	18% Crude Protein Diet		16% Crude Protein Diet		14% Crude Protein Diet	
Crude Protein	18	%	16	%	14	%
Metabolizable Energy (ME)	3082	kcal/kg	3124	kcal/kg	3089	kcal/kg
Lysine	1.05	%	0.75	%	0.60	%
Crude Fat	1.04	%	3.45	%	2.50	%
Crude Fiber	3.80	%	4.50	%	5.00	%
Calcium	0.50	%	0.60	%	0.45	%
Calcium	0.90	%	1.00	%	0.85	%
Phosphorus	0.57	%	0.75	%	0.55	%
Salt	0.20	%	0.40	%	0.40	%
Salt	0.60	%	0.80	%	0.80	%
Selenium	0.30	PPM	0.30	PPM	0.30	PPM
Zinc	150	PPM	150	PPM	150	PPM

Assignment of piglets at weaning to dietary treatment based on birth weight

At weaning (21 d of age), pigs were assigned to dietary treatment based on their birth weight, either IUGR or normal body weight. Pigs that were below 1.1 kg in birth weight were designated as IUGR and pigs that weighed 1.1 kg or more were designated as normal pigs. The IUGR and normal pigs were then assigned randomly to either the control or the treatment diet. Pigs in the treatment groups received 1% glycine (Gly) plus 0.19% cornstarch, and those in the control groups received 1.19% alanine (Ala) as an isonitrogenous control. The four treatment groups were:

Group 1: IUGR – Supplemented with 1% Gly + 0.19% cornstarch (n=7)

Group 2: IUGR – Supplemented with 1.19% Ala (Control) (n=7)

Group 3: Normal – Supplemented with 1% Gly + 0.19% cornstarch (n=10)

Group 4: Normal – Supplemented with 1.19% Ala (Control) (n=10)

Pigs had free access to their respective diets and were weighed biweekly to monitor the effects of Gly supplementation on body weight gain throughout the experiment.

Termination of feeding trial and assessment of carcass quality

The feeding trial was terminated once pigs reached 188 d of age. Pigs were slaughtered according to HAACP protocols at Texas A&M University's Rosenthal Meat Science Center and tissues collected for further analyses. Graduate students in the meat science section then processed the pig carcasses according to standard pork processing protocols to allow for assessment of meat quality by members of the Meat Science section of the Department of Animal Science, Texas A&M University.

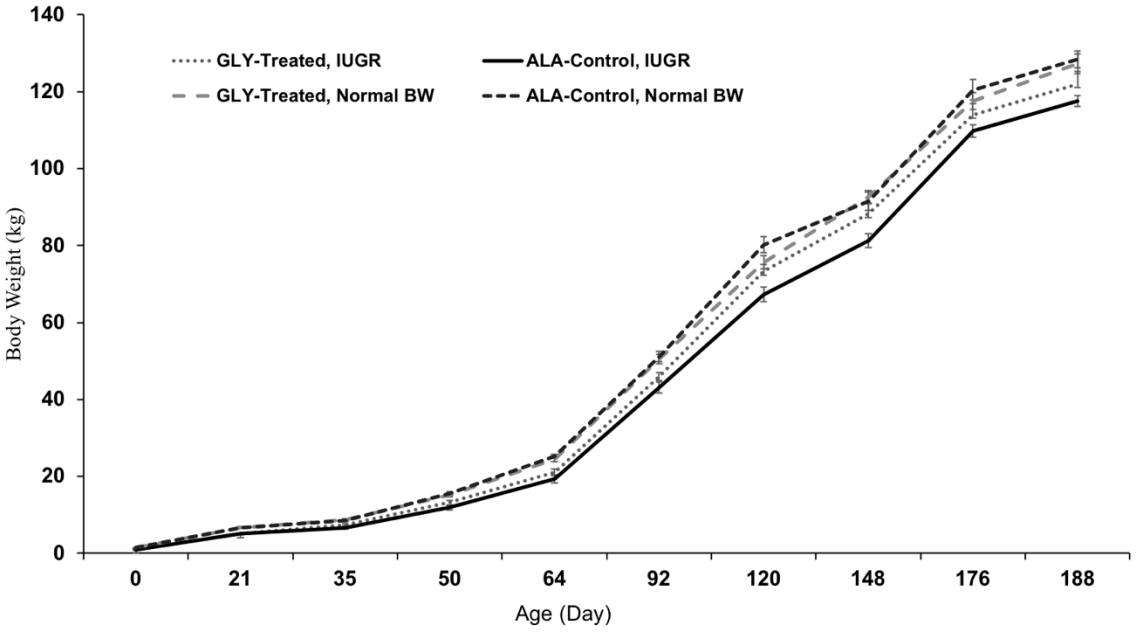
Statistical Analyses

Results are expressed as means ± SEM. Analyses of data were performed using JMP 15 Pro software for two-way analysis of variance using General Linear Models procedures and testing for main effects of birthweight status ("IUGR" vs "normal"), dietary treatment and their interaction. Differences due to treatment effects were determined using the Duncan multiple comparison test. Data were first tested for normality using the Shapiro-Wilk W Test and confirmed by a probability of > 0.05. A probability value for ANOVA of $P \leq 0.05$ was taken to indicate statistical significance.

Results

The effects of dietary supplementation with 1% Gly on the growth of IUGR pigs and normal birthweight pigs are summarized in Figure 2.1. Dietary Gly supplementation enhanced growth of the IUGR pigs from d 21 to d 148, 127 days post-weaning ($P < 0.05$),

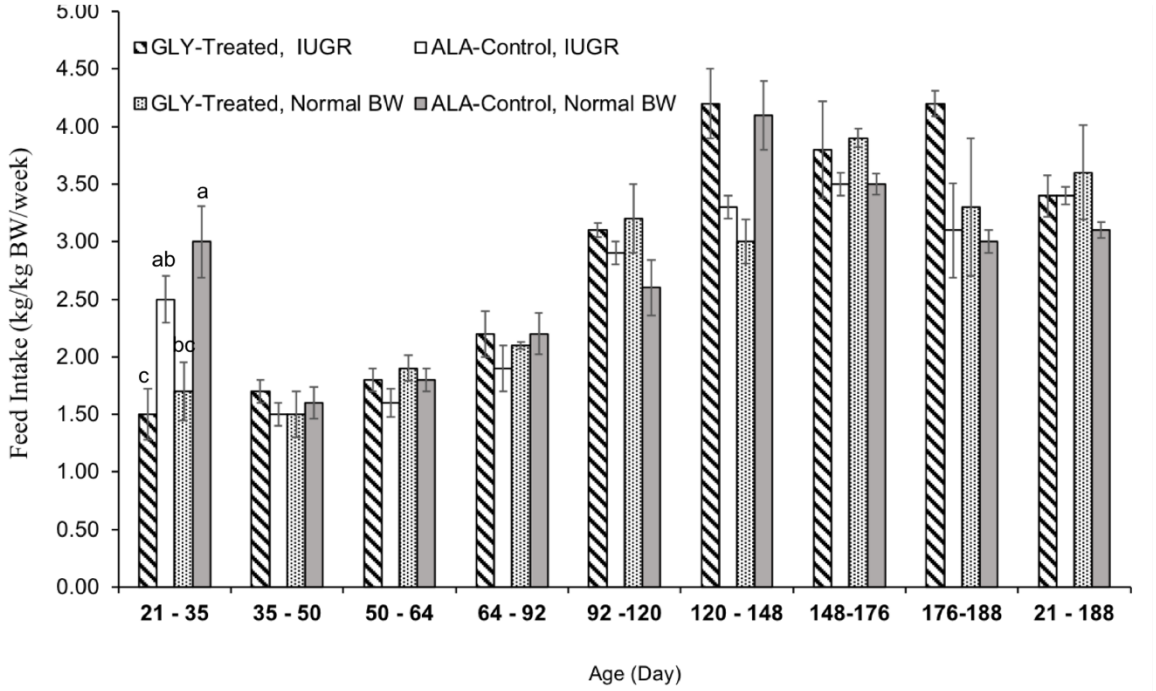
and continued to follow this same trend to d 188. Dietary supplementation of Gly did not have an effect on the body weight of normal birthweight pigs ($P > 0.05$) from d 0 to d 188. As feed intake and body weight are the factors associated with feed efficiency, these data were further analyzed to reveal that there was also no effect on feed efficiency following dietary supplementation with Gly ($P > 0.05$). This is illustrated in Figure 2.3. It should be noted that the decrease in gain:feed ratio during the d120-d148 time period was likely due to a change in type of feeder. This change had to be made to accommodate growing pigs, but did have a brief impact on feed intake as well as growth.



Mean body weights (kg) with standard errors of the means					P Values of Treatment Effects		
	IUGR		Normal Birth Weight				
Age of Pigs	Gly-Treated (n=6)	Ala-Control (n=7)	Gly-Treated (n=10)	Ala-Control (n=9)	IUGR Effect	Gly Effect	IUGR x Gly Effect
Day 0	1.03 ± 0.03^{a}	0.95 ± 0.06^{a}	1.45 ± 0.03^{b}	1.47 ± 0.02^{b}	<0.0001	0.618	0.359
Day 21	5.04 ± 0.22^{a}	5.04 ± 0.24^{a}	6.66 ± 0.19^{b}	6.65 ± 0.18^{b}	0.001	0.887	0.866
Day 35	7.32 ± 0.42^{a}	6.70 ± 0.33^{a}	8.75 ± 0.3^{b}	8.46 ± 0.23^{b}	0.001	0.279	0.754
Day 50	13.4 ± 0.81^{a}	12.0 ± 0.62^{a}	15.2 ± 0.56^{b}	15.6 ± 0.43^{b}	0.001	0.283	0.492
Day 64	21.1 ± 0.81^{a}	19.3 ± 1.1^{a}	24.6 ± 0.69^{b}	25.2 ± 0.52^{b}	0.0004	0.288	0.384
Day 92	45.9 ± 1.0^{a}	43.1 ± 1.5^{a}	50.5 ± 1.2^{b}	51.2 ± 1.3^{b}	0.002	0.160	0.333
Day 120	73.3 ± 2.0^{b}	67.3 ± 1.9^{c}	75.6 ± 1.7^{ab}	80.2 ± 2.1^{a}	0.003	0.378	0.033
Day 148	88.2 ± 2.7^{a}	81.3 ± 1.8^{b}	92.7 ± 1.6^{a}	91.6 ± 2.4^{a}	0.001	0.025	0.100
Day 176	114.0 ± 2.8^{bc}	109.8 ± 1.6^{c}	120.0 ± 2.1^{ab}	120.5 ± 2.7^{a}	0.005	0.787	0.203
Day 188	122.0 ± 3.2^{bc}	117.6 ± 1.4^{c}	127.3 ± 2.6^{ab}	128.5 ± 2.2^{a}	0.007	0.7420	0.384

a-c: Within a row, means not sharing the same superscript letter are different ($P < 0.05$).

Figure 2.1. The body weights of IUGR and normal birthweight pigs from birth (Day 0) to market weight (Day 188). Following dietary supplementation with either glycine (Gly, 1% of the diet) or Ala (1% of the diet as isonitrogenous control) from weaning at 21 d of age to slaughter at 188 d of age. These results indicated that Gly supplementation allows IUGR pigs to reach market at weights (d 188) comparable to those for normal pigs. The compensatory gain for IUGR pigs is detectable by d120 of treatment (approximately 100 days of age). Values are expressed as means ± SEM. Different superscript letters indicate statistical difference ($P < 0.05$) according to analysis by two-way ANOVA linear model of multiple effects. Day 0 = 21 days of age.



Feed Intake Per Kilogram of Bodyweight (kg/kg BW/week)					P-Value of Treatment Effects		
	IUGR		Normal Birth Weight				
Age of Pigs	Gly-Treated (n=6)	Ala-Control (n=7)	Gly-Treated (n=10)	Ala-Control (n=9)	IUGR Effect	Gly Effect	IUGR x Gly Effect
Days 21- 35	1.5 ± 0.22^{c}	2.5 ± 0.20^{ab}	1.7 ± 0.25^{bc}	3.0 ± 0.31^{a}	0.185	0.002	0.409
Days 35-50	1.7 ± 0.10	1.5 ± 0.10	1.5 ± 0.20	1.6 ± 0.14	0.808	0.713	0.867
Days 50-64	1.8 ± 0.10	1.6 ± 0.12	1.9 ± 0.11	1.8 ± 0.10	0.201	0.171	0.961
Days 64-92	2.2 ± 0.20	1.9 ± 0.10	2.1 ± 0.03	2.2 ± 0.18	0.505	0.510	0.150
Days 92-120	3.1 ± 0.06	2.9 ± 0.10	3.2 ± 0.30	2.6 ± 0.24	0.716	0.079	0.237
Days 120-148	4.2 ± 0.30	3.3 ± 0.10	3.0 ± 0.19	4.1 ± 0.30	0.875	0.876	0.246
Days 148-176	3.8 ± 0.42	3.5 ± 0.10	3.9 ± 0.08	3.5 ± 0.09	0.886	0.136	0.847
Days 176-188	4.2 ± 0.11	3.1 ± 0.41	3.3 ± 0.60	3.0 ± 0.10	0.258	0.092	0.277
Days 21-188	3.4 ± 0.18	3.4 ± 0.08	3.6 ± 0.41	3.1 ± 0.07	0.848	0.333	0.354

a-c: Within a row, means not sharing the same superscript letter differ ($P < 0.05$).

Figure 2.2. Feed intake for IUGR and normal birthweight pigs was determined from 2 weeks into treatment (Age: 35 d) to d 127 post-weaning (Age: 148 d) when fed a diet supplemented with either (Gly, 1% of diet) or the isonitrogenous control, alanine (Ala, 1% of diet) beginning at weaning (Age: Day 21). No effect of diet on feed intake was detected for either IUGR or normal BW pigs. This can be seen beginning at Day 64 of age (approximately 6 weeks of treatment). Values are expressed as means ± SEM. Superscripts (a-c) on means in a row without a common letter indicate a statistical difference (P < 0.05) according to analysis by two-way ANOVA linear model of multiple effects.

Table 2.2 Body weight gain for IUGR and normal birthweight pigs

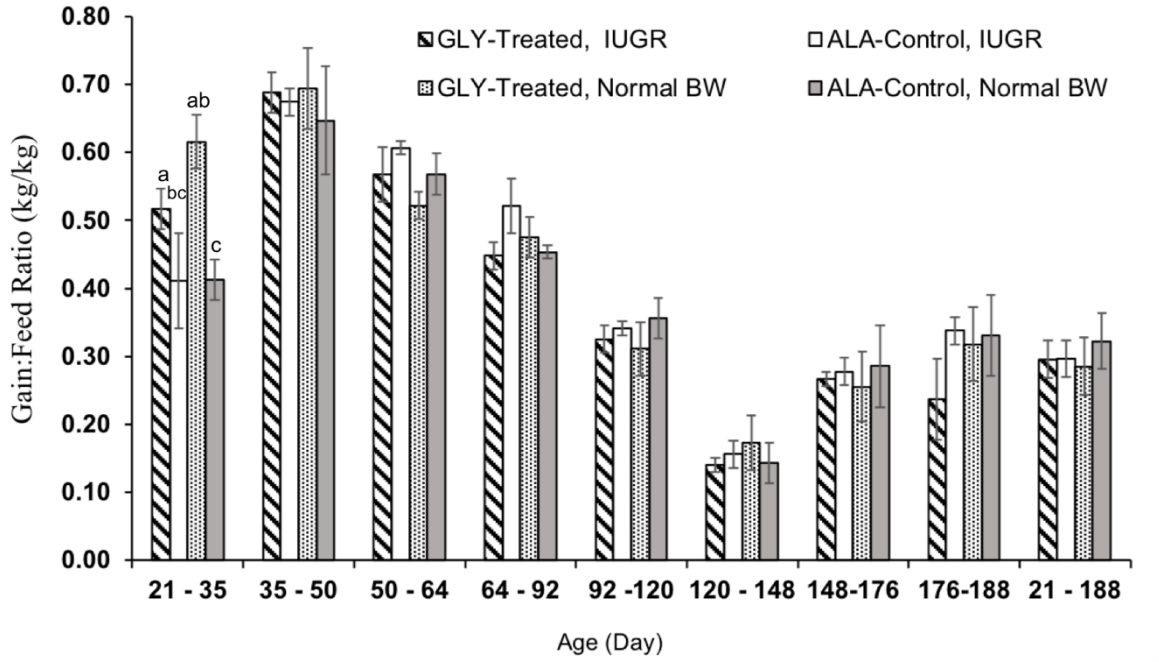
Body weight gains (kg/pig)					P-Values of Treatment Effect		
	IUGR		Normal Birth Weight				
Age of Pigs	Gly-Treated (n=6)	Ala-Control (n=7)	Gly-Treated (n=10)	Ala-Control (n=9)	IUGR Effect	Gly Effect	IUGR x Gly Effect
Day 21-35	2.19 ± 0.65	1.68 ± 0.53	2.43 ± 0.89	1.86 ± 0.87	0.292	0.010	0.605
Day 35-50	5.81 ± 1.6^{ab}	5.18 ± 0.46^{b}	6.72 ± 3.0^{a}	7.16 ± 2.1^{a}	0.015	0.840	0.292
Day 50-64	7.91 ± 1.8^{ab}	7.11 ± 0.74^{b}	9.49 ± 1.9^{a}	9.54 ± 1.8^{a}	0.014	0.565	0.539
Day 64-92	25.7 ± 6.7	24.1 ± 0.93	26.2 ± 6.7	25.7 ± 5.1	0.350	0.344	0.615
Day 92-120	27.19 ± 6.1	24.5 ± 1.1	25.3 ± 4.4	29.0 ± 8.4	0.072	0.212	0.006
Day 120-148	13.6 ± 2.6	13.9 ± 1.6	15.9 ± 5.4	11.7 ± 7.8	0.943	0.507	0.399
Days 148-176	26.2 ± 7.5	27.8 ± 0.70	27.2 ± 8.6	31.41 ± 4.0	0.470	0.253	0.710
Days 176-188	7.63 ± 2.6	10.1 ± 1.2	9.80 ± 0.59	10.7 ± 1.0	0.324	0.223	0.543
Day 21-188	116 ± 2.0	107 ± 0.82	121 ± 3.1	127 ± 5.0	0.128	0.700	0.050

a-c: Within a row, means not sharing the same superscript letter differ ($P < 0.05$).

Table 2.3. Feed intake for IUGR and normal birthweight pigs

Feed Intake (kg/pig)					P-Values of Treatments Effect		
	IUGR		Normal Birth Weight				
Age of Pigs	Gly-Treated (n=6)	Ala-Control (n=7)	Gly-Treated (n=10)	Ala-Control (n=9)	IUGR Effect	Gly Effect	IUGR x Gly Effect
Days 21- 35	4.24 ± 0.16	4.09 ± 0.19	3.95 ± 0.54	4.50 ± 0.42	0.875	0.591	0.367
Days 35-50	8.44 ± 0.15^{a}	7.67 ± 0.50^{a}	9.68 ± 0.64^{a}	11.1 ± 0.97^{a}	0.007	0.647	0.130
Days 50-64	13.9 ± 1.2^{a}	11.7 ± 2.1^{a}	18.2 ± 1.6^{a}	16.8 ± 0.25^{a}	0.012	0.241	0.778
Days 64-92	57.4 ± 6.0	46.2 ± 2.6	55.1 ± 1.2	56.7 ± 1.8	0.267	0.199	0.101
Days 92-120	83.6 ± 0.73	71.7 ± 0.78	81.5 ± 2.9	81.3 ± 5.7	0.282	0.100	0.106
Days 120-148	95.3 ± 12.4	105 ± 21.3	92.3 ± 6.2	81.4 ± 3.2	0.540	0.325	0.793
Day 148 - 176	98.3 ± 3.3	100 ± 2.9	106 ± 7.7	110 ± 2.2	0.254	0.7284	0.898
Day 176 -188	32.2 ± 6.2	30.0 ± 2.2	30.8 ± 2.2	32.2 ± 2.2	0.907	0.907	0.617
Days 21-188	394 ± 14.0	360 ± 13.4	398 ± 14.1	394 ± 13.8	0.367	0.378	0.481

a-c: Within a row, means not sharing the same superscript letter differ ($P < 0.05$).



Gain:Feed Ratio (kg/kg)					P Values for Treatment Effects		
	IUGR		Normal Birth Weight				
Age of Pigs	Gly-Treated (n=6)	Ala-Control (n=7)	Gly-Treated (n=10)	Ala-Control (n=9)	IUGR Effect	Gly Effect	IUGR x Gly Effect
Days 21-35	0.52 ± 0.03[a]	0.41 ± 0.07[bc]	0.62 ± 0.04[ab]	0.41 ± 0.03[c]	0.432	0.007	0.914
Days 35-50	0.69 ± 0.03[a]	0.67 ± 0.02[a]	0.69 ± 0.06[a]	0.65 ± 0.08[a]	0.991	0.635	0.803
Days 50-64	0.57 ± 0.04[a]	0.61 ± 0.01[a]	0.52 ± 0.02[a]	0.57 ± 0.03[a]	0.188	0.169	0.806
Days 64-92	0.45 ± 0.02[a]	0.52 ± 0.04[a]	0.48 ± 0.03[a]	0.45 ± 0.01[a]	0.393	0.410	0.140
Days 92-120	0.33 ± 0.02[a]	0.34 ± 0.01[a]	0.31 ± 0.04[a]	0.36 ± 0.03[a]	0.484	0.080	0.232
Days 120-148†	0.14 ± 0.01[a]	0.13 ± 0.02[a]	0.17 ± 0.04[a]	0.14 ± 0.03[a]	0.923	0.671	0.656
Day 148-176	0.27 ± 0.01	0.28 ± 0.02	0.26 ± 0.05	0.29 ± 0.06	0.743	0.126	0.701
Day 176-188	0.24 ± 0.06	0.34 ± 0.02	0.32 ± 0.05	0.33 ± 0.06	0.357	0.241	0.273
Days 21-188	0.30 ± 0.03[a]	0.30 ± 0.03[a]	0.29 ± 0.04[a]	0.33 ± 0.04[a]	0.653	0.349	0.338

a-c: Within a row, means not sharing the same superscript letter differ ($P < 0.05$).

† During this time the type of feeder had to be changed to accommodate pig growth. This impacted feed intake and growth measurements for a short time while pigs acclimated.

Figure 2.3. Feed efficiency (gain:feed ratio) of IUGR and normal birthweight pigs measured two weeks into treatment (Age: 35 d) to Day 127 post-weaning (Age:148 d) following treatment with either glycine (Gly, 1% of diet) or the isonitrogenous control, alanine (Ala, 1% of diet) beginning at weaning (Age: 21 d). These results indicate that Gly treatment has no effect on the feed efficiency of either IUGR or normal BW pigs. This can be seen beginning at 64 d of age (approximately 6 weeks of treatment). Values are expressed as means ± SEM. Different superscript letters indicate statistical difference (P < 0.05) according to analysis by two-way ANOVA linear model of multiple effects.

Meat Quality Effects

Table 2.4 shows the effects of dietary Gly supplementation from weaning to market weight on overall meat quality following processing.

Table 2.4 Effects of Glycine on Meat Quality					P-Values for Treatment Effects		
	IUGR		Normal Birth Weight				
	Gly-Treated (n=6)	Ala-Control (n=7)	Gly-Treated (n=10)	Ala-Control (n=9)	IUGR Effect	Gly Effect	IUGR x Gly Effect
Dressing, %	73.7 ± 0.64[b]	73.5 ± 0.59[b]	75.0 ± 0.73[a]	75.0 ± 0.43[a]	0.0264	0.7564	0.9209
Backfat* (cm)	1.78 ± 0.13[b]	2.54 ± 0.48[a]	2.18 ± 0.18[ab]	2.31 ± 0.20[ab]	0.9715	0.0459	0.0838
Loineye Area *(cm^2)	49.9 ± 2.7	47.2 ± 2.8	46.5 ± 1.8	51.7 ± 1.7	0.9389	0.7846	0.1418
Muscle Score	2.50 ± 0.22[a]	2.14 ± 0.14[ab]	2.00 ± 0.00[b]	2.29 ± 0.16[ab]	0.1301	0.6208	0.0405
FLC ** (%)	44.4 ± 1.7[a]	43.8 ± 1.1[a]	49.7 ± 0.85[b]	54.5 ± 2.0[b]	0.0001	0.3835	0.2359
Side weight (%)	99.0 ± 0.82	99.9 ± 0.38	99.6 ± 0.22	99.1 ± 0.44	0.9811	0.4122	0.2734
pH	5.46 ± 0.03	5.43 ± 0.03	5.45 ± 0.03	5.48 ± 0.04	0.7779	0.1584	0.0641

a-c: Within a row, means not sharing the same superscript letter differ ($P < 0.05$).

* 10th rib backfat; ** Four Lean Cuts (FLC)

Dietary supplementation with Gly had a positive effect on meat quality by decreasing back fat thickness for both normal birth weight pigs and IUGR pigs (Table 2.2). The backfat for normal birthweight pigs that received dietary Gly supplementation was 6% less ($P < 0.10$), compared to control pigs that received the isonitrogenous control (Ala). There was also a difference in backfat thickness in the IUGR animals that had a 30% decrease ($P < 0.10$) in backfat thickness in response to Gly supplementation. The average backfat thickness for the Gly supplemented IUGR pigs was the lowest among the four treatment groups. Additional measures of meat quality did not reveal negative effects of dietary supplementation with Gly when compared to Ala. Additionally, overall muscle score and loineye area tended to increase, though not statistically significantly for IUGR pigs supplemented with Gly. Considering that carcass quality from IUGR pigs is usually typified by lower meat yield and

increased fat deposition, these results indicate only positive effects of dietary Gly supplementation on meat quality.

Discussion

IUGR pigs represent 20-25% of all pigs born in the swine industry each year (Wu et al. 2010). Because of this, IUGR pigs represent a considerable loss to pork producers as the animals are culled at birth due low rates of survival and an inability to achieve maximum growth rates to market weight. Glycine synthesis in IUGR pigs is insufficient to meet metabolic demands (Hu et al., 2017). Concentrations of glycine are also low in plant products, including the traditional corn-and-soybean-meal-based feeds used by pork producers (Hou et al. 2019; Li at el. 2011; Li and Wu 2020). As glycine is the most abundant amino acid in tissues of pigs (Li and Wu 2018; Zhang et al. 2021) and is essential for synthesis of both nucleotides and muscle protein (Wu 2013), a glycine deficiency results in adverse effects on IUGR animals that have underdeveloped gastrointestinal tracts and skeletal muscle protein from birth. Therefore, implementing a feeding strategy of supplementing glycine at 1% of dietary intake would have large potential impact for improving body weight gain, survivability, and carcass quality.

The supplementation of 1% glycine in the traditional corn-and-soybean-meal based diets of IUGR pigs enhanced their body weight gain, compared to IUGR pigs that received only the isonitrogenous Ala control diet ($P < 0.05$). As stated previously, glycine plays a vital role in protein synthesis, especially collagen and elastin proteins, as glycine accounts for 1/3 of the amino acids in collagen and elastin (Li and Wu 2018). Glycine is also the precursor for many metabolites that improve overall growth and development. Two metabolites of note are serine and nucleic acids. Glycine contributes to serine synthesis and

the one-carbon metabolism pathway (Bazer et al. 2021), and both glycine and serine can play a direct role in protein synthesis. Additionally, through the one carbon metabolism pathway, glycine is the precursor for nucleic acids, required for growth and development. Furthermore, glycine is required for the synthesis of glutathione (a major antioxidant) and heme (a component of hemoglobin and other heme proteins). Therefore, it is likely that the dietary supplementation of glycine stimulated one or more of these pathways and contributed to the overall growth of the IUGR pigs, allowing them to attain similar market weights at the same time as normal pigs while consuming similar amounts of feed, as their normal birthweight, non-supplemented littermates.

Additionally, the carcasses of glycine supplemented IUGR pigs scored higher on key aspects of meat quality grading, indicating that the increase in body weight gain is not simply due to fat deposition. Indeed, the key positive effects of Gly supplementation to IUGR pigs included: decreased back fat thickness ($P=0.018$), increased loineye area, and increased muscle score, when compared to carcasses of non-supplemented pigs. The biochemical mechanisms whereby glycine reduced white adipose tissue in swine are unknown, but this amino acid has been reported to alleviate fat accretion and obesity in rats by improving anti-oxidative and anti-inflammatory responses (Chen et al. 2021). Glycine can stimulate lipolysis in white adipocytes and the subsequent oxidation of fatty acids to CO_2 through alleviating inflammation, thereby reducing the amount of triacylglycerols in the body. Consistent with its chemistry and physiology (Wu 2013), there were no negative effects of dietary supplementation with glycine on meat quality. Improving the quality of the final end-product, that is the carcasses of pigs, further increases the number of benefits of dietary glycine supplementation to IUGR pigs following weaning. Currently, IUGR piglets are

discarded at birth or weaning, due to the lack of intervention methods that would allow them to attain market weight at the similar rate, and with the similar feed intake, as their normal birthweight littermates. The ease and inexpensive strategy of dietary glycine supplementation makes this an attractive option for producers to utilize in managing IUGR piglets.

However, the greatest concern producers have before implementing any intervention is the overall cost and their net gain. Glycine supplementation is very inexpensive and compared to the profit lost each year due to discarding IUGR animals, the net profit gained is considerable, as summarized in Table 2.5.

Table 2.5. Summary of direct and indirect net gain from rescuing IUGR piglets at birth

Variable	No Gly supplementation	Gly supplemented to IUGR pigs from weaning to market weight
A. Direct net gain from Gly supplementation to postweaning IUGR pigs for 127 days[1]		
Amount of Gly supplemented /IUGR pig/year (kg)[2]	0.0	2.56
Cost of Gly supplementation/IUGR pig/year (US $)[3]	0.0	-2.56
Body weight gain due to Gly supplementation (kg/pig)	0.0	6.9
Value of BW gain due to Gly supplementation (US $/kg)[4]	0.0	6.9
Net Benefit/IUGR pig due to Gly supplementation (US $/pig)	0.0	+4.34
B. Indirect benefit from Gly supplementation through savings in time and labor (i.e., decrease of 10 days for raising an IUGR pig to 90 kg market weight) (US $/IUGR pig/10 days	0.0	7.5
C. Indirect benefit of saving $ on sow production loss by rescuing her IUGR offspring (US $/pig)[5]	0.0	5.5
D. Total benefit from rescuing IUGR postweaning pig and raising to market weight (US $/pig)	0.0	17.34
E. Total net benefit from saving and raising postweaning IUGR pigs/year worldwide (US $)[6]	0.0	2.51×10^9

[1]According to the FAO (2018), there are 33.4×10^6 sows and 966×10^6 newborn piglets per year worldwide. Assuming that IUGR piglets represent 15% of total newborn pigs, there are 145×10^6 IUGR pigs per year worldwide (i.e., 15% x 966×10^6).
[2] The previously explained feed intake data indicated that each IUGR pig consumed 2.56 kg Gly during a 127-day period.
[3] The cost of Gly is $1/kg (Dalian Chem Imp. & Exp Group Co., Ltd.)
[4] The average live-weight market price for pigs is US $1/kg body weight.
[5] The cost of raising a sow during gestation is $66/sow. For a litter size of 12, the cost of producing a newborn pig is $5.5/piglet. In this study, all IUGR piglets that received Gly supplementation survived the critical post-weaning period.
[6] Calculated as US $17.34/IUGR pig x 145×10^6 IUGR pigs/year worldwide.

Glycine is a very inexpensive amino acid to be used as a dietary supplement, costing between $0.40-$1.50 for 1 kg (Dalilan Chemical Import & Exp. Group Co., Ltd). The cost of supplementing Gly for the entire growing phase, when the greatest changes occurred in

this study (127 days following weaning), would cost approximately $2.56/pig. One pig represents approximately $220 as an average liveweight sale price of $1/kg BW. Since the average body weight gain due to Gly supplementation for IUGR pigs was 6.9 kg/ IUGR pig, the net profit following glycine supplementation would be $4.34/pig. Since 20-25% of all pigs born are IUGR pigs, it can be assumed that 20-25% of the piglets in all litters will be IUGR. Assuming an average litter size of 12 piglets, it is assumed that there will be 2-3 IUGR piglets per litter. Thus, the total net profit from pigs saved by dietary Gly supplementation will be between $8-13/litter. According to the Food and Agriculture Organization of the United Nations (FAO 2018), there are 33.4×10^6 sows in the world, and 966×10^6 newborn piglets (including IUGR piglets, which represent 145×10^6 pigs in that population). Remembering that each IUGR piglet represents $4.34 in net profit, dietary Gly supplementation represents $\$629 \times 10^6$ net profit for the global pork industry each year. It is worth noting that this only represents direct net profits. When considering the indirect profits from decreasing the usual amount of time it takes to feed an IUGR pig to market weight and through saving IUGR piglets at birth, the profits increase considerably. Decreasing the amount of time it takes to feed IUGR pigs to market weight by 10 days, less the cost of Gly supplementation for that pig, results in a net profit of $4.94/IUGR pig and $\$716 \times 10^6$ net profit globally. The resulting profit of saving IUGR pigs at birth, rather than culling the animals is extrapolated from the cost it takes to feed and maintain a sow during gestation, which is approximately $66. For a litter of 12 piglets, this represents a cost of $5.50/piglet to produce one litter. Since 2-3 piglets in each litter will be IUGR, the total profit lost due to IUGR piglets being culled at birth is between $11.00 and $13.50. Saving the IUGR piglets at birth will result in approximately $26-$40 indirect net profit per sow per year (assuming

2.4 farrowings per year that is common for productive sows), and a global savings of between $882 x 10^6 and $132 x 10^7.

In conclusion, dietary glycine supplementation is a viable intervention method for rescuing IUGR piglets at birth. Dietary glycine supplementation not only improves survivability of the IUGR piglets by getting them through the critical postweaning period, but also improves their final body weight at market age. IUGR pigs reached market weight at the same time as their normal birthweight littermates and did so without increasing the deposition of excessive fat. This led to improved meat quality for IUGR pig carcasses following processing. Additionally, IUGR pigs did not consume increased amounts of feed compared to their normal birthweight littermates to increase their bodyweight gain and carcass quality. Rescuing these IUGR pigs at birth, instead of culling them as is traditionally done in pork production, also leads to improved net savings for pork producers, instead of the unrealized loss of income currently experienced. Producers can gain net profit from IUGR pigs simply with the low-cost intervention of providing dietary glycine supplementation to traditional corn-and-soybean-meal-based diets. Overall, results of this study indicate that dietary Gly supplementation can lead to considerable benefits and should be considered a feasible intervention protocol for pork production enterprises to profit from management of IUGR piglets.

CHAPTER III

ANALYSIS OF EFFECTS OF DIETARY GLYCINE SUPPLEMENTATION ON CONCENTRATIONS OF CREATINE AND CREATINE PHOSPHATE IN TISSUES FROM IUGR AND NORMAL PIGS

Introduction

Glycine (Gly) is a proteinogenic amino acid that also has many functional roles in growth and overall health. While pathways for synthesis of Gly are present in swinefrom serine, threonine, choline, and 4-hydroxyproline, these synthesis pathways are inadequate for meeting daily Gly demands for growth and metabolism. Additionally, IUGR pigs have severely diminished capabilities to synthesize Gly from available precursors (Hu et al. 2017). Therefore, Gly should be classified as a conditionally essential amino acid (CEAA) and considered for supplementation in diets for all growing swine, but especially for postweaning IUGR pigs.

One of the many roles that Gly plays in overall health is as a primary substrate for creatine (CR) synthesis (Wu 2013a). Creatine is essential for proper energy metabolism in the skeletal muscle and brain and also improves survivability by aiding in membrane stability and exerting antioxidant effects (Almeida et al .2006; Bender et al. 2005; Fortalezas et al. 2018; Wu 2013a). In mammals, CR synthesis occurs via two primary enzymes, arginine:glycine amidinotransferase (AGAT) and guanidinoacetate N-methyltransferase (GAMT) located primarily in the kidneys and the liver, respectively (Da Silva et al. 2009; Wu 2013a). For each mol of CR synthesized via the renal-hepatic axis, one molecule of Gly is required. This means that 87% of dietary Gly goes to CR synthesis each day (Wu and

Morris 1998). Traditional diets for growing pigs are deficient in Gly, as they based on plant-based products such as corn and soybean meal, which have very low concentrations of Gly (Hou et al. 2019).

Creatine is stored primarily (95%) in skeletal muscle as creatine phosphate (CRP), with only 5% existing as free CR (Brosnan and Brosnan 2007). In skeletal muscle, CR as CRP participates in cellular energy metabolism and helps promote skeletal muscle growth (Wyss and Kaddurah-Daouk 2000). Additionally, both CRP and CR undergo spontaneous cyclization under physiological conditions, resulting in production of the waste product creatinine at a rate of approximately 1.7% of total CR each day. This process is irreversible, and CR or CRP that is converted creatinine will be filtered by the kidneys and excreted in the urine (Brosnan and Brosnan 2007; Cockcroft and Gault 1976; Kan et al. 2006; Wyss and Kaddurah-Daouk 2000). To gain a complete understanding of the effects of dietary supplementation on CR synthesis, total CR content (CR + CRP) in tissues must be measured.

The endogenous synthesis of CR may place a particularly large burden on rapidly growing young animals, such as growing-finishing pigs (Brosnan et al. 2009; Riedijk et al. 2007). However, direct provision of dietary CR does not have an effect on skeletal muscle accretion in physiologically normal, non-exercising animals (Marzuca-Nassr et al. 2019; Dolan et al. 2019) and may lead to an overall decrease in concentrations of tissue CR due to the strong inhibition that CR exerts on the CR synthesis pathway through inhibition of AGAT (Da Silva et al. 2008). An increase substrate availability (such as Gly) increases the activity of AGAT, and in turn increases the synthesis of CR (Edison et al. 2007). Therefore, the present study was conducted to determine the effects of dietary supplementation with

Gly on total CR content (CR+CRP) in tissues of IUGR pigs and normal birthweight pigs at the end of the growing and finishing phases of production.

Materials and Methods

Animals and diets

The experimental protocol for this study was approved prior to beginning the study by the Texas A&M University Animal Care and Use Committee (IACUC).

Animals, diets, and feeding regimens are the same as described in Chapter II.

Termination of feeding trial and tissue collection

The feeding trial was terminated at 188 days of age, and pigs were slaughtered as described in Chapter II. During the slaughter process, the following tissues were collected in triplicate from each pig for further laboratory analyses: whole blood, kidneys, liver, small intestine (jejunum, washed with 1x Phosphate Buffer Solution [PBS]), pancreas, longissimus dorsi muscle, and semimembranosus (thigh) muscle. Tissues were snap frozen in liquid nitrogen at the time of collection and stored at -80 C for subsequent analyses.

Tissue Processing

Tissues that had been snap frozen in liquid nitrogen were individually cut and weighed into 100 mg aliquots and homogenized in 0.5 mL of 1.5 M $HClO_4$ using a glass tissue homogenizer. This was done to deproteinize the tissues. Tissue homogenate was then transferred to a plastic 15 mL tube and the glass homogenizer was rinsed with an additional 0.5 mL of 1.5 M $HClO_4$; this was also added to tube with the homogenate. After 1 min, 0.5 mL of 2 M K_2CO_3 was added to neutralize the homogenate solution. This homogenate solution was centrifuged at 600 g for 10 min. The supernatant fluid was then removed and

was used for analysis of creatine and creatine phosphate content in each tissue. Tissue homogenate supernatants were stored at -80 C.

Creatine Analysis

Tissue sample supernatants and creatine standards were used for analyses of creatine (CR). The following CR standards were used: 1 mM CR (13.1 mg/100 mL of deionized water) and 100 µM CR (0.1 mL of 1 mM CR standard + 0.9 mL of deionized water). 20 µL of each sample supernatant and each standard were added to individual Waters HPLC micro vials (Waters SKU: 186002805) containing 80 µL of HPLC-grade water. Vials were then analyzed for creatine content using Waters HPLC with an autosampler for derivatization with 30 mM Benzoin (Boppana and Rhodes 1990; Buchberger and Ferdig 2004), as described by Li and Wu (2020). The autosampling program was programed to run a 16-min protocol for CR analysis following auto-additions of 30 mM Benzoin (5 µL), 100 mM β-mercaptoethanol + 200 mM sodium sulfite (5 µL), 2 M KOH (10 µL), and a 2-min delay before the sample was allowed to run on the column. The Supelco C18 analytical column and Supelco C18 guard column were used for this protocol. The solvents used for liquid chromatography were Solvent A (0.1 M NaAC made with HPLC-grade water and HPLC-grade 100% methanol) and Solvent B (100% HPLC-grade methanol). Detection was at an excitation wavelength of 325 nm and an emission wavelength of 425 nm. Creatine peak elutes at 9.5 min and this was confirmed using the 1mM and 100 µM creatine standards compared to blank vials run on the same protocol.

Creatine Phosphate Analysis

The principle for this analysis is based on the following equation:

$$\text{Creatine Phosphate} + \text{ADP} \rightleftharpoons \text{Creatine} + \text{ATP} + H^+ \quad (\text{Creatine Kinase}, Mg^{2+})$$

The supernatant fluid of processed samples or 1 mM and 100 µM creatine phosphate (CRP) standards was incubated for 30 min with creatine kinase (CK) and 10 mM ADP/100 mM $MgCl_2$ at 37°C. This allowed for CRP present in the samples to be converted to CR, which was detectable through HPLC analysis, as described previously. At the end of the 30 min incubation period, the reaction was terminated by adding 1.5 M $HClO_4$ and then neutralized with 2 M K_2CO_3. The sample and standard tubes were centrifuged at 10,000 g for 5 min and the supernatant was taken and used for HPLC analysis for creatine utilizing the autosampler protocol as described in "Creatine Analysis," and the CRP value was determined using the following equation:

$$\text{Creatine Phosphate} = \text{Amount of Creatine with Creatine Kinase} + \text{Amount of Creatine without Creatine Kinase}$$

The value for CR determined in "Creatine Analysis" was subtracted from the HPLC value of CR obtained after incubation with creatine kinase, to indicate the amount of CRP present in the sample (nmol/g tissue).

Statistical Analyses

Statistical analyses were performed on normalized data using the methods described in Chapter II.

Results

The effects of dietary supplementation with 1% Gly on the concentration of CR present in various tissues are shown in Table 3.1. Creatine is an amino acid metabolite

synthesized endogenously via the renal-hepatic axis (Chapter 1). The two enzymes required for synthesis of creatine are AGAT and GAMT in the kidneys and liver, respectively. But, other tissues throughout the body may synthesize CR. Dietary Gly supplementation increased concentrations of CR in all tissues and plasma, with the greatest increases in the kidneys ($P < 0.01$), pancreas ($P < 0.01$), and semimembranosus muscle ($P < 0.05$) of Gly-treated animals, regardless of birthweight status. The liver of Gly-supplemented IUGR pigs specifically, also had a significant increase in CR ($P < 0.05$). These data are shown in Figures 3.1 - 3.3.

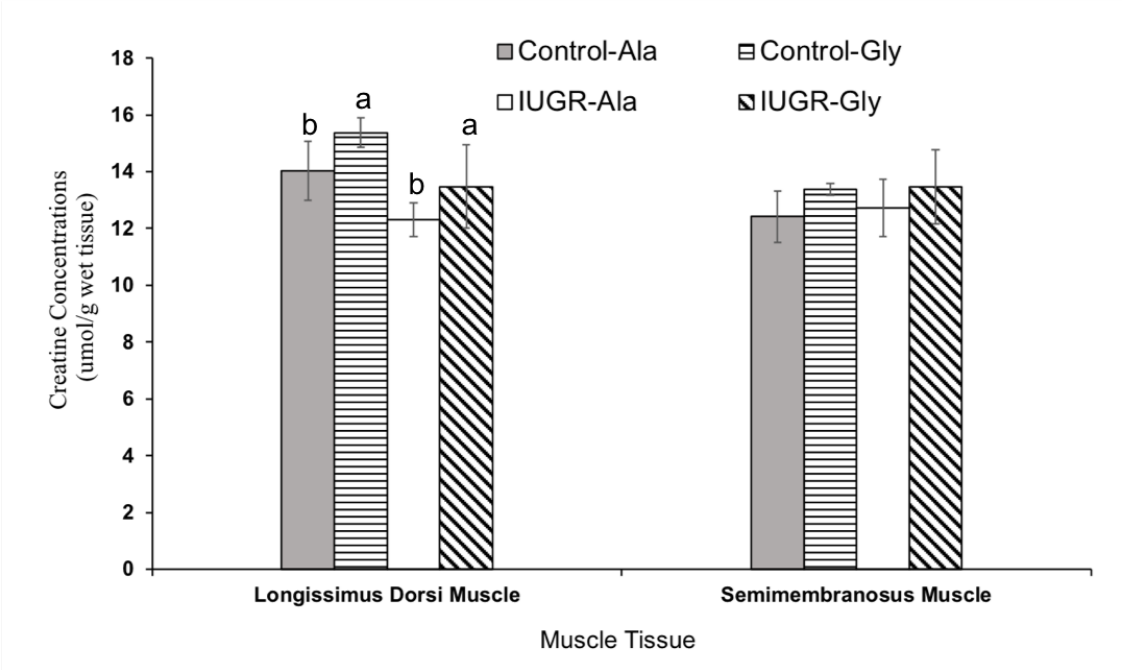


Figure 3.1. The creatine content in skeletal muscle from IUGR and normal (control) birthweight pigs at the time of slaughter following dietary supplementation with either glycine (Gly, 1% of the diet) or Ala (1% of the diet as isonitrogenous control) from weaning at 21 d of age to slaughter at 188 d of age. Dietary Gly supplementation increased concentrations of creatine in both types of skeletal muscle, but the effect was only significant for concentrations of creatine in longissimus dorsi muscle, regardless of birthweight status. Statistical differences were determined by analysis using two-way ANOVA linear model of multiple effects. For a tissue, means not sharing the same superscript letter are different ($P < 0.05$).

Table 3.1 Creatine Content of Tissues in IUGR and Normal Birthweight Pigs

Creatine Concentration (μmol/g of wet tissue)					P – Values for Treatment		
	Normal Birthweight		IUGR		IUGR Effect	Gly Effect	IUGR x Gly Effect
	Ala-Control (n=9)	Gly-Treated (n=10)	Ala-Control (n=7)	Gly-Treated (n=6)			
Kidney	1.2 ± 0.1^{b}	1.8 ± 0.1^{a}	1.2 ± 0.1^{b}	1.6 ± 0.1^{a}	0.701	0.009	0.792
Liver	1.3 ± 0.1^{b}	1.2 ± 0.1^{b}	1.2 ± 0.1^{b}	1.8 ± 0.2^{a}	0.137	0.224	0.018
Pancreas	1.9 ± 0.1^{b}	2.3 ± 0.2^{a}	1.8 ± 0.2^{b}	2.5 ± 0.2^{a}	0.945	0.006	0.232
Longissimus Dorsi Muscle	14.0 ± 1.0^{b}	16.1 ± 0.5^{a}	12.5 ± 0.6^{b}	13.5 ± 1.5^{a}	0.725	0.027	0.111
Semimembranosus Muscle	12.4 ± 0.9	13.4 ± 0.2	12.7 ± 1.0	13.4 ± 1.3	0.4473	0.560	0.502
Creatine Concentration (nmol/mL)							
	Normal Birthweight		IUGR				
	Ala-Control (n=9)	Gly-Treated (n=10)	Ala-Control (n=7)	Gly-Treated (n=6)			
Plasma	289 ± 25^{a}	309 ± 29^{a}	321 ± 32^{a}	344 ± 48^{a}	0.324	0.983	0.512

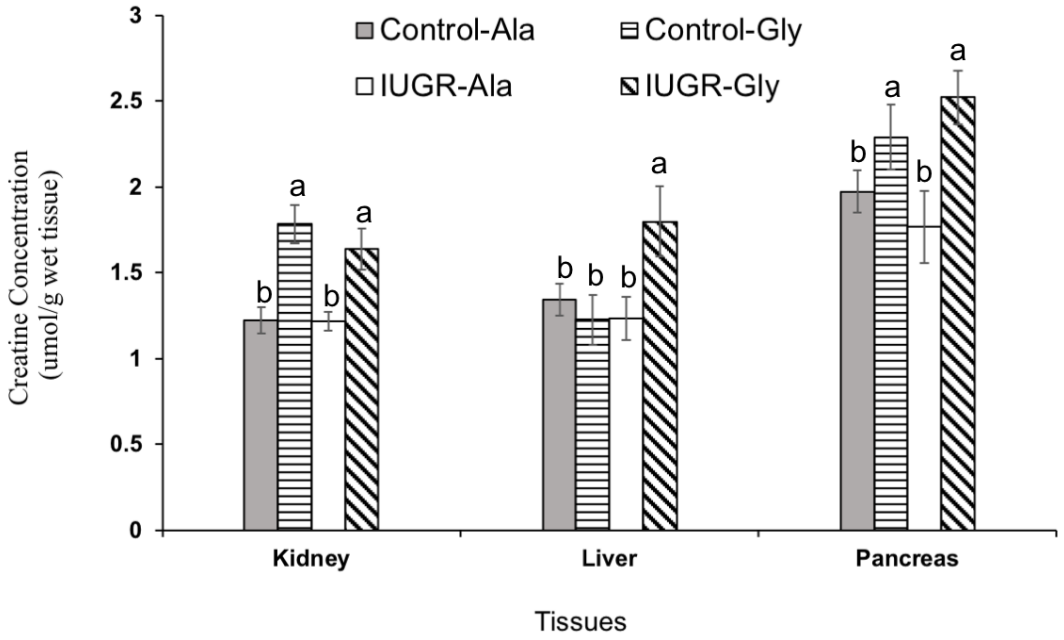


Figure 3.2. The creatine content of non-skeletal muscle tissue from IUGR and normal (control) birthweight pigs at the time of slaughter, following dietary supplementation with either glycine (Gly, 1% of the diet) or Ala (1% of the diet as isonitrogenous control) from weaning at 21 d of age to slaughter at 188 d of age. These results indicated that Gly supplementation increased concentrations of CR in the pancreas and kidneys, regardless of birthweight status, and in the liver of IUGR pigs. For a tissue, different superscript letters indicate statistical difference ($P < 0.05$) according to analysis by two-way ANOVA linear model of multiple effects.

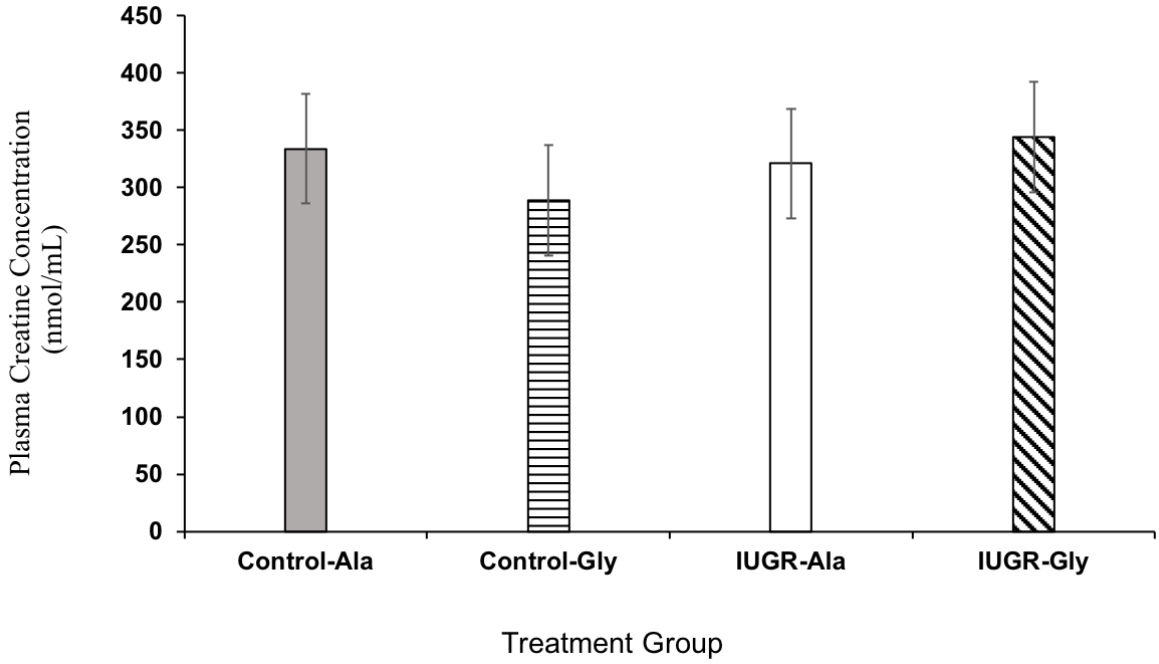


Figure 3.3. The concentrations of creatine in plasma from IUGR and normal (control) birthweight pigs at time of slaughter, following dietary supplementation with either glycine (Gly, 1% of the diet) or Ala (1% of the diet as isonitrogenous control) from weaning at 21 d of age to slaughter at 188 d of age. Dietary Gly supplementation had no significant effect on concentrations of creatine in the plasma of either IUGR or normal birthweight pigs, according to analysis by two-way ANOVA linear model of multiple effects.

Creatine plays many functional roles within the body, one of which is cellular energy metabolism. In this role, CR must first be converted to creatine phosphate (CRP) by creatine kinase. Creatine phosphate then creates a dynamic "phosphate pool" for rapid cellular energy turnover where the phosphate group of CRP will be transferred to a molecule of ADP, converting it to ATP during times of intense energetic demands. The effects of dietary supplementation with 1% Gly on concentrations of CRP in tissues of pigs are summarized in Figures 3.6 and 3.7 and Table 3.3. Overall, dietary supplementation of Gly increased CRP content in both skeletal muscle (Fig. 3.7) and non-skeletal muscle tissues (Fig 3.6) regardless

of birthweight status of the pigs, but the effects were greater for IUGR pigs ($P < 0.05$). The concentration of CRP in the semimembranosus muscle of Gly-supplemented IUGR pigs increased more than 3.5-fold, compared to non-supplemented IUGR pigs ($P < 0.05$). Dietary Gly supplementation also increased concentrations of CRP in the kidneys from non-detectable (ND) in non-supplemented pigs to approximately 6 umol/g tissue (greater than any other non-skeletal muscle tissue), regardless of birthweight status. The CRP content of the pancreas also increased ($P < 0.05$) regardless of bodyweight following Gly supplementation. Additionally, concentrations of CRP were increased in the longissimus dorsi and semimembranosus muscles of Gly-supplemented IUGR pigs that increased final body weight of the pigs ($P < 0.05$ and $P < 0.10$, respectively), indicating that concentrations of CRP may be indicative of increased energy metabolism required for increased growth of Gly-supplemented IUGR pigs (see Chapter II).

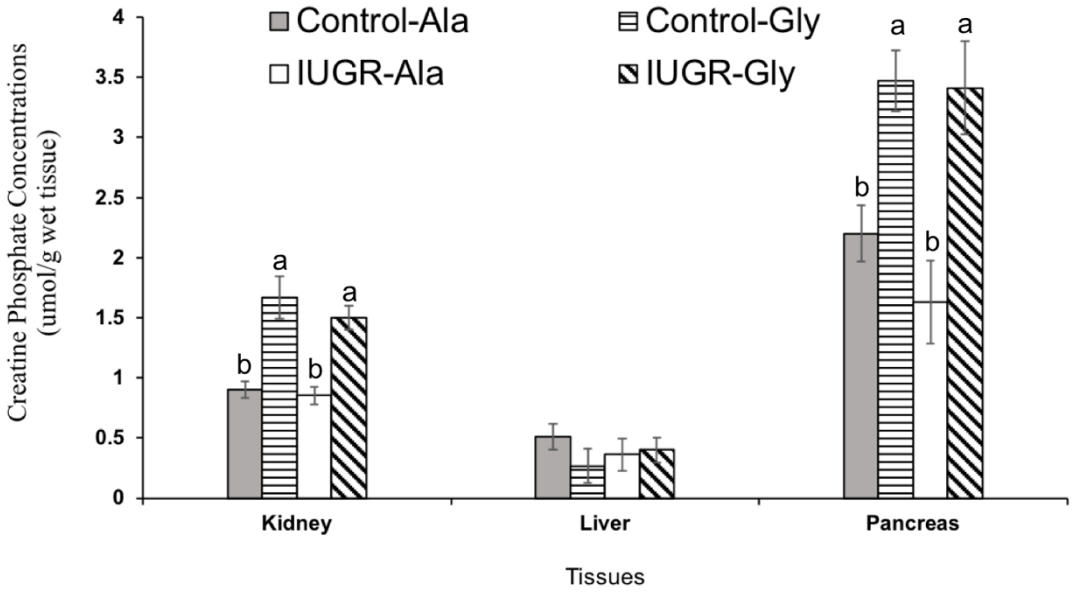


Figure 3.4. The concentrations of creatine phosphate in kidney, liver, and pancreas from IUGR and normal (control) birthweight pigs at time of slaughter, following dietary supplementation with either Gly or Ala (as isonitrogenous control) from weaning at 21 d of age to slaughter at 188 d of age. Dietary Gly supplementation had no significant effect on creatine phosphate in liver but did have a significant effect on concentrations of creatine phosphate in the kidneys and pancreas of IUGR and normal birthweight pigs. Different superscript letters indicate statistical difference ($P < 0.05$) according to analysis by two-way ANOVA linear model of multiple effects. For a tissue, means not sharing the same letter differ ($P < 0.05$).

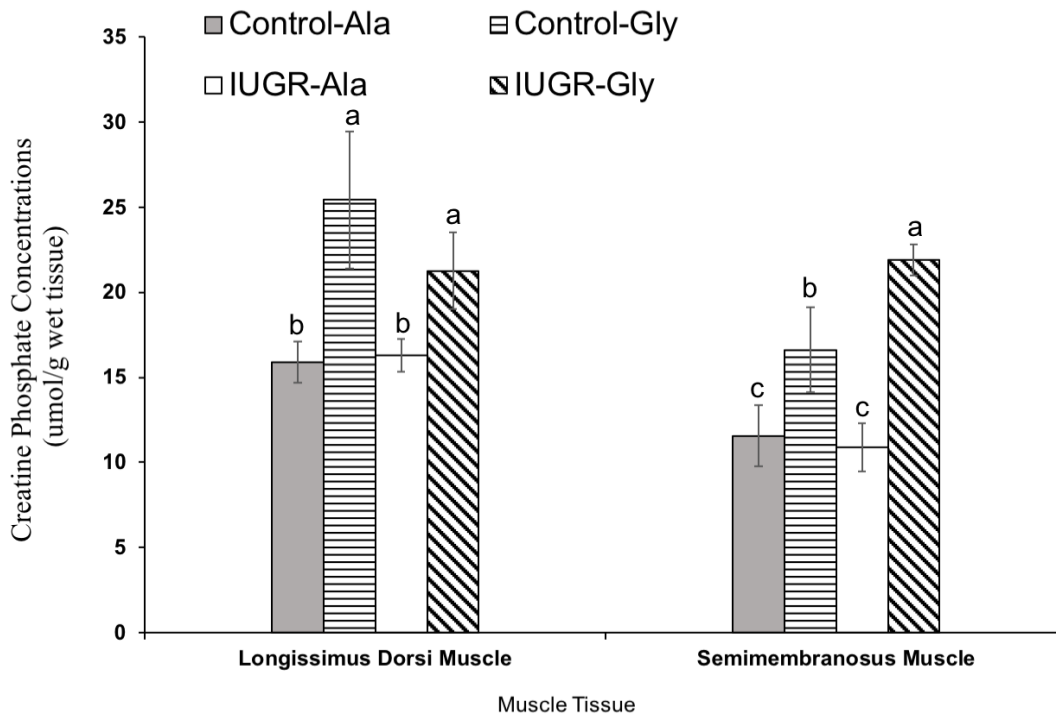


Figure 3.5. The concentrations of creatine phosphate in skeletal muscle from IUGR and normal (control) birthweight pigs at the time of slaughter, following dietary supplementation with either Gly or Ala (as isonitrogenous control) from weaning at 21 d of age to slaughter at 188 d of age. Dietary Gly supplementation had significant effects on concentrations of creatine phosphate in both types of skeletal muscle from IUGR and normal birthweight pigs. For a tissue, means with different letters indicate statistical difference ($P < 0.05$) according to analysis by two-way ANOVA linear model of multiple effects.

Table 3.2 Creatine Phosphate Content of Tissues in IUGR and Normal Birthweight Pigs

	Creatine Phosphate Concentration (μmol/g of tissue)				P – Values for Treatment Effects		
	Normal Birthweight		IUGR				
	Ala-Control (n=9)	Gly-Treated (n=10)	Ala-Control (n=7)	Gly-Treated (n=6)	IUGR Effect	Gly Effect	IUGR x Gly Effect
Kidney	0.9 ± 0.07^{b}	1.7 ± 0.18^{a}	0.8 ± 0.07^{b}	1.5 ± 0.10^{a}	0.757	<0.0001	0.034
Liver	0.27 ± 0.13^{a}	0.51 ± 0.10^{a}	0.36 ± 0.14^{a}	0.40 ± 0.11^{a}	0.962	0.522	0.465
Pancreas	2.2 ± 0.39^{b}	3.5 ± 0.35^{a}	1.6 ± 0.25^{b}	3.4 ± 0.23^{a}	0.370	0.0002	0.465
Longissimus Dorsi Muscle	15.9 ± 1.2^{c}	25.4 ± 4.0^{ab}	16.3 ± 0.96^{bc}	21.1 ± 2.2^{a}	0.049	0.005	0.982
Semimembranosus Muscle	11.6 ± 1.8^{c}	16.6 ± 2.5^{b}	10.9 ± 1.3^{c}	21.9 ± 2.5^{a}	0.304	0.002	0.187

For a tissue, differing superscripts across a row (a-c) indicates statistical significance ($P < 0.05$).

Discussion

Creatine is an amino acid metabolite synthesized endogenously by two enzymes via the renal-hepatic axis, AGAT and GAMT (Wu 2013a). These enzymes are found in the kidneys and liver, respectively, but also in many other tissues throughout the body, leading to localized CR synthesis in some organs (Da Silva et al. 2014). CR is synthesized from three amino acids, Gly, arginine, and methionine, as *S*-adenosylmethionine (SAM). Pigs have all necessary enzymes to synthesize CR *de novo*, given that the necessary substrates are provided in adequate amounts. This is especially important as CR has many functional roles throughout the body, including being an antioxidant, regulating brain function, improving nitric oxide availability for tissues, and improving stability of cell membranes (Wu 2020).

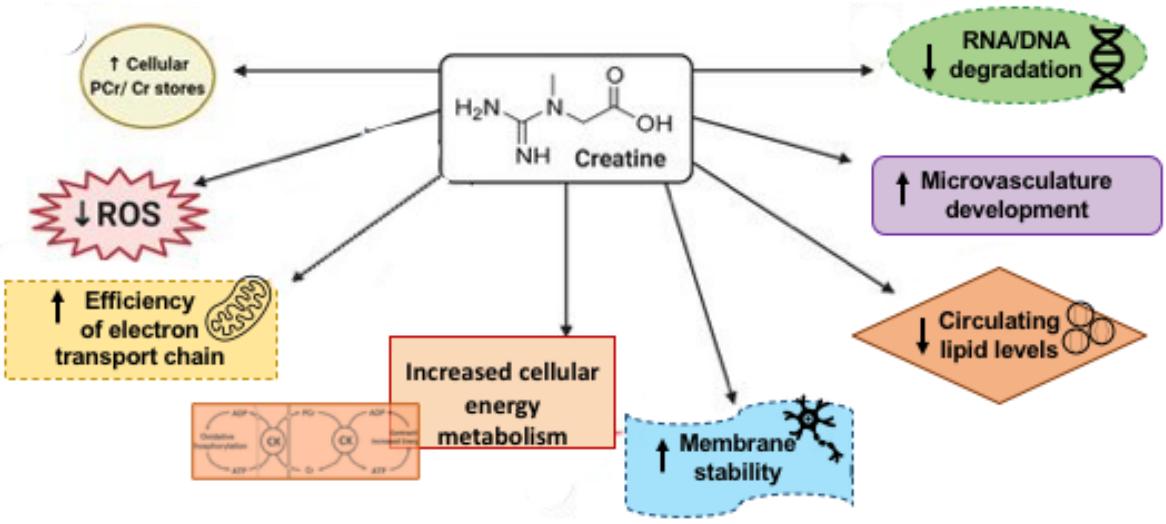


Figure 3.6. Summary of cellular functions of creatine in animals. All of these functions indicate that creatine is a vital metabolite for maintenance of health and metabolism. Adapted from Clarke et al. 2021.

The most important role CR plays is in cellular energy metabolism, where CR is used to synthesize CRP, the major donor of phosphate for replenishment of ATP during periods of intense demand for energy. This is why CR is most often associated with growth and skeletal muscle function. Additionally, 95% of body CR is stored in skeletal muscle (Brosnan & Brosnan 2007). Without an adequate supply of Gly, endogenous CR synthesis is not sufficient to meet daily demands. IUGR pigs have diminished capacity for Gly synthesis (Hu et al. 2019) and traditional corn-and-soybean-meal-based (plant-based) diets fed to growing pigs are very low in Gly content (Li et al. 2011); therefore, IUGR pigs require additional dietary supplementation of Gly to thrive and reach peak growth potential. Dietary Gly supplementation can provide this essential substrate for CR synthesis so that growth and survivability of IUGR pigs can be improved. Results of this study indicate that dietary Gly supplementation increased concentrations of CR in most tissues analyzed. This indicates the potential for dietary Gly supplementation to enhance cellular energy metabolism and improved overall growth and survivability of IUGR pigs as noted in this study. Additionally, as reported in Chapter II, there was a clear effect of Gly supplementation to improve growth of IUGR pigs ($P < 0.05$) and to improve overall carcass quality by decreasing back fat thickness and increasing loin eye area. This is especially important as IUGR pig carcasses tend to have greater fat accumulation, than normal birthweight pigs. Results of this study also revealed that Gly supplementation increased CR in muscle ($P < 0.05$) as a potential mechanism to explain the compensatory gain in skeletal muscle and decrease in back fat in Gly-supplemented IUGR pigs.

The majority (95%) of CR within skeletal muscle tissue is stored as phosphocreatine/creatine phosphate (CRP) (Brosnan and Brosnan 2007). CR is converted to CRP by creatine kinase (CK) when the muscle is at a resting state, as shown in the reaction below. At rest, CK transfers the energy from the γ-phosphate bond in ATP to one mol of CR. ATP is a highly energetic compound and, therefore, very unstable. This reaction allows for the energy to be stored as an energy reserve in the form of a "phosphate pool," within skeletal muscle, as CRP. The affinity for CK is much greater for ATP than for CR (Wu 2013a); however, the concentration of CRP is usually 3- to 4-times greater than that of ATP in skeletal muscle. This large difference in concentrations of substrate and differences in enzyme affinity allows for this "energy reservoir" to be easily accessed during strenuous exercise or during times of increased demand for energy, such as in the case of growth of animals (Wu 2013a).

$$\text{ATP} + \text{Creatine} \rightleftharpoons \text{Creatine Phosphate} + \text{ADP} + H^{+} \qquad (\text{Creatine Kinase}, Mg^{2+})$$

In the present study, dietary Gly supplementation increased CRP in the kidneys, pancreas and longissimus dorsi muscle in both IUGR and normal birthweight pigs, without an effect in the liver. In the kidneys, this increase was especially interesting as the concentration of CRP in non-supplemented pigs was non-detectable (ND), but increased to 6 to 5 μmol/g tissue with dietary Gly supplementation (Fig. 3.6), regardless of birthweight status of pigs ($P < 0.05$). There was a similar increase in both skeletal muscles (Fig 3.7) with an increase of approximately 10 μmol/g tissue for the longissimus dorsi muscle, and the concentration of CRP in the semimembranosus muscle from IUGR pigs essentially doubled following

dietary Gly supplementation ($P < 0.05$). The smallest change was in the pancreas (Fig. 3.6), with a slight increase from 1 to 2 μmol/g tissue to 3.5 μmol/g tissue following dietary Gly supplementation, regardless of birthweight status of pigs. Overall, these results indicate that dietary Gly supplementation has a profound effect on total tissue content of CR (CR + CRP), especially within skeletal muscle and kidney tissues, regardless of birthweight status of pigs. As 95% of all CR in the body is in skeletal muscle (Brosnan and Brosnan 2007), those results indicate a large overall increase in total CR present in tissues in response to dietary Gly supplementation. Interestingly, previously results from studies of skeletal muscle from rats showed that muscles rich in type I or "white" fibers had 45% less total CR than those that had low proportions of type I fibers (Murphy et al. 2001). However, the concentrations of CR in the semimembranosus muscle and the longissimus dorsi muscle were nearly identical for pigs in each treatment group($P > 0.10$). As the semimembranosus muscle in swine has a much greater density of type I fibers than the longissimus dorsi muscle (Hwang et al. 2019), these results are particularly interesting. Thus, Gly supplementation increased CR synthesis allowing for more CRP for use in cellular energy metabolism and stabilization of cellular membranes in these high activity tissues. Collectively, these results indicate that the conventional corn- and soybean meal-based diet did not provide sufficient Gly for sufficient synthesis of CR in pigs.

In conclusion, dietary Gly supplementation increased concentrations of CR in tissues ($P < 0.05$), but not in plasma. Additionally, metabolites of CR metabolism, such as CRP increased with dietary Gly supplementation. The tissues most affected by these increases varied by metabolite measured, but skeletal muscle was most dramatically affected, especially in IUGR pigs, followed by the kidney. Furthermore, the pancreas was the tissue

most affected by substrate availability for increasing overall concentration of CR ($P < 0.01$). This is likely due to the pancreatic islets containing all enzymes necessary for CR synthesis and being able to synthesize CR independently of any other organs and not being subjected to the same regulatory pathways as other organs engaged in synthesis of creatine (Da Silva et al. 2014; Nasrallaht al. 2010). These results provide further insight into the Gly-creatine pathway in IUGR growing pigs and support the notion that Gly deficiency in these animals reduced CR availability in their skeletal muscles. These results and those from CH II indicate that the potential CR deficiency in IUGR pigs has negative effects on optimal growth performance, and that it can be attenuated by dietary supplementation with Gly.

CHAPTER IV

ANALYSIS OF EFFECTS OF DIETARY GLYCINE SUPPLEMENTATION ON ENZYMES FOR CREATINE SYNTHESIS IN TISSUES FROM IUGR AND NORMAL PIGS

Introduction

Glycine (Gly) plays many roles as both a structural component of protein (especially collagen and elastin protein, where it constitutes one-third of the total amino acid content) representing 11.5% of total body amino acids, and as a functional amino acid (Wu 2013a). Glycine has physiological functions in nutrition, metabolism, and overall health, and is required for synthesis of many essential metabolites such as heme, purines, glutathione, and bile salts (Amelio et al. 2014; Hall 1998; Li et al. 2016; Matilla et al. 2002; Mudd et al. 2007; Petrat et al. 2012). However, it is the synthesis of creatine (CR) that utilizes a large proportion of total Gly with 14.5% of total Gly (dietary + endogenous) and 87% of dietary Gly being used for CR synthesis (Melendez-Hevia et al. 2009; Wu and Morris 1998). This is why recent studies have indicated that CR synthesis may represent a substantial burden to young, rapidly growing animal, such as growing-finishing pigs (Brosnan et al. 2009; Riedijk et al. 2007). As IUGR pigs have inadequate pathways for endogenous synthesis of Gly (Hu et al. 2017) and conventional swine diets are low in Gly content (Hou e al. 2019), this burden is even more substantial for IUGR pigs.

In terrestrial mammals, CR synthesis occurs via the renal-hepatic axis and utilizes AGAT to synthesize guanidinoacetate and ornithine from Gly and arginine, and GAMT to synthesize CR through methylation of guanidinoacetate with the cofactor *S*-

adenosylmethionine (SAM) (Da Silva et al. 2008). This interorgan metabolism allows for regulation at different steps in the CR synthesis pathway based on the enzymes and the tissue in which they are located. The primary site of regulation for CR synthesis is the rate-limiting enzyme, AGAT (Da Silva et al. 2008, Da Silva et al. 2014; Nasrallah et al. 2010). While there are other forms of regulation on AGAT activity, such as intracellular concentrations of ornithine and circulating levels of thyroid hormone and growth hormone (Walker 1972; Van Pilsum et al. 1982; Wu and Morris 1998), product feedback inhibition is the primary regulatory mechanism for AGAT. One of the strongest forms of regulation of the AGAT enzyme is feedback inhibition from CR, especially dietary supplementation of CR (Da Silva et al. 2014; Nasrallah et al. 2010; Wu and Morris 1998). This is likely due to a biochemical mechanism to conserve valuable arginine, Gly and methionine when CR is present in sufficient amounts, as regulation occurs at the pre-translation level to downregulate expression of AGAT mRNA in the kidneys, but not in the pancreas (Da Silva et al. 2014). One method to increase CR production is through increased provision of substrates, such as Gly in the diet (Nasrallah et al. 2010).

The current study was to determine effects of dietary Gly supplementation on the activities AGAT and GAMT as key enzymes in the CR synthesis pathway. This feeding experiment with IUGR and normal birthweight pigs was to determine the extent to which dietary Gly supplementation benefited growth and development of IUGR pigs with diminished Gly synthesis capacity compared to pigs with a normal capacity for Gly synthesis. This study tested the hypothesis that dietary Gly supplementation would successfully upregulate expression of enzymes in the CR synthesis pathway in IUGR pigs,

and that this would contribute to overall improvements in growth and survivability of postweaning IUGR pigs.

Materials and Methods

Animals and diets

The experimental protocol for this study was approved prior to beginning the study by the Texas A&M University Animal Care and Use Committee (IACUC).

Animals, diets, and feeding regimens utilized are the same as described in Chapter II.

Termination of feeding trial and tissue collection

The feeding trial was terminated at 188 days of age, and pigs were slaughtered as described in Chapter II and tissues were collected at time of slaughter, as described in Chapter III.

Tissue Processing

Tissues snap frozen in liquid nitrogen at time of slaughter were individually distributed into 100 mg aliquots for homogenization in 0.5 mL of 80 mM sodium phosphate using a glass homogenizer. This homogenization allowed for enzymes within the snap frozen tissues to remain active for later determination of their activity. The homogenates were then subjected to two cycles of freezing (-80°C) and thawing (37°C) to break down cellular and mitochondrial membranes to release mitochondrial enzymes within the homogenate.

Arginine:guanidinoacetate amidinotransferase (AGAT) Activity Analysis

The principle for this assay was based on the following equation:

$$\text{L-Arginine} + \text{Glycine} \rightleftharpoons \text{Guanidinoacetate (GA)} + \text{L-Ornithine}$$

The activity of AGAT in homogenates was analyzed over a 30 min period. Samples and blank tubes (those used to model no enzyme activity during the incubation time) were all incubated with a 30 mM L-arginine/glycine substrate in 80 mM sodium phosphate buffer solution (pH 7.5) for 30 min at 37°C. Enzymes present within the homogenates of the blank tubes were denatured prior to the addition of the precursors by adding 1.5 M $HClO_4$ prior to the incubation. After the sample tubes were incubated, 1.5 M $HClO_4$ was also added to the sample tubes to stop the reaction and 2 M K_2CO_3 was added to all tubes to neutralize samples and blanks. All tubes were centrifuged at 10,000 x *g* for 1 min and supernatant transferred to a new tube for further analysis. All tubes were then subjected to the same boiling method as described in "Creatinine Analysis" in Chapter III. This allowed for detection of the guanidinoacetate (GA) peak during HPLC analysis (HPLC method described in Chapter III). Additionally, total creatine was analyzed in these samples to detect any possible further conversion of GA to creatine, creatinine, or creatine phosphate. No changes were detected for concentrations of these metabolite among samples and blanks; therefore, GA was the only metabolite utilized for calculations. The following tissues were analyzed for AGAT activity: kidneys, liver, pancreas, small intestine (jejunum), and longissimus dorsi muscle.

Guanidinoacetate N-methyltransferase (GAMT) Activity Analysis

The principle for this analysis is based on the following equation:

S-adenosyl-methionine + Guanidinoacetate ⇌ *S*-adenosyl-homocysteine + Creatine

The activity of GAMT was analyzed using a modified method first described by Verhoeven et al. (2004). Samples and blanks were analyzed for GAMT activity over a 30 min incubation

period. Sample and blank tubes were incubated with a 2 mM guanidinoacetate/*S*-adenosylmethionine substrate in an 80 mM sodium phosphate buffer solution (pH 7.5) for 30 min at 37°C. Enzymes present within the homogenates of the blank tubes were denatured prior to the addition of the precursors by adding 1.5 M $HClO_4$ prior to incubation. After incubation, 1.5 M $HClO_4$ was added to sample tubes to halt the reaction and 2 M K_2CO_3 was added to all tubes to neutralize samples and blanks prior to HPLC analysis. HPLC analysis of CR production was analyzed using the methods described in Chapter III, "Creatine Analysis". Additionally, as for AGAT activity analysis, total creatine was analyzed in these samples to detect any possible further conversion of creatine to creatinine or creatine phosphate. The following tissues were analyzed for GAMT activity: kidneys, liver, pancreas, longissimus dorsi muscle, and small intestine (jejunum).

Statistical Analyses

Statistical analyses were performed on normalized data using the methods described in Chapter II.

Results

AGAT Enzyme Activity Effect

The effects of 1% dietary Gly supplementation on AGAT activity in tissues of interest are summarized in Figure 4.1 and Table 4.

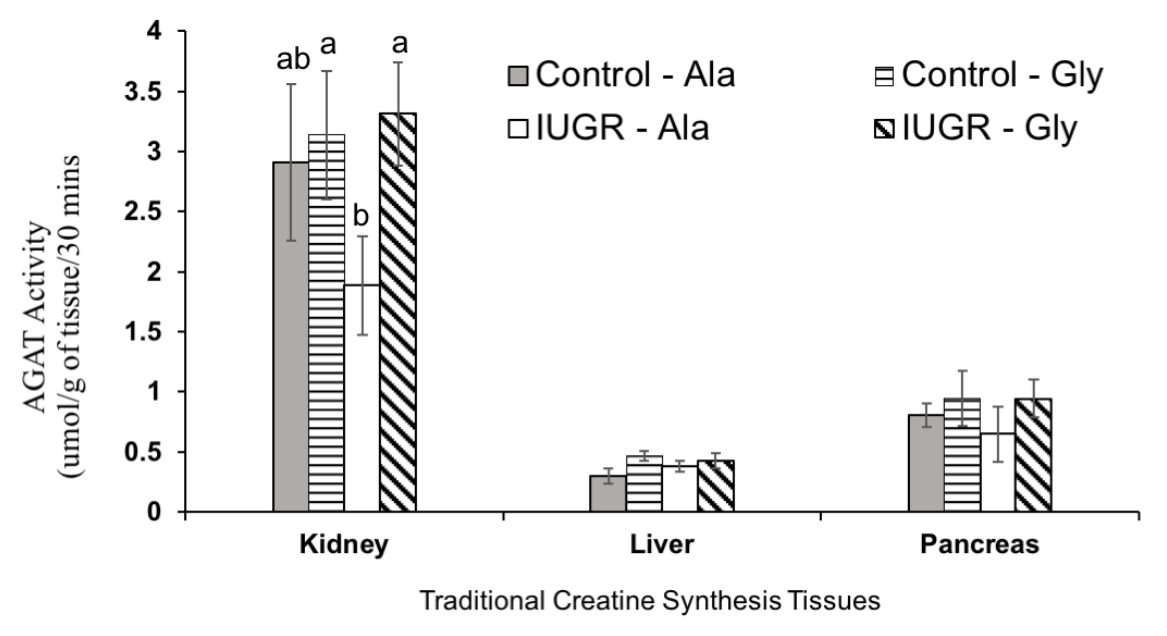


Figure 4.1. The AGAT activity in traditional tissues for creatine synthesis from IUGR and normal (control) birthweight pigs at time of slaughter, following dietary supplementation with either glycine (Gly, 1% of the diet) or Ala (1% of the diet as isonitrogenous control) from weaning at 21 d of age to slaughter at 188 d of age. The results indicated that Gly supplementation significantly increased AGAT activity in kidneys of IUGR pigs and there were marginal increases in activity in the pancreas of IUGR and normal birthweight pigs. For a tissue, different superscript letters indicate statistical difference ($P < 0.05$) according to analysis by two-way ANOVA linear model of multiple effects.

The kidney is the primary site for AGAT activity. This enzyme catalyzes the initial reaction in the interorgan metabolism pathway for CR synthesis by transferring the amidino group of arginine onto Gly to synthesize guanidinoacetate and ornithine. However, this enzyme is also present in other organs such as the pancreas and testes (Brosnan et al. 2011; Da Silva et al. 2014). Overall, dietary Gly supplementation had the greatest effect on AGAT activity in kidneys of IUGR pigs ($P < 0.05$), though there was a trend towards increased activity in the liver ($P=0.078$), regardless of birthweight status (Fig 4.1). However, the overall activity of AGAT in the liver was very low compared to that in the pancreas and especially in the kidneys, the primary tissue for AGAT activity in the creatine synthesis

pathways (Da Silva et al. 2014). AGAT activity increased in pancreatic tissues of both IUGR and normal birthweight pigs supplemented with Gly, but these changes were not significant. AGAT activity was not detected in either skeletal muscle tissue or small intestine (jejunum) regardless of birthweight or dietary treatment (Table 4.1). Those tissues do not traditionally express AGAT, so these results were expected.

Table 4.1 AGAT Enzyme Activity in Tissues of IUGR and Normal Birthweight Pigs

AGAT Activity (μmol/g of tissue/30 mins)					P-Values for Treatment		
	Normal Birth Weight		IUGR		IUGR Effect	Gly Effect	IUGR x Gly Effect
	Ala-Control (n=9)	Gly-Treated (n=10)	Ala-Control (n=7)	Gly-Treated (n=6)			
Kidney	2.9 ± 0.7^{ab}	3.1 ± 0.5^{a}	1.9 ± 0.4^{b}	3.3 ± 0.4^{a}	0.454	0.151	0.292
Liver	$0.3x \pm 0.04^{a}$	$0.5x \pm 0.06^{a}$	$0.4x \pm 0.06^{a}$	0.42 ± 0.04^{a}	0.740	0.078	0.280
Pancreas	0.8 ± 0.2^{a}	0.9 ± 0.1^{a}	0.6 ± 0.2^{a}	0.94 ± 0.2^{a}	0.687	0.268	0.676
Longissimus Dorsi Muscle	ND	ND	ND	ND	--	--	--
Small Intestine	ND	ND	ND	ND	--	--	--

For a tissue, different superscripts (a-c) across a row indicate statistical significance ($P < 0.05$).

GAMT Enzyme Activity Effect

The results of 1% dietary Gly supplementation on GAMT activity are summarized in Figure 4.2 and Table 4.2.

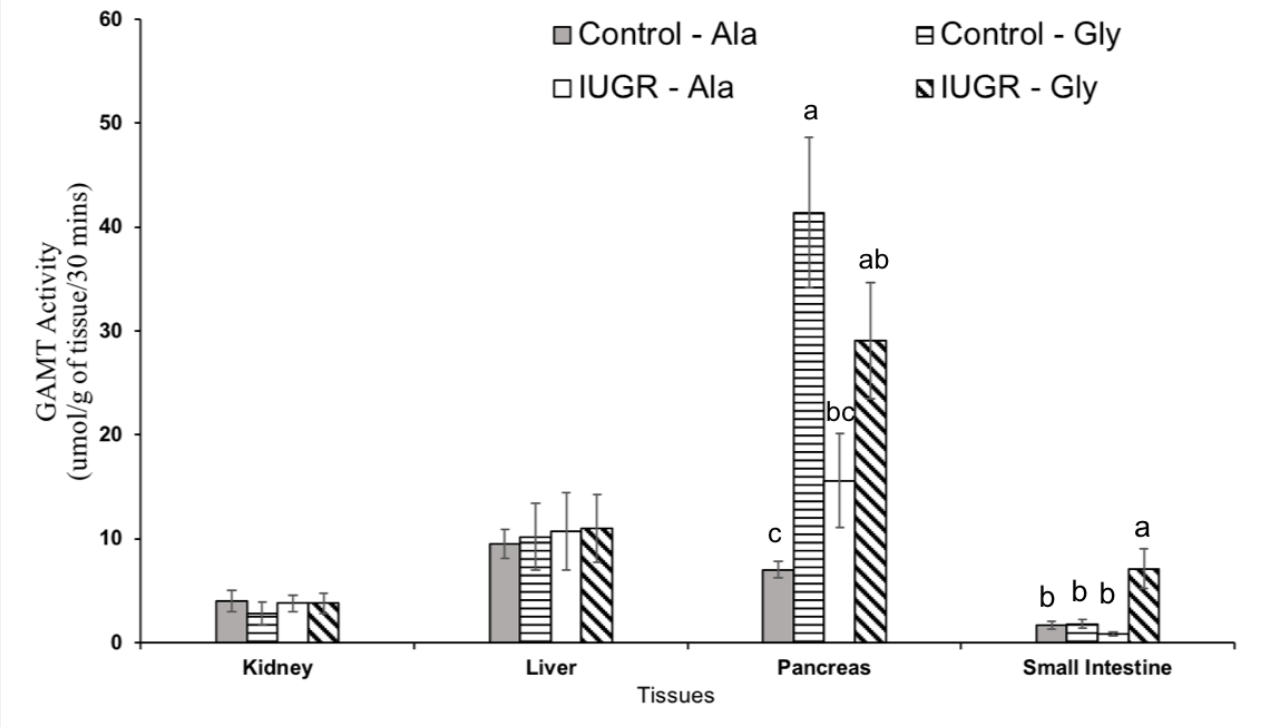


Figure 4.2 The GAMT activity in various tissues collected from IUGR and normal (control) birthweight pigs at time of slaughter, following dietary supplementation with either glycine (Gly, 1% of the diet) or Ala (isonitrogenous control) from weaning at 21 d of age to slaughter at 188 d of age. These results indicated that Gly supplementation significantly increased GAMT activity in the pancreas, regardless of birthweight status, and in the small intestine of IUGR pigs. There was no effect of dietary Gly on GAMT activity in the kidneys or liver of pigs in either birthweight status. For a tissue, different superscript letters indicate statistical difference ($P < 0.05$) according to analysis by two-way ANOVA linear model of multiple effects.

The liver is the primary site for GAMT activity and the second step in the creatine synthesis pathway. This enzyme catalyzes the methylation of guanidinoacetate to form creatine. There are other tissues with GAMT activity such as spleen and brain (Brosnan and Brosnan 2007). These results clearly indicate a significant effect of dietary Gly supplementation to increase

GAMT activity in the small intestine and pancreas, but not liver and kidney. While GAMT activity in the liver and kidney of pigs from either birthweight status was not affected by Gly supplementation, pancreatic tissues and jejunum were significantly affected (Fig 4.2). GAMT activity in the small intestine of Gly-supplemented IUGR pigs increased 6-fold compared to both non-supplemented IUGR pigs and normal birthweight pigs regardless of dietary treatment. GAMT activity in pancreatic tissues from normal birthweight pigs was also significantly affected, increasing 5-fold compared to non-supplemented normal birthweight pigs (Fig. 4.2) The GAMT activity in the pancreatic tissue of Gly-supplemented IUGR pigs also trended towards significance ($P < 0.10$) (Table 4.2).

Table 4.2 GAMT Activity in Tissues of IUGR and Normal Birthweight Pigs

	GAMT Activity (μmol/g of tissue/30 mins)				P-Values for Treatment Effects		
	Normal Birth Weight		IUGR				
	Ala-Control (n=9)	Gly-Treated (n=10)	Ala-Control (n=7)	Gly-Treated (n=6)	IUGR Effect	Gly Effect	Gly x IUGR Effect
Kidney	4.0 ± 1.0^{a}	2.8 ± 1.1^{a}	3.8 ± 0.81^{a}	3.8 ± 1.0^{a}	0.693	0.559	0.543
Liver	9.5 ± 1.4^{a}	10.2 ± 3.2^{a}	10.7 ± 3.7^{a}	11.0 ± 3.3^{a}	0.739	0.862	0.947
Pancreas	7.0 ± 0.8^{c}	41.4 ± 7.2^{a}	15.6 ± 4.5^{bc}	29.1 ± 5.6^{ab}	0.729	0.0002	0.067
Small Intestine	1.7 ± 0.36^{b}	1.8 ± 0.40^{b}	0.81 ± 0.18^{b}	7.1 ± 1.9^{a}	0.032	0.003	0.003
Longissimus Dorsi Muscle	ND	ND	ND	ND	--	--	--

For a tissue, different superscripts (a-c) across a row indicate statistical significance ($P < 0.05$).

Discussion

Conventional diets for growing swine are low in Gly content, as they are largely corn-and-soybean-meal-based feeds and Gly is very low in all plant-based products (Hou at al. 2019). This indicates a potential for a severe Gly deficiency in the diets of growing pigs with undesirable for growth and development of normal birthweight pigs, but even more detrimental for IUGR pigs. IUGR pigs already suffer from low birthweight status, failure to reach maximum growth potential, and low survivability due to impaired nutrient absorption caused by poor gut development. IUGR pigs also have severely diminished capabilities for synthesizing Gly endogenously (Hu et al. 2017). Dietary supplementation with Gly improves growth of IUGR pigs in the early post-weaning period proposed to be due to improved hydroxyproline synthesis pathways and stimulation of the mTOR pathway for protein synthesis (Hu et al. 2017). However, Gly has many other roles as both a functional amino acid and as a precursor for important bioactive molecules within the body including nitric oxide, free radical scavenging as a direct and indirect antioxidant, membrane stability, and energy metabolism via the synthesis of CRP (Bender et al. 2005; Fortalezas et al. 2018; Wyss and Kaddurah-Daouk 2000; Wu 2013a).

The renal-hepatic axis is primarily engaged in endogenous synthesis of CR with Gly and arginine undergoing a two-step reaction catalyzed by AGAT to yield guanidinoacetate (see Figure 4.3). It is estimated that 14.5% of all Gly (dietary + endogenous) is used for synthesis of CR each day (Melendez-Hevia et al. 2009) with 87% of dietary Gly alone being used to support CR synthesis (Wu and Morris 1998). Therefore,

it is clear that without adequate Gly provision or synthesis, synthesis of CR will also be impaired. Results of this study indicate that IUGR pigs have a greatly reduced capability to synthesize CR (Fig. 4.1) as AGAT activity is lower than for all other treatment groups ($P < 0.05$), including non-supplemented normal birthweight pigs. This finding supports that of Hu et al. (2017). With Gly supplementation, however, AGAT activity increased in IUGR pigs to that for normal birthweight littermates, regardless of dietary Gly supplementation ($P > 0.05$). Thus, dietary Gly supplementation increases synthesis of creatine in IUGR pigs, which suggests that a deficiency in Gly is limiting synthesis of CR via AGAT and GAMT activities in IUGR pigs (Fig. 4.1)

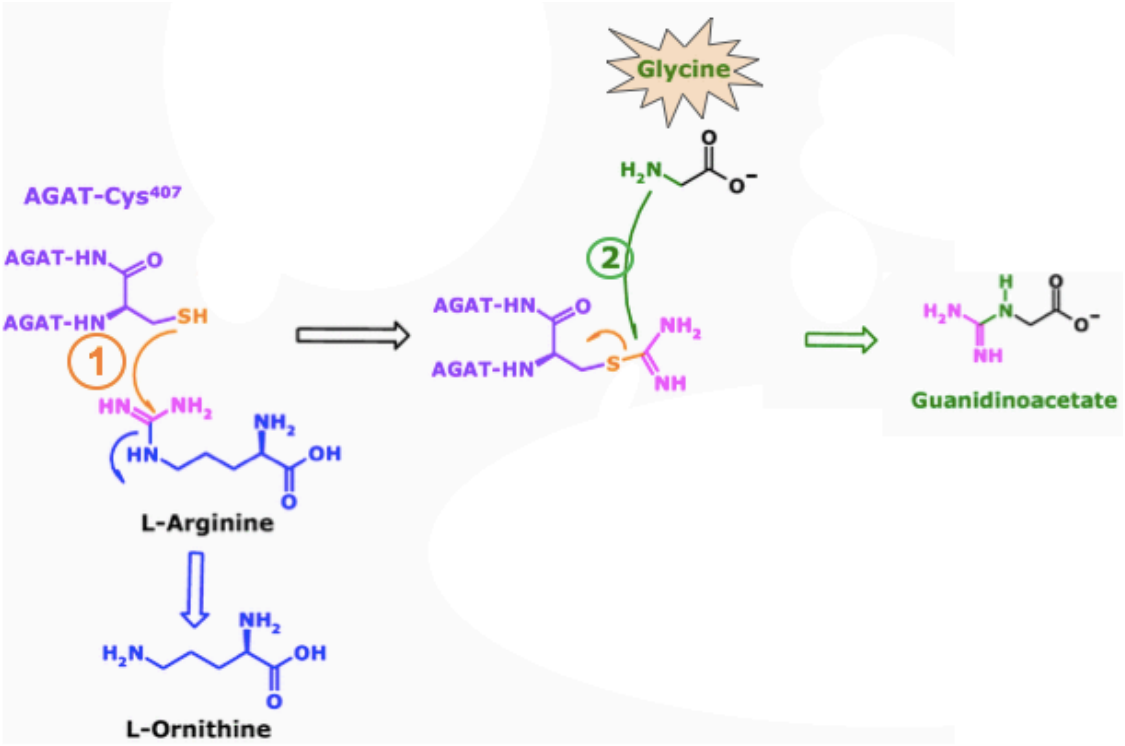


Figure 4.3. The reaction mechanism for arginine:glycine amidinotransferase (AGAT). The reaction begins with the removal of the amidino group from arginine by the AGAT enzyme (1), releasing ornithine. The amidino group is then transferred from the AGAT enzyme site to the amino terminal end of Gly (2), forming guanidinoacetate. Adapted from Tsikas and Wu 2015.

The second step in creatine synthesis is catalyzed by GAMT and involves *S*-adenosylmethionine (SAM) cofactor for methylation. Guanidinoacetate, from the AGAT reaction, is methylated by GAMT, resulting in synthesis of CR and the production of the demethylated compound, *S*-adenosylhomocysteine (SAH), which will be regenerated to SAM through the one-carbon metabolism pathway that includes Gly. GAMT is present in many tissues of the body, however, in the pig, the most metabolically important site of GAMT activity is the liver. Results of this study indicate that GAMT activity in the liver and kidneys is not affected significantly by either Gly supplementation or bodyweight

status ($P > 0.05$) (Fig 4.2) and that the pancreas has the greatest GAMT activity of all tissues evaluated ($P < 0.05$) (Table 4.2). This is metabolically interesting because dietary Gly supplementation increased GAMT activity in the pancreas ($P < 0.05$). However, while the pancreas has the greatest GAMT activity in this study, it is a much smaller organ than the liver. For comparison, the pancreas and liver of market weight (120-130 kg live weight) pigs weighed 54 g and 2115 g, respectively, making the liver nearly 40-fold larger than the pancreas by mass (Loeffel and Koch 1970). Therefore, when comparing enzyme activity/g of tissue, the size of the organ must be taken into account to allow for proper understanding of their relative total contributions to the synthesis of CR. GAMT activity in the jejunum of the small intestine was nearly undetectable in pigs in all treatment groups and bodyweight groups, but for IUGR pigs supplemented with dietary Gly, GAMT activity increased from 1 to 2 μmol/g tissue/30 min for Ala control IUGR pigs and both groups of normal birthweight pigs, to 7 μmol/g tissue/30 min in Gly-supplemented IUGR pigs ($P < 0.05$). Thus, GAMT enzymatic activity is very responsive to dietary Gly supplementation, particularly in the small intestine of IUGR pigs and the pancreas of both IUGR and normal birthweight pigs. These results differ from published results indicating that the GAMT enzyme is not a site for regulation for CR synthesis (Da Silva et al. 2008; Nasrallah et al. 2010). This is a novel finding in that dietary Gly supplementation exerted a regulatory effect on GAMT activity in non-traditional tissues for CR synthesis, such as the small intestine and the pancreas. However, more research is needed to confirm these novel findings.

Results of the present study indicate that dietary Gly supplementation increases activation of both AGAT and GAMT for increased endogenous synthesis of creatine in organs associated with this pathway (the renal-hepatic axis). Further, there was an increase in expression of AGAT and GAMT in other organs, such as the small intestine and pancreas, in response to dietary Gly supplementation. Although the pancreas and small intestine represent less tissue mass, they can influence the synthesis of CR in support of growth and development of IUGR and normal birthweight pigs. Based on results noted in Chapter III, it can be confirmed that increases in activities of AGAT and GAMT result in increased concentrations of creatine (and its metabolite creatine phosphate) throughout the body, especially in the skeletal muscle, kidney, and pancreas (CH III).

In conclusion, while all growing pigs likely suffer from some degree of Gly deficiency, IUGR pigs are subject to greater difficulties due to this deficiency. Dietary Gly supplementation is a reasonable and easy dietary intervention that can improve growth and survivability of IUGR pigs by improving energy metabolism via increasing activity of the creatine synthesis pathways. Further, dietary Gly supplementation increased activities of both AGAT and GAMT in multiple tissues, including the traditional renal-hepatic axis of the creatine synthesis pathway. The IUGR pigs were confirmed to have diminished AGAT activity in the kidneys, indicating that a Gly deficiency is contributing to a deficiency in synthesis of CR that reduces growth rates of IUGR pigs. Additionally, a potentially novel finding was that GAMT, the second enzyme in the CR synthesis pathway, is subject to regulatory mechanisms that can increase activity in non-traditional tissues engaged in synthesis of CR. This differs from the current understanding that AGAT

is the only site of regulation for CR synthesis within the renal-hepatic axis and may indicate that additional pathways for CR synthesis exist to maintain CR homeostasis and, as such, are not subjected to the same regulatory mechanisms as that in the renal-hepatic axis. Finally, results of this study advance understanding of the mechanisms whereby dietary Gly supplementation successfully rescues IUGR pigs and allows them to achieve maximum growth potential and survivability to improve efficiencies and profitability in swine production enterprises worldwide.

CHAPTER V

ANALYSIS OF EFFECTS OF DIETARY GLYCINE SUPPLEMENTATION ON EXPRSSION OF CREATINE-SYNTHETIC ENZYMES, CREATINE KNASE, AND MTOR GENES IN TISSUES OF IUGR AND NORMAL PIGS

Introduction

Intrauterine growth restriction (IUGR) is a naturally occurring phenomena in swine production that affects 20-25% of all pigs born each year (Wu et al. 2010). These pigs are born with underdeveloped skeletal muscle tissue, as well as small intestines and weigh only one-half to one-third of that for their normal birthweight siblings (Wang et al. 2008). The survivability of IUGR pigs to weaning is very low and they will also experience greatly reduced growth rates and lower feed efficiencies than normal birthweight pigs, meaning that they are usually culled at birth by producers (Ji et al. 2017). These pigs also have disrupted metabolic pathways, such as a decreased ability to synthesize glycine (Gly) from 4-hydroxyproline, the primary substrate for endogenous Gly synthesis in pigs (Hu et al. 2017). This results in pronounced Gly deficiency in IUGR pigs that likely contributes to their decreased growth and decreased synthesis of creatine (CR), a metabolically vital product synthesized from arginine, methionine, and Gly. Creatine synthesis utilizes 87% of dietary Gly each day (Wu and Morris 1998) and 14.5% of total Gly (dietary + endogenous) (Melendez-Hevia et al. 2009), representing the function of the majority of dietary Gly intake. However, due to very low levels of dietary Gly in traditional plant-based swine diets (Hou et al. 2019), this places the responsibility

for obtaining the majority of Gly on the underdeveloped pathways for synthesis in IUGR pigs. Due to the importance of Gly overall, and the severely diminished supply, Gly is a conditionally essential amino acid (CEAA) and should be considered for dietary supplementation for management strategies, such as for growing IUGR pigs.

Dietary Gly supplementation has been shown to affect gene expression in different tissues. Dietary Gly supplementation improved the growth performance of both normal birthweight sow-reared pigs (Wang et al. 2014), as well as IUGR sow-reared pigs (Hu et al. 2017) through stimulation of mechanistic target of rapamycin (mTOR). It is unknown what effects dietary Gly supplementation may have on mTOR activity in postweaning pigs. Provision of substrates such as Gly stimulate the creatine-synthetic enzyme AGAT at the pre-translational level (Edison et al. 2007). This increases downstream synthesis of CR that can improve overall growth of skeletal muscle and improve neonatal survival through its metabolic functions. Increased expression of one or both of these proteins in the creatine synthesis pathway in response to dietary Gly supplementation would improve survivability, and growth rate of IUGR pigs, with the potential to improve skeletal muscle mass in normal birthweight pigs as well. Therefore, the goal of this study was to determine the effects of dietary Gly supplementation on expression of genes for (1) CR synthesis (AGAT*, GAMT),* (2) creatine kinase muscle-type (*CKM*), and (3) mechanistic target of rapamycin (*MTOR*). This was done to test the hypothesis that dietary supplementation of Gly will upregulate expression of genes for both enzymes of the creatine synthetic pathway due to increased substrate provision, and subsequently result in increased expression of CKM as more CR is synthesized and must be taken up by the skeletal

muscle. Additionally, it is hypothesized that the trends seen in previous studies of Gly supplementation stimulation of mTOR in preweaning piglets will be detected for growing and finishing pigs, regardless of birthweight status.

Materials and Methods

Animals and diets

The experimental protocol for this study was approved prior to beginning the study by the Texas A&M University Animal Care and Use Committee (IACUC).

Animals, diets, and feeding regimens utilized are the same as described in Chapter II.

Termination of feeding trial and tissue collection

The feeding trial was terminated at 188 days of age, and pigs were slaughtered as described in Chapter II and tissues were collected at time of slaughter, as described in Chapter III.

RNA Extraction and cDNA Synthesis

RNA was extracted from liver, kidney, small intestine (jejunal), longissimus dorsi muscle, and semimembranosus muscle using Trizol reagent and RNeasy kits (Qiagen Cat. No. 74106, Life Technologies, Carlsbad, CA) according to manufacturer's instructions. RNA quality and quantity were initially assessed by Nanodrop prior to cDNA synthesis. First-strand cDNA was synthesized using SuperScript^{TM} First-Strand Synthesis System for RT-PCR (Invitrogen Cat. No. 11904-018, Life Technologies) according to

manufacturer's instructions. First-strand cDNA was diluted 10-fold and then used for all quantitative PCR (qPCR) reactions.

Gene Analysis

Before beginning analyses to determine expression of genes of interest, adequate reference genes were established for each tissue. Reference genes showed uniform expression across all groups without significant effects ($P > 0.05$). Primers for reference genes for qPCR analysis were designed using the National Center for Biotechnology Information (NCBI) Genbank sequences and Primer-BLAST (http://ncbi.nlm.nih.gov/). Primers were submitted to BLAST to test for specificity against the *Sus scrofa* (porcine) genome. Primer information for reference genes and other gene sequences used can be found in Supplemental Table S1.

Quantitative PCR

The qPCR assays were performed using PerfeCta SYBR Green Mastermix (Quanta Biosciences, Gaithersburg, MD) in 10 μL reactions with 2.5 mM of each primer, on a Roche 480 Lightcycler (Roche, Basel, Switzerland) using approximately 10 ng of cDNA per reaction. The PCR program began with 5 min at 95º C followed by 40 cycles of 95º C denaturation for 10 sec and 60º C annealing/extension for 30 sec. A melt curve was produced with every run to verify a single gene-specific peak. Standard curves with serial dilutions from 50% to 0.39% of cDNA pool of all cDNA for a given tissue were run to determine primer efficiencies for reference genes and genes of interest. All primer

correlation coefficients were greater than 0.90 and efficiencies were 95%-102%. The mean of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) and hypoxanthine phosphoribosyltransferase 1(*HPRT1*) was used to normalize data on genes of interest in all tissues. These genes were chosen as reference genes based on previous studies and also confirmed as having uniform expression across all treatment groups ($P > 0.05$) (Nygard et al. 2007). Primer sequences for genes of interest were based on previous work from Borchel et al. (2019) for the following genes and verified using NCBI and Primer-BLAST as described previously: glycine amidinotransferase *(GATM/*AGAT), guanidinoacetate *N*-methyltransferase (*GAMT)*, and creatine kinase (*CKM*). Primer sequences for mechanistic target of rapamycin kinase (*MTOR*) were designed as described previously. The $2^{-\Delta\Delta Ct}$ method was utilized to normalize data and fold changes were utilized for statistical analyses.

Statistical Analyses

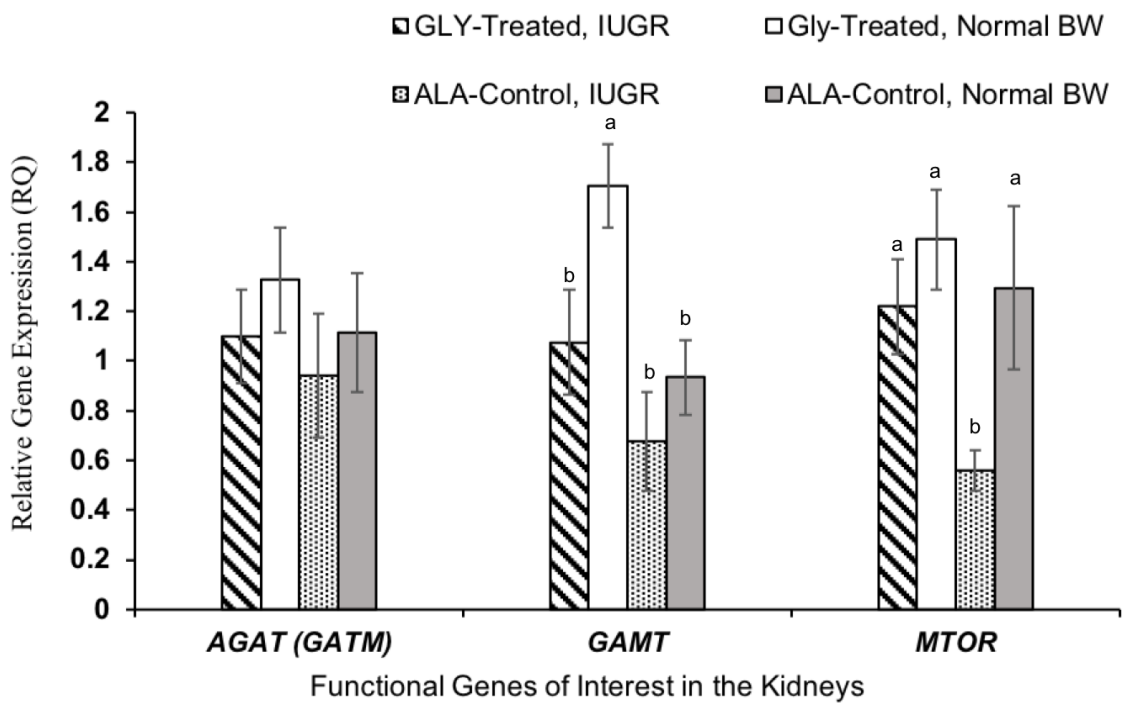
Statistical analyses were performed on normalized qPCR data using the methods described in Chapter II.

Results

The effects of dietary supplementation of Gly on expression of key genes for creatine metabolism and protein metabolism are summarized in Figures 5.1 to 5.4. The data are summarized by tissue type for ease of comparison of multiple genes of interest within individual tissues. Overall Gly supplementation had a significant effect on expression of multiple genes across all of the tissues analyzed.

Gene Expression Effects in Kidney

The kidney is the primary site for AGAT activity that catalyzes the conversion of arginine and glycine to ornithine and guanidinoacetate. Expression of AGAT mRNA was not affected by either dietary Gly supplementation or birthweight status of pigs ($P > 0.05$). However, the expression of *GAMT* mRNA, but not MTOR did increase with Gly treatment in kidneys of normal birthweight pigs ($P < 0.05$) and IUGR pigs ($P = 0.03$), respectively (Figure 5.1). AGAT is involved with creatine synthesis and MTOR is involved in mRNA translation and protein synthesis, respectively.

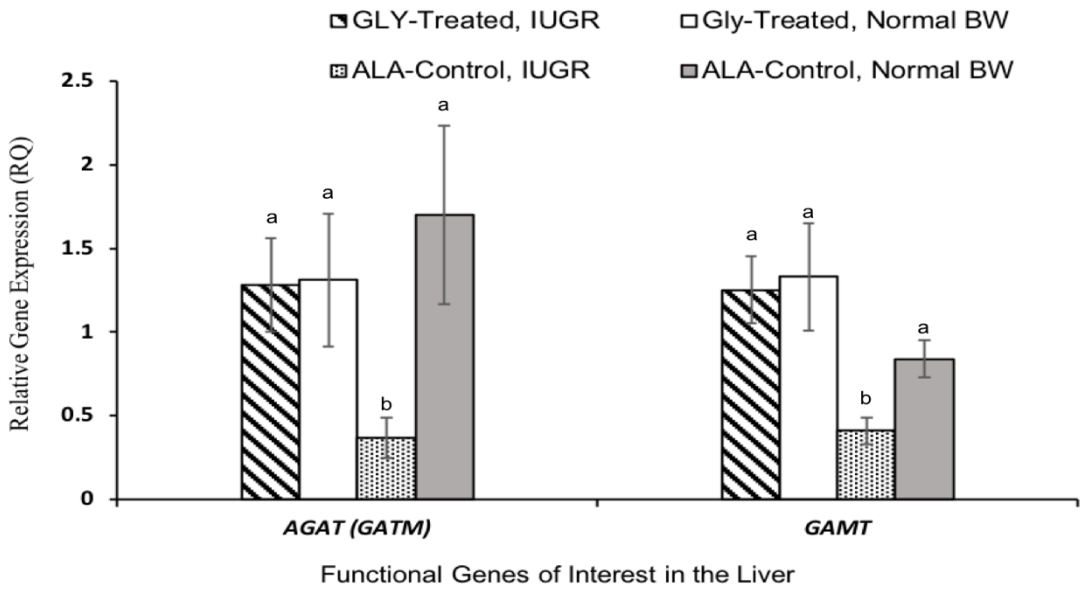


Relative Gene Expression in Kidney Tissue					P-Values for Treatment Effects		
	Normal Birth Weight		IUGR				
Gene	Ala-Control (n=9)	Gly-Treated (n=10)	Ala-Control (n=7)	Gly-Treated (n=6)	IUGR Effect	Gly Effect	Gly x IUGR Effect
GATM (AGAT)	1.1 ± 0.24	1.3 ± 0.21	0.94 ± 0.25	1.1 ± 0.19	0.3952	0.5166	0.8599
GAMT	0.94 ± 0.15^{b}	1.7 ± 0.17^{a}	0.70 ± 0.20^{b}	1.1 ± 0.21^{b}	0.0280	0.0051	0.3391
MTOR	1.3 ± 0.33^{a}	1.9 ± 0.08^{a}	0.56 ± 0.08^{b}	1.2 ± 0.19^{a}	0.1681	0.2110	0.9463

Figure 5.1. The relative expression of genes for creatine synthetic enzymes (AGAT, GAMT) and protein synthesis, mTOR, in kidney tissue of IUGR and normal birthweight pigs at the time of slaughter, following dietary supplementation with either Gly or Ala (isonitrogenous control) from weaning at 21 d of age to slaughter at 188 d of age. These results indicated that Gly supplementation significantly increased expression of *GAMT* mRNA in the kidneys of Gly-supplemented normal birthweight pigs, but not IUGR pigs. Gly supplementation also significantly increased relative expression of *MTOR* mRNA in kidneys of IUGR pigs. For a gene, different letters indicate statistical difference ($P < 0.05$) according to analysis by two-way ANOVA linear model of multiple effects.

Gene Expression Effects in Liver

The liver is the primary site for activity of GAMT that catalyzes the methylation of guanidinoacetate to form creatine. Supplemental dietary Gly increased expression ($P < 0.01$) of *GAMT* mRNA in livers of IUGR pigs, while expression of AGAT mRNA was decreased in control IUGR pigs ($P < 0.05$) compared to Gly-supplemented IUGR pigs and normal birthweight pigs, regardless of treatment status ($P > 0.05$). AGAT is not typically associated with the liver.



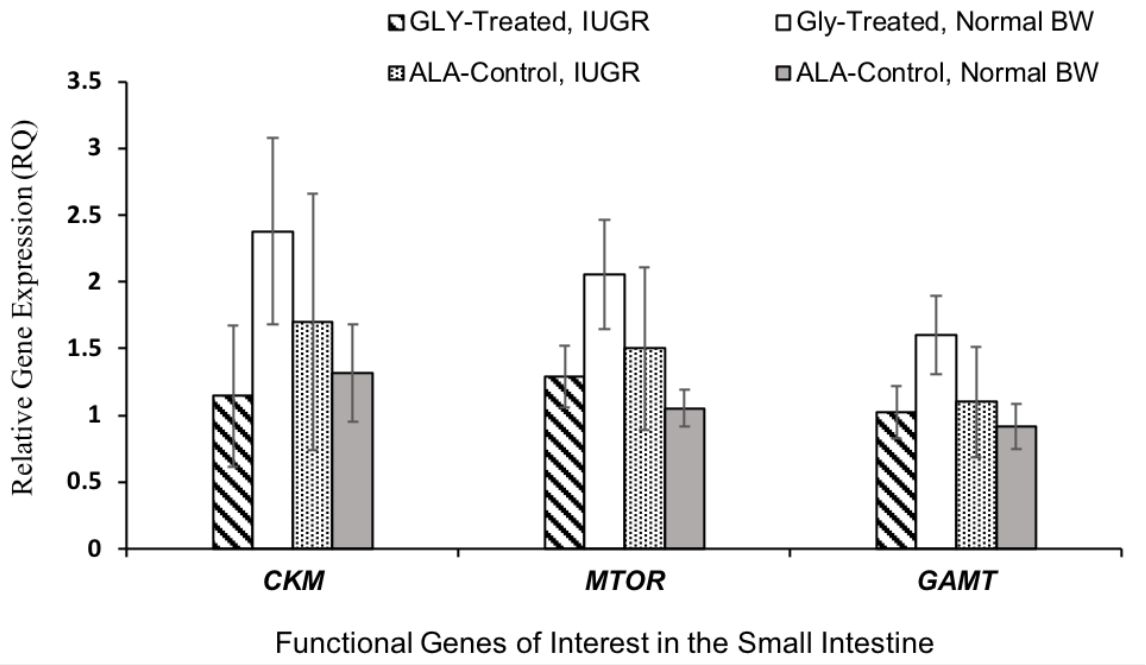
Relative Gene Expression in Liver Tissue					P-Values for Treatment Effects		
	Normal Birth Weight		IUGR				
Gene	Ala-Control (n=9)	Gly-Treated (n=10)	Ala-Control (n=7)	Gly-Treated (n=6)	IUGR Effect	Gly Effect	Gly x IUGR Effect
*GATM (*AGAT)	1.7 ± 0.53^{a}	1.3 ± 0.39^{a}	0.37 ± 0.12^{b}	1.3 ± 0.28^{q}	0.0744	0.0790	0.0388
GAMT	0.84 ± 0.11^{ab}	1.3 ± 0.32^{a}	0.41 ± 0.08^{b}	1.3 ± 0.20^{a}	0.2296	0.0729	0.0059

Figure 5.2. The relative gene expression of creatine synthetic enzymes (AGAT, GAMT) in livers from IUGR and normal birthweight pigs at time of slaughter, following dietary supplementation with either Gly (1% of diet) or Ala (isonitrogenous control) from weaning at 21 d of age to slaughter at 188 d of age. These results indicated that supplemental dietary Gly significantly increased expression of AGAT and *GAMT* mRNAs in livers of IUGR pigs but was without a significant effects for pigs with normal birthweights. Different superscript letters indicate statistical difference ($P < 0.05$) and different asterisks indicate greater statistical difference ($P < 0.01$) according to analysis by two-way ANOVA linear model of multiple effects.

Gene Expression Effects in the Small Intestine (Jejunum)

The small intestine of IUGR pigs is typically underdeveloped and atrophied, with reduced protein and energy metabolism. The mTOR transcriptional complex and CKM (creatine kinase muscle-type) are associated with protein and energy metabolism, respectively and are encoded for by the genes *MTOR* and *CKM.* There was no apparent effect of Gly supplementation or birthweight status on expression of *CKM*, *MTOR*, or

GAMT mRNAs (P > 0.05) in the small intestine (jejunum). There was a trend towards increased expression of *MTOR* mRNA in IUGR and normal birthweight pigs (P < 0.10).



Relative Gene Expression in Small Intestine Tissue					P-Values for Treatment Effects		
	Normal Birth Weight		IUGR				
Gene	Ala-Control (n=9)	Gly-Treated (n=10)	Ala-Control (n=7)	Gly-Treated (n=6)	IUGR Effect	Gly Effect	Gly x IUGR Effect
CKM	1.3 ± 0.36	2.4 ± 0.70	1.7 ± 0.96	1.1 ± 0.53	0.2523	0.7271	0.6480
MTOR	1.1 ± 0.14	2.1 ± 0.41	1.5 ± 0.61	1.3 ± 0.23	0.6277	0.2293	0.0714
GAMT	0.92 ± 0.17	1.6 ± 0.29	1.1 ± 0.41	1.0 ± 0.20	0.4802	0.2735	0.1722

Figure 5.3. The relative gene expression of creatine metabolism enzymes (creatine kinase, GAMT) and mTOR, in small intestine (jejunum) tissue from IUGR and normal birthweight pigs at the time of slaughter, following dietary supplementation with either Gly or Ala (isonitrogenous control) from weaning at 21 d of age to slaughter at 188 d of age. There were no effects ($P > 0.05$) of dietary glycine supplementation on expression of the mRNAs of interest. Different letters across a row indicate statistical difference ($P < 0.05$) according to analysis by two-way ANOVA linear model of multiple effects.

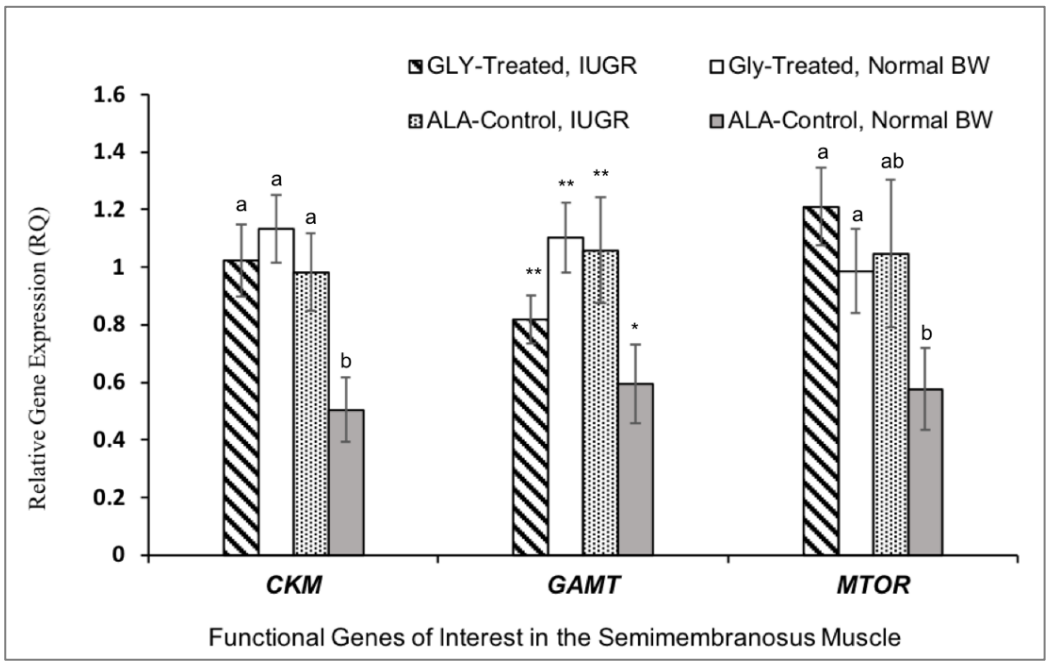
Skeletal Muscle Gene Expression Effects

Skeletal muscle is a primary site of creatine energy metabolism , as well as protein metabolism. The longissimus dorsi muscle and the semimembranosus muscle are both skeletal muscles, but they are functionally different as the former is primarily a support muscle and the latter is a muscle for locomotion. Therefore, their muscle fiber types are different. This could indicate a potential for differential effects on *CKM*, and mTOR, encoded by *MTOR.* Additionally, there are multiple locations throughout the body, outside of the primary renal-hepatic axis that express GAMT (Brosnan and Brosnan 2007).

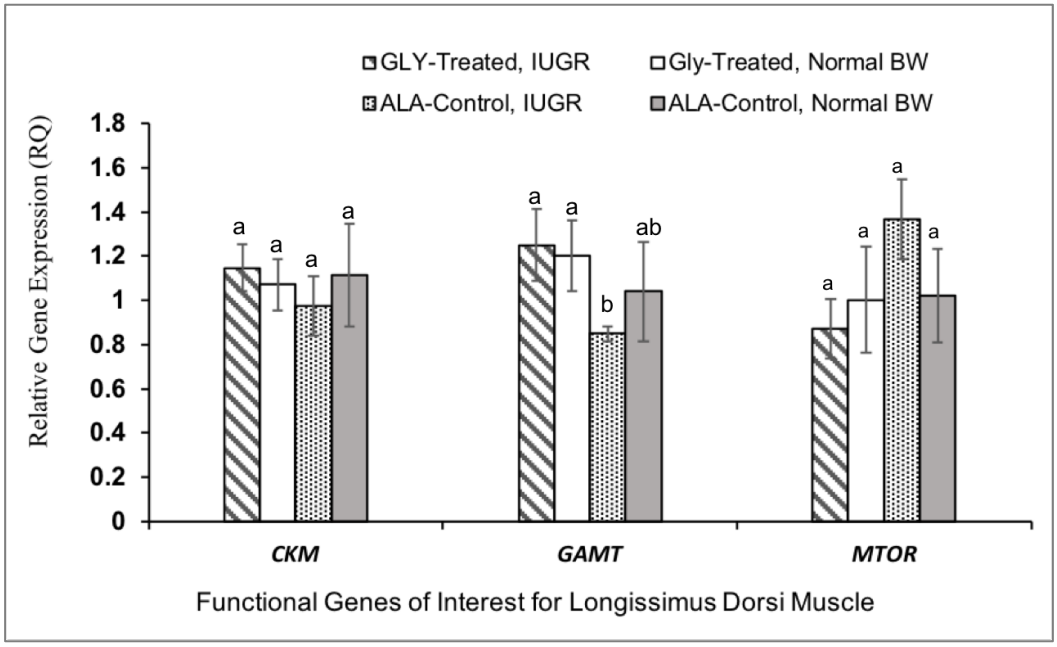
In the semimembranosus muscle (Figure 5.4) expression of mRNAs for CKM, MTOR and GAMT increased in response to dietary Gly supplementation in normal birthweight pigs, but there was no effect of supplemental dietary Gly on expression of the three genes in the semimembranosus muscle from IUGR pigs.

Expression of mRNAs for GAMT, CKM and MTOR in the longissimus dorsi muscle differed from that for the semimembranosus muscle. The expression of *CKM* and *MTOR* mRNAs was not affected by supplemental dietary Gly regardless of birthweight status ($P > 0.10$). But, expression of *GAMT* mRNA expression increased ($P<0.05$) in longissimus dorsi muscle of IUGR pigs fed dietary glycine compared to that for expression in longissimus dorsi muscle from normal birthweight pigs.

A


GLY-Treated, IUGR
Gly-Treated, Normal BW
ALA-Control, IUGR
ALA-Control, Normal BW
Relative Gene Expression (RQ)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
a
b
**
**
**
*
a
ab
a
b
CKM
GAMT
MTOR
Functional Genes of Interest in the Semimembranosus Muscle

B


GLY-Treated, IUGR
Gly-Treated, Normal BW
ALA-Control, IUGR
ALA-Control, Normal BW
Relative Gene Expression (RQ)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
a
a
a
a
b
ab
a
a
a
a
CKM
GAMT
MTOR
Functional Genes of Interest for Longissimus Dorsi Muscle

Relative Gene Expression in Skeletal Muscle Tissue					P-Values for Treatment Effects		
	Normal Birth Weight		IUGR				
Gene	Ala-Control (n=9)	Gly-Treated (n=10)	Ala-Control (n=7)	Gly-Treated (n=6)	IUGR Effect	Gly Effect	Gly x IUGR Effect
	Semimembranosus Muscle Tissue (**A**)						
CKM	0.51 ± 0.11^{b}	1.1 ± 0.12^{a}	0.98 ± 0.13^{a}	1.0 ± 0.13^{a}	0.1492	0.0126	0.0262
GAMT	0.60 ± 0.14^{b}	1.0 ± 0.13^{a}	1.1 ± 0.18^{a}	0.82 ± 0.08^{a}	0.5066	0.3217	0.0098
MTOR	0.58 ± 0.14^{b}	0.99 ± 0.15^{a}	1.0 ± 0.25^{ab}	1.2 ± 0.13^{a}	0.0801	0.0051	0.9869
	Longissimus Dorsi Muscle Tissue (**B**)						
CKM	1.1 ± 0.23	1.1 ± 0.12	0.98 ± 0.13	1.1 ± 0.11	0.6348	0.2593	0.2913
GAMT	1.0 ± 0.23^{ab}	1.2 ± 0.16^{a}	0.85 ± 0.03^{b}	1.3 ± 0.16^{a}	0.3964	0.2168	0.4876
MTOR	1.0 ± 0.21	0.87 ± 0.24	1.4 ± 0.18	0.87 ± 0.13	0.4286	0.1891	0.8842

Relative Gene Expression in Skeletal Muscle Tissue					P-Values for Treatment Effects		
	Normal Birth Weight		IUGR				
Gene	Ala-Control (n=9)	Gly-Treated (n=10)	Ala-Control (n=7)	Gly-Treated (n=6)	IUGR Effect	Gly Effect	Gly x IUGR Effect
	Semimembranosus Muscle Tissue (**A**)						
CKM	0.51 ± 0.11^{b}	1.1 ± 0.12^{a}	0.98 ± 0.13^{a}	1.0 ± 0.13^{a}	0.1492	0.0126	0.0262
GAMT	0.60 ± 0.14^{b}	1.0 ± 0.13^{a}	1.1 ± 0.18^{a}	0.82 ± 0.08^{a}	0.5066	0.3217	0.0098
MTOR	0.58 ± 0.14^{b}	0.99 ± 0.15^{a}	1.0 ± 0.25^{ab}	1.2 ± 0.13^{a}	0.0801	0.0051	0.9869
	Longissimus Dorsi Muscle Tissue (**B**)						
CKM	1.1 ± 0.23	1.1 ± 0.12	0.98 ± 0.13	1.1 ± 0.11	0.6348	0.2593	0.2913
GAMT	1.0 ± 0.23^{ab}	1.2 ± 0.16^{a}	0.85 ± 0.03^{b}	1.3 ± 0.16^{a}	0.3964	0.2168	0.4876
MTOR	1.0 ± 0.21	0.87 ± 0.24	1.4 ± 0.18	0.87 ± 0.13	0.4286	0.1891	0.8842

Figure 5.4. The relative expression of mRNAs for creatine metabolism (*CKM, GAMT*) and protein metabolism (*MTOR*) in skeletal muscle of IUGR and normal birthweight pigs at time of slaughter, following dietary supplementation with either Gly or Ala (isonitrogenous control) from weaning at 21 d of age to slaughter at 188 d of age. These results indicated that in (**A**) semimembranosus tissue Gly had a significant effect on increasing *MTOR*, *GAMT,* and *CKM* expression in normal birthweight pigs, but not IUGR pigs. In longissimus dorsi muscle (**B**), there was no change in expression for *CKM* or *MTOR*, regardless of birthweight or treatment status, but there was a significant increase in *GAMT* expression for IUGR pigs. Different superscript letters indicate statistical differences ($P < 0.05$) according to analysis by two-way ANOVA linear model of multiple effects.

Discussion

Gly synthesis pathways are known to be diminished in IUGR pigs (Hu et al. 2017). Gly has many functional roles including those related to energy metabolism through

creatine synthesis and protein metabolism via stimulation of the mTOR (Hu et al. 2017). As Gly can have many effects on overall health, it has been used previously as a dietary supplement for improving growth and survivability, primarily by targeting stimulation of mTOR to increase protein synthesis and cellular metabolism (Hu et al. 2017). However, this study aimed to elucidate other potential pathways influenced by Gly that improve growth and survivability of IUGR pigs, namely the creatine synthetic pathway and related energy metabolism pathways involving creatine. Results of this study indicate that dietary Gly supplementation increases expression of selected genes for creatine and protein metabolism across different tissues from in both IUGR and normal birthweight pigs.

Dietary Gly supplementation influences on renal-hepatic axis creatine synthesis

The endogenous synthesis of creatine begins primarily in the kidneys with conversion of arginine and glycine into ornithine and guanidinoacetate via the transfer of the amidino group of arginine to Gly by AGAT, the rate-limiting step in the creatine synthesis pathway. Guanidinoacetate is transported to the liver where it is converted to creatine by GAMT in concert with SAM. Creatine is then released into the hepatic vein for delivery to extrahepatic tissues. Results of this study revealed that expression of AGAT mRNA is not affected by dietary Gly supplementation or birthweight of pigs (Fig 5.1) ($P > 0.05$), expression of GAMT mRNA was affected significantly by dietary Gly supplementation in both IUGR and normal birthweight pigs. These effects are stratified across the renal-hepatic axis. In renal tissues, expression of AGAT mRNA is unchanged ($P > 0.05$), whereas expression of GAMT mRNA increased in Gly supplemented normal birthweight pigs ($P < 0.05$), but there was only a trend ($P < 0.10$) for dietary Gly

supplementation to increase expression of GAMT mRNA in IUGR pigs (Fig. 5.1). Interestingly, this trend mostly held true for expression of *GAMT* mRNA in hepatic tissues (Fig. 5.2) with the increase in expression being greater for Gly-supplemented IUGR pigs ($P > 0.05$) than Gly-supplemented normal birthweight pigs. Interestingly, expression of AGAT mRNA was detectable in hepatic tissues of all treatment groups and was equally expressed across all groups except for non-supplemented IUGR pigs ($P > 0.05$). The IUGR control pigs have diminished expression of AGAT mRNA ($P < 0.05$) that was restored to that of normal birthweight pigs following Gly supplementation ($P > 0.05$). There are tissues other than the kidneys that do have AGAT activity including the pancreas and the testes (Brosnan & Brosnan, 2007). However, non-renal tissues may express AGAT mRNA (*GATM*) but may not encode for a functional enzyme (Da Silva et al. 2014). Additionally, in previous *in situ* studies of enzymes in these same tissues, there was a significant increase in AGAT activity in Gly-supplemented IUGR pigs that restored AGAT activity to that for their normal birthweight counterparts following Gly supplementation (see Chapter IV). In these same studies, AGAT activity was nearly undetectable in liver tissues for all treatment groups, and GAMT activity was similarly low for kidney tissues across all treatment groups. GAMT activity within the small intestine was also nearly undetectable for all except Gly-supplemented pigs, which, again, does not mirror the mRNA expression profile for *GAMT.* Overall, this suggests that mRNA expression does not necessarily denote enzyme functionality or capability for all tissues and demonstrates the importance of *in situ* experiments to corroborate results from mRNA expression profiles.

Dietary Gly supplementation influences creatine energy metabolism and protein metabolism mRNA expressions

Creatine plays many functional roles throughout the body, including antioxidant capabilities, regulating brain function, improving nitric oxide availability for tissues, and improving stability of cellular membranes (Wu 2013a, Wu 2020). However, creatine is most often associated with cellular energy metabolism via its conversion to creatine phosphate by mitochondrial and cytoplasmic forms of creatine kinase (CK). Creatine is phosphorylated by CK within the mitochondria and within the cytoplasm, which allows for an accumulation of a dynamic "phosphate pool" within the cell. This pool of phosphate is rapidly accessed in times of intense energetic demand for rapid conversion of ADP to highly energetic ATP. The phosphate group of creatine phosphate is removed by CK and ADP is phosphorylated to form ATP by this same enzyme, albeit a different isoform, which is also tissue-dependent.

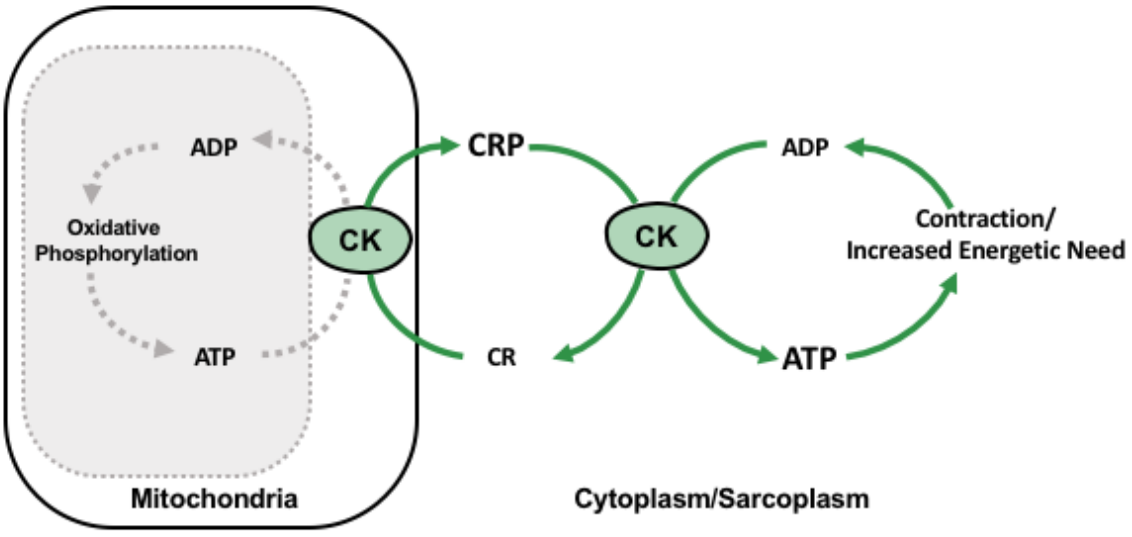


Figure 5.5. The creatine phosphate – creatine "shuttle" mechanism that allows for rapid ATP replenishment during times of increased cellular need. Adapted from Neubauer 1998.

The ATP can then be used to meet the increased energetic needs of the cell and creatine is regenerated and available for phosphorylation once again. This is an important energetic mechanism for cells in tissues that are undergoing increased work such as skeletal muscle, or periods of intense growth and cellular division or locomotion, such as during growth and development. In this way, creatine is a potentially potent compound for improving both growth and development of IUGR pigs, which face barriers to survivability and reaching maximum growth potential due to underdeveloped small intestine mucosa and disrupted skeletal muscle tissue turnover. In this study, Gly supplementation increased expression of *MTOR* in the small intestine of normal birthweight pigs ($P < 0.10$), but there was no corresponding increase in its expression in the small intestine of IUGR pigs. However, it should be noted that there was no statistical difference between expression of *MTOR* mRNA in either IUGR group compared to either non-supplemented normal birthweight pigs or Gly-supplemented pigs (Fig 5.3). This is possibly due simply to the low level of expression of *MTOR* in the small intestine in normal birthweight pigs compared to the other three treatment groups in this study. However, increased mTOR activity within the mucosa of the small intestine is a potential indicator of increased cellular proliferation and improved protein synthesis due to dietary Gly supplementation for normal birthweight pigs (Kaur and Moreau 2019; Kim et al. 2012; Saxton and Sabatini 2017). There was also no change in overall expression of *CKM* in the small intestine regardless of treatment or birthweight status ($P > 0.05$) (Fig 5.3), which is likely due to this transcript not being commonly found in the small intestine tissue. However this was analyzed in this tissue regardless, as previous results had shown expression of this isoform

in non-muscle tissue such as the liver and kidneys (Borchel et al. 2019) . *MTOR* was highly expressed in the kidneys of IUGR pigs in response to dietary Gly supplementation ($P < 0.05$) and trended towards increased expression in normal birthweight pigs ($P < 0.10$). This indicates the potential for increased protein synthesis in the renal tissue. This could be indicative of increased enzyme synthesis activity in response to dietary Gly supplementation, which would correspond with the increase in *in situ* AGAT enzyme activity in kidneys of IUGR pigs (see Chapter IV).

Other indications of effects of Gly supplementation on energy and protein metabolism are the differential expression profiles for *CKM* and *MTOR* in two types of skeletal muscle. Within the longissimus dorsi muscle there was no effect of Gly supplementation on *CKM* or *MTOR* expression (Fig 5.4 B), although there was a minor increase in expression of *GAMT* mRNA, but enzymatic activity in the tissue was not determined. In contrast, there was an increase in *CKM* and *MTOR* expression in the semimembranosus muscle of normal birthweight pigs ($P < 0.05$) (Fig 5.4 A). Additionally, expression of *MTOR* mRNA in semimembranosus muscle tissue from Gly-supplemented IUGR pigs was greater than for all other treatment groups ($P < 0.05$). While corresponding results were not detected for expression of *CKM* mRNA in Gly-supplemented IUGR pigs, the benefits of Gly supplementation are clear for improving muscle protein synthesis in IUGR pigs. The difference between the expression profiles for these two skeletal muscle

tissues is likely due to differences in their primary functions, as well as differences in muscle fiber type.

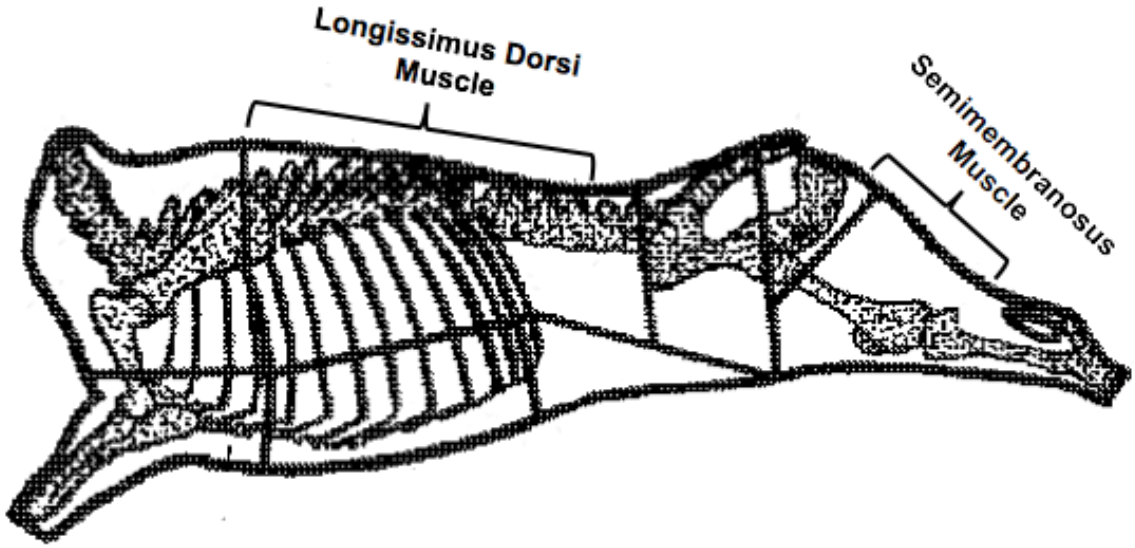


Figure 5.6 General locations of the longissimus dorsi (LD) and semimembranosus skeletal muscles in swine. As can be inferred from functional anatomy, the LD muscle is primarily used for structural support while the semimembranosus muscle is used for locomotion. Adapted from Zobrinsky et al. 1954.

The longissimus dorsi muscle runs along the back of the pig, near the spine. This muscle is primarily a stabilizing muscle that supports the spine and, therefore, is not often subjected to an increased workload or endurance demands. It has a much lower type 1 (oxidative or slow twitch) fiber density than the semimembranosus muscle (Hwang et al. 2019). The semimembranosus muscle is a locomotion muscle located within the thigh or "ham" of the pig. Therefore, this muscle is subjected to much more endurance-based workloads and has a higher type I fiber density than the longissimus dorsi muscle. *CKM* encodes for the cytoplasmic enzyme, creatine kinase muscle-type (CKM), which is highly responsive to creatine substrate availability within the myocyte, and *MTOR* encodes for mTOR, which is responsive to exercise (such as locomotion and long periods of standing)

and Gly signaling for muscle protein synthesis (Bolster et al. 2003; Davis et al. 2010; Sun et al. 2016). Because there is a higher density of type 1 fibers in the myocytes of the semimembranosus muscle, they would have a greater response to Gly for stimulation of mTOR, which would further be increased by exercise achieved through locomotion. Additionally, muscle tissue rich in oxidative (type I) fibers have an increased ability to transport creatine into the cell, due to increased abudnacne of creatine transporter protein (CreaT), as shown in rats (Murphy et al. 2001). Therefore, the semimembranosus muscle, with high type I (oxidative) muscle fiber enrichment, and its role as a locomotor muscle, would be primed to respond the most readily to changes in creatine concentration and mTOR activation due to Gly supplementation. The results of this study indicate a clear effect of Gly supplementation in rescuing IUGR pig growth by improving protein metabolism via increased expression of *MTOR* and *CKM* in skeletal muscles.

In conclusion, expression of genes associated with creatine metabolism is affected by dietary supplementation of 1% Gly across many tissues in IUGR and normal birthweight pigs (Fig. 5.1 – 5.4). The expression of mRNAs for specific creatine-synthetic genes increased in response to Gly supplementation within tissues of the renal-hepatic axis. However, expression did not increase for AGAT, the enzyme traditionally associated with renal function. *GAMT* expression did increase in the liver in response to Gly supplementation, most notably in IUGR pigs. There was also expression of AGAT in the liver, but it may not encode for a functional enzyme since previous in situ experiments concluded that functional AGAT is not detectable in the liver of pigs, regardless of treatment or birthweight status. Similarly, other tissues had an increase in expression of

GAMT mRNA, but enzymatic activity was not confirmed. Therefore, further work with fresh tissues should be done to confirm the activity of GAMT in the various tissues. Additionally, dietary Gly supplementation increased expression of *CKM* and *MTOR* mRNA to a greater extent in normal birthweight pigs, but to a lesser degree in IUGR pigs. These enzymes are heavily involved in energy metabolism and protein synthesis, respectively. Therefore, an increase in expression in these enzymes in key tissues such as the small intestine, kidney, and skeletal muscle would indicate an increase in synthesis of the enzymes (improving overall function of these organs), and an increase in protein deposition (improving growth and development). However, it should be noted that these expression profiles differed significantly between the two types of skeletal muscle. This is likely due to the differences in muscle fiber type between support-type muscles and locomotor muscles regarding creatine transporter availability, usage, and responsiveness to Gly for enzymes such as mTOR. Therefore, with its impacts on protein synthesis and creatine synthesis and subsequent effects of creatine on energy metabolism, dietary Gly supplementation is likely to significantly enhance animal health and profitability in the commercial swine industry.

CHAPTER VI

SUMMARY AND DIRECTION OF FUTURE RESEARCH

While swine production gives producers unique advantages over other livestock production enterprises due to high farrowing rates and large litter sizes, it also comes with unique challenges. Due to the naturally occurring phenomenon of IUGR, 20-25% of all pigs born in the swine industry will not make it to market (Wu et al. 2010). The IUGR pigs suffer from low birthweights, underdeveloped skeletal muscles and gastrointestinal tracts, and lower weaning weights (< 1.1 kg) (Wang et al. 2008). The IUGR pigs also suffer from decreased feed efficiency, severely reduced growth potential, and high mortality rates. This results in approximately 76% of IUGR pigs dying before weaning or being culled by producers at birth (Ji et al. 2017). Therefore, to avoid this waste of animals, time, and profit, novel management strategies must be developed to rescue IUGR pigs. These strategies should improve survivability as well as improve growth performance so that IUGR pigs reach market weight at the same rate and time as their normal birthweight siblings, while also being cost effective and easy for producers to implement. The ideal strategy should be targeted to address supplementation of specific nutrients that are lacking in both dietary sources and capacity for endogenous synthesis.

Glycine (Gly) is a structural and functional amino acid that is vital to maintaining overall health and metabolism. It is one of the most abundant amino acids in body protein (making up one-third of all collagen and elastin proteins, which in turn makes it up one-third of total body proteins) and plays many important functional roles including being a

precursor for creatine synthesis (Wang et al. 2013; Wu et al. 1999). However, Gly content is low in conventional plant-based diets for growing pigs, leaving most of the burden for Gly provision on endogenous pathways for synthesis (Hou et al. 2019). Additionally, recent research has revealed that endogenous pathways for synthesis of Gly, such as synthesis from hydroxyproline, are underdeveloped in IUGR pigs and are, therefore, inadequate for meeting daily Gly requirements (Hu et al. 2017). Failure to meet daily requirements for Gly can dramatically affect growth potential and survivability of IUGR pigs and result in a deficiency in endogenous synthesis of CR.

Gly is a precursor for endogenous CR synthesis. For each molecule of CR synthesized, an entire molecule of Gly is incorporated. This places a large demand on Gly availability, with 87% of dietary Gly and 14.5% of total Gly (endogenous + dietary) being utilized for CR synthesis each day in human models (Melendez-Hevia et al. 2009; Wu and Morris 1998). Creatine is known to aid in growth performance and enhance survivability by improving skeletal muscle accretion, aiding in stabilization of cellular membranes, and decreasing systemic inflammation and tissue damage through its antioxidant activity (Alemida et al. 2006; Bender et al. 2005; Fortalezas et al. 2018; Posey et al. 2021; Wang 2014b; Wu 2013a; Wu 2020). Therefore, a deficiency in Gly in IUGR pigs due to inadequate endogenous synthesis and low dietary provision will lead to a deficiency in available CR. Inadequate CR concentrations have the potential to dramatically impact growth performance and survival of post-weaning and growing IUGR pigs. Direct dietary provision of CR has not been shown to have beneficial effects in normal non-exercising animals and may, in fact, downregulate the enzymes for endogenous synthesis of CR.

However, dietary supplementation of substrates for CR synthesis, such as Gly, have resulted in positive outcomes for growth performance (Brosnan 2011; Dolan et al. 2019; Guthmiiller et al. 1994; Hu et al. 2017; Marzuca-Nassr et al. 2019; Wang et al. 2014c).

Thus, the major purpose of the research for this dissertation was to investigate the novel intervention of dietary Gly supplementation to post-weaning growing and finishing IUGR pigs to improve growth performance and survival through creatine-based mechanisms. Gly alone stimulates growth in preweaning normal and IUGR pigs as it is a potent stimulator of skeletal muscle cell growth (Hu et al. 2017; Powell et al. 2011; Sun et al. 2015; Wang et al. 2013a; Wang et al. 2014bc; Wu et al. 2014). However, little was known about the effects of Gly supplementation to improve CR availability and what effects that could have on growth and development of IUGR pigs. Additionally, a considerable amount of Gly goes to CR synthesis, and CR has long been known to exert both growth-promoting and cell-protective effects on many different tissues including skeletal muscle and brain (Almeida et al. 2006; Bender et al. 2005; Brosnan and Brosnan 2007; Fortalezas et al. 2018; Posey et al. 2021; Wu 2013a; Wu 2020). Finally, dietary Gly and endogenous Gly synthesis are not adequate to meet IUGR pig requirements, especially for CR synthesis (Brosnan et al. 2009: Hou et al. 2019; Hu et al. 2017; Riedijk et al. 2007). Therefore, this study was designed to investigate the effects of dietary provision of Gly to improve CR synthesis in growing and finishing IUGR pigs to improve overall growth and skeletal muscle accretion. Results of this study showed that IUGR pigs have significantly lower concentrations of total CR in all tissues analyzed, as well as insufficient activation of CR synthetic pathways. However, the activity of these pathways and subsequent

increases in concentrations of CR can be restored through daily dietary Gly supplementation throughout the growing and finishing phases of swine production systems. Additionally, Gly supplementation allowed IUGR pigs to reach market weight at the same rate and with the same amount of nutritional support as their normal birthweight counterparts.

The first part of this study was designed to investigate the effects of dietary Gly supplementation on the overall growth and carcass quality of IUGR and normal birthweight pigs. Results showed that Gly improved final live weight of IUGR pigs and that IUGR pigs reached market weight at the same time as their normal birthweight siblings, but there were no significant effects of dietary Gly supplementation for normal birthweight pigs. Carcass quality of IUGR pigs was also improved following Gly supplementation, with decreased back fat thickness, increased loineye area, and an overall increase in muscle score. Thus, Gly supplementation improved total body weight gain through skeletal muscle accretion and not fat deposition. This is notable as IUGR carcasses have traditionally scored much lower in quality due to increased fat deposition and lower carcass yields (Nissen and Oksbjerg 2011).

The question of the mechanisms leading to increased skeletal muscle accretion following Gly supplementation was also addressed. Gly increases growth by stimulating the mTOR pathway for protein synthesis in IUGR and normal pigs (Hu et al. 2017; Wang 2014b); therefore, it was hypothesized that additional mechanisms were involved , such as increased synthesis of CR to increased total CR in tissues such as skeletal muscle. The second part of this study was designed to test the hypothesis that dietary Gly

supplementation was contributing to overall increases in total CR (CR + CRP + CRN) by upregulating the activities of enzymes required for synthesis of CR. Overall, total CR increased in most tissues analyzed, especially in skeletal muscle, and particularly in IUGR pigs. Skeletal muscle is the primary storage site for CR as CRP in the body (95 % of CR), thus indicating a large increase in total body CR. Other tissues affected by Gly supplementation were the kidneys and the pancreas, both of which contain the AGAT enzyme. Additionally, the pancreas expresses GAMT, the second enzyme in the pathway for CR synthesis, and it was affected significantly by dietary Gly supplementation with the greatest net increase in concentration of CR compared to all other measured tissues. This is likely due to the ability of the pancreas to respond directly to increases in available Gly independent of other organs and regulatory mechanism present in the kidneys (Da Silva et al. 2014; Nasrallah et al. 2010). This suggests a key role for the pancreas in creatine synthesis in Gly-supplemented IUGR pigs. However, due to its limited tissue mass compared to the tissue masses of the traditional renal-hepatic axis tissues for creatine-synthesis, the overall contribution of the pancreas to total CR production may be low. Nevertheless, the pancreas likely has a role in maintaining CR homeostasis when other tissues are subjected to the potent feedback inhibition from increasing concentrations of CR , as reported for pancreas in rats (Da Silva et al. 2014).

Once it was confirmed that dietary Gly supplementation increased concentrations of total CR, the activities of the creatine synthesis enzymes were determined. In mammals (including pigs, humans, and rats), CR synthesis requires interorgan cooperation with activities of enzymes partitioned within the kidneys and the liver. This metabolic pathway

also allows nutritional and hormonal regulation of CR synthesis, and the conservation of essential amino acids such as Gly, arginine, and methionine when CR levels are adequate. The renal-hepatic axis requires AGAT in the kidneys, and GAMT in the liver. It also requires folate and one-carbon metabolism pathways to provide *S*-adenosylmethionine (SAM). AGAT catalyzes the amidino group transfer from arginine to Gly, resulting in the synthesis of guanidinoacetate and ornithine, and subsequent release of guanidinoacetate from the kidneys into the blood. Guanidinoacetate is transported to the liver where it is methylated by GAMT and SAM to form creatine (CR) and SAH. The results of this study showed that dietary Gly supplementation increased activities of both CR synthetic enzymes in the renal-hepatic axis and in other tissues. The kidneys of IUGR pigs provided the strongest response to dietary Gly supplementation, with a large increase in AGAT activity detected. There were some changes in AGAT activity in the pancreas, but it was minimal regardless of birthweight status and treatment. . Conversely, the activity of GAMT in the pancreas of both IUGR and normal birthweight pigs responded very strongly to Gly supplementation, with pancreatic GAMT activity increasing in both Gly-supplemented birthweight groups above that of the other tissues analyzed, including the liver. This is interesting because it indicates that the pancreas of pigs responds to increases in Gly availability and is not subjected to negative feedback regulation that decreases activity of AGAT. Thus, the pig pancreas upregulates GAMT activity to continue to synthesize CR similar to that previously reported for the pancreas in rats (Da Silva et al. 2014).

In the small intestine tissue, there was no detectable activity of AGAT in any of the treatment groups, while GAMT activity was detectable at very low levels in small intestine from pigs in all treatment groups. Gly supplementation increased GAMT activity in the small intestine, but, like the pancreas, the overall contribution of the small intestine to total body CR may be minimal. GAMT is not the rate limiting enzyme in the renal-hepatic pathway, so its lack of change in activity in the liver was not surprising, given the dramatic change in AGAT activity in the kidneys. Additionally, the greater mass of the liver suggests that it is contributing more to the increase in total CR. Interestingly, IUGR pigs that were not subjected to dietary Gly supplementation had significantly lower AGAT activity compared to all other treatment groups. This suggests a limited ability of growing IUGR pigs to synthesize CR without dietary intervention and specific management strategies, validating the hypothesis that Gly deficiency in IUGR pigs leads to CR deficiencies that retard growth and feed efficiency.

Subsequent analyses were to determine whether effects of dietary Gly supplementation were active pre-translation to increase expression of AGAT, GAMT and mTOR mRNAs or post-translationally at the protein level. Dietary CR inhibits AGAT activity at the pre-translational step in the kidneys, but not the pancreas (Da Silva et al. 2014; Nasrallah et al. 2010). Therefore, it was of interest to determine at which level was activation of gene expression occurring following dietary Gly supplementation, and what effects did birthweight status have on overall gene expression for enzymes responsible for synthesis of CR and CRP. The genes of interest were creatine kinase muscle-type (*CKM*), mTOR (MTOR), AGAT *(GATM),* and *GAMT.* There was an increased response to Gly

supplementation in the livers of both birthweight groups that did not affect expression of AGAT mRNA in any treatment group; however, for non-supplemented IUGR pigs, expression of *GAMT* mRNA in the liver was reduced compared to that for the other three treatment groups, indicating that both steps in the CR synthesis pathway are underdeveloped in IUGR pigs. But expression of GAMT mRNA was rescued by dietary Gly supplementation.

GAMT gene expression was detectable in both types of skeletal muscle and small intestine, indicating their potential to contribute to CR synthesis. Skeletal muscle of rodents express guanidinoacetate transporter (GAT2) (Zhou et al. 2012), suggesting the possibility that GAT2 or another isoform exists in swine skeletal muscle to allow guanidinoacetate in blood to be taken up into myocytes to compliment CR synthesis in myocytes. Little is known about GAT2 in swine, so future research will be needed to confirm this possibility.

The expression of the CK gene (*CKM*) was unchanged in the longissimus dorsi muscle and was similar to that in the semimembranosus muscle across all treatment groups. These results indicate that IUGR pigs can store CR in skeletal muscle as CRP, where the majority (95%) of CR is stored. The expression of *CKM* mRNA was not affected by treatment in IUGR pigs, but there was an increase in expression in semimembranosus muscle of normal birthweight pigs. However, dietary Gly supplementation increased expression of *MTOR* mRNA in kidneys, small intestine, and semimembranosus muscle of both IUGR and normal birthweight pigs, but not in the longissimus dorsi muscle. These

results confirmed previous findings that Gly supplementation stimulates *MTOR* in pigs in general and include postweaning growing pigs (Hu et al. 2017; Wang et al. 2014b).

Collectively, results of this research confirm that inadequate Gly from dietary sources and reduced capability of IUGR pigs to synthesize Gly endogenously leads to a deficiency in total CR present in their tissues. Additionally, dietary Gly supplementation clearly reverses these deficiencies and improves overall growth and skeletal muscle accretion in IUGR pigs. Further studies are needed to confirm the roles of creatine synthesis in the pancreas and skeletal muscle and contributions of transporters for guanidinoacetate in tissues that may compliment mechanisms for synthesis of CR/CRP in various tissues in swine. It would also be interesting to determine the possible regulatory mechanism(s) that exist in tissues for regulating expression of enzymes such as GAMT, which have not been shown to be subject to regulation. Further, analysis of the effects of dietary provision of other substrates for CR synthesis (e.g., arginine and methionine) would also be of interest, to determine if they have the same or similar effects on CR synthesis or if Gly has a unique role in enhancing synthesis of CR/CRP. The results of this dissertation add further evidence in support of the classification of Gly as a conditionally essential amino acid for growing IUGR pigs that has important implications for the swine industry. Dietary Gly supplementation is a an easily implemented management strategy for rescuing IUGR pigs in the swine industry. This research also contributes to furthering basic knowledge of nutritional biochemistry regarding substrate provision and creatine synthesis, as well as questions regarding alternative regulatory mechanisms and synthesis pathways for CR/CRP. Finally, these results impact human health and nutrition because

the IUGR pig is an excellent model for studying premature human infant nutrition and growth, and CR is known to affect brain function, muscle development, and growth in humans. Results of this study may be used to formulate diets fortified in Gly for premature and IUGR human infants. Therefore, the findings of this dissertation represent a dual contribution to the biomedical community and animal agriculture for intervention strategies that can have significant impacts on many areas of biochemical nutrition and, importantly, human and animal health.

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APPENDIX A

PRIMER SEQUENCES USED FOR ANALYSIS OF GENE EXPRESSION

Table A.1. Primer pair sequences for selected genes

Gene Symbol	Accession Number	Forward Primer Sequence (5' – 3")	Reverse Primer Sequence (5' – 3')
YWAHZ	DQ845179	TGATGATAAGAAAGGGATTGTGG	GTTCAGCAATGGCTTCATCA
HPRT1	DQ845174	GGACTTGAATCATGTTTGTG	CAGATGTTTCCAAACTCAAC
*GATM**	NM_001128442	CCTGATTTTGAGTCTACGGGTTT	CCCACGGCGGAAGTAGTCTTT
*GAMT**	XM_003353975.4	TGCAATGACGGCGTCTTCCAG	CCTCAGACAGCGGGTACGTG
MTOR	XM_003127584.6	AGGCAGCGCTAGAGACAGTG	TGGTCCAGGGTTCGCACAAT
*CKM**	NM_001129949	AGAACCTCAAGGGTGGAGACG	CTTGAACTCTCCCGTCAGGCT

*Sequences for *GATM*, *GAMT*, and *CKM* were adapted from Borchel et al. 2018.

Table A.2. Gene symbols and names for selected genes

Gene Symbol	**Gene Name**
YWAHZ	Tyrosine 3-monooxygenase/tryptophan5-monooxygenase activation protein
HPRT1	Hypoxanthine phophoribosyltransferase 1
GATM	Glycine amidinotransferase/arginine:glycine amidino transferase (AGAT)
GAMT	Guanidinoacetate N-methyltransferase (GAMT)
MTOR	Mechanistic target of rapamycin
CKM	Creatine kinase muscle-type