Advances in Experimental Medicine and Biology 1285

Guoyao Wu *Editor*

Amino Acids in Nutrition and Health

Amino Acids in the Nutrition of Companion, Zoo and Farm Animals

Springer

Advances in Experimental Medicine and Biology

Volume 1285

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2019 Impact Factor: 2.450 5 Year Impact Factor: 2.324

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-030-54461-4 ISBN 978-3-030-54462-1 (eBook)
https://doi.org/10.1007/978-3-030-54462-1

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

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1 One-Carbon Metabolism and Development of the Conceptus During Pregnancy: Lessons from Studies with Sheep and Pigs

Fuller W. Bazer, Heewon Seo, Gregory A. Johnson, and Guoyao Wu

Abstract

The pregnancy recognition signal from the conceptus (embryo/fetus and associated membranes) to the mother is interferon tau (IFNT) in ruminants and estradiol, possibly in concert with interferons gamma and delta in pigs. Those pregnancy recognition signals silence expression of interferon stimulated genes (ISG) in uterine luminal (LE) and superficial glandular (sGE) epithelia while inducing expression of genes for transport of nutrients, including glucose and amino acids, into the uterine lumen to support growth and development of the conceptus. In sheep and pigs, glucose not utilized immediately by the conceptus is converted to fructose. Glucose, fructose, serine and glycine in uterine histotroph can contribute to one carbon (1C) metabolism that provides one-carbon groups for the synthesis of purines and thymidylate, as well as *S*-adenosylmethionine for epigenetic methylation reactions. Serine and glycine are transported into the mitochondria of cells and metabolized to formate that is transported into the cytoplasm for the synthesis of purines, thymidine and *S*-adenosylmethionine. The unique aspects of one-carbon metabolism are discussed in the context of the hypoxic uterine environment, aerobic glycolysis, and similarities in metabolism between cancer cells and cells of the rapidly developing fetal-placental tissues during pregnancy. Further, the evolution of anatomical and functional aspects of the placentae of sheep and pigs versus primates is discussed in the context of mechanisms to efficiently obtain, store and utilize nutrients required for rapid fetal growth in the last one-half of gestation.

Keywords

Pregnancy · Placenta · One-carbon metabolism · Formate · Glycine · Serine · Glucose · Fructose

Abbreviations

1C	one carbon
AFT4	activating transcription factor 4
GE	glandular epithelium
IFNT	interferon tau
ISG	interferon stimulated gene
LE	luminal epithelium
MTHFD	methylenetetrahydrofolate dehydrogenase
MTOR	Mechanistic target of rapamycin
PFK	phosphofructokinase-1
PHGDH	phosphoglyceride dehydrogenase

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1285, https://doi.org/10.1007/978-3-030-54462-1_1

PPP	pentose phosphate pathway
PSAT	phophoserine aminotransferase
PSPH	phosphoserine phosphatase
sGE	superficial glandular
SHMT	serine hydroxymethyltransferase
TCA	tricarboxylic acid
THF	tetrahydrofolate
Tr	trophectoderm
αKG	α-ketoglutarate

1.1 Introduction

Reproduction is essential to the propagation of all species. Accordingly, diverse species employ multiple options regarding mechanisms for pregnancy recognition signaling, implantation, placentation and the initiation of parturition. Livestock species and primates differ in mechanisms for implantation and placentation and these differences impact how nutrients are acquired, stored and utilized. Implantation is invasive in primates, but diffuse and superficial in livestock species (Perry 1981). Before ovine and porcine blastocysts develop into a conceptus, they "hatch" from the zona pellucida on Days 6–7 of pregnancy and then undergo a remarkable transition to filamentous forms in preparation for implantation and placentation (Bazer and Johnson 2014; Johnson et al. 2018). Sheep blastocysts are spherical on Days 4 (0.14 mm) and 10 (0.4 mm), elongate to the filamentous form between Days 12 (1.0 by 33 mm) and 15 (1 by 150–190 mm), and extend through the uterine body into the contralateral uterine horn by Days 16–17 of pregnancy while attaching to the uterine luminal epithelium (LE) to initiate implantation. Pig blastocysts are 0.5–1 mm diameter spheres when they "hatch" from the zona pellucida and increase in size to Day 10 of pregnancy (2–6 mm) before undergoing a morphological transition to large spheres of 10–15 mm diameter and then tubular (15 mm by 50 mm) and filamentous (l by 100–200 mm) forms on Day 11. During the transition from tubular to filamentous forms, pig conceptuses elongate at 30–45 mm/h, primarily by cellular remodeling and proliferation of trophectoderm cells. However, hyperplasia is responsible for subsequent growth and elongation of the conceptus to 800–1000 mm length by Day 15 of pregnancy as implantation progresses. Elongation of ovine and porcine conceptuses is a prerequisite for central implantation that involves the trophectoderm achieving maximum surface area contact with uterine epithelia that secrete and/or transport nutrients into the uterine lumen. As conceptuses elongate they metabolize and are responsive to significant concentrations of molecules supplied in the form of histotroph within the uterine lumen. Histotroph is a complex mixture of molecules either secreted or transported into the uterine lumen and includes hormones, enzymes, growth factors, cytokines, transport proteins, adhesion factors, nutrients and other substances that plays roles in conceptus nourishment, implantation and placentation (Bazer et al. 2015). The invasive implantation for primates results in the spherical blastocyst invading into the uterine stroma wherein it establishes intimate contact with maternal blood vessels that directly supply it with nutrients and other molecules essential for growth and development in preparation for placentation (Huppertz and Borges 2008).

Placentae evolved independently among the livestock species leading to substantial differences in morphology, vascularization, folding of the chorioallantois and associated uterine endometrium, development of placentomes (uterine caruncles and placental cotyledons in ruminants), development of areolae to absorb secretions directly from uterine glands for transport into the fetal-placental circulation, and development of the allantois (Seo et al. 2019, 2020a). The allantois is connected to the fetal bladder via the urachus that allows molecules cleared via the fetal kidney to enter the bladder and then move, via the urachus, into allantoic fluid within the allantoic sac. Those molecules that accumulate in allantoic fluid include nutrients, growth factors and hormones that can be reabsorbed across the allantoic epithelium into the fetal-placental vasculature. Thus, the allantois is a repository from which recirculation of nutrients, hormones, growth factors, cytokines and other molecules

occurs to meet demands for placental development and exponential growth of the fetus. Placental weight in sheep increases from 5 to 435 g between Days 25 and 80 of gestation while fetal weight increases from 0.2 to 257 g during the same period, but fetal weight is tenfold greater on Day 140 (2956 g) of the 147 day period of gestation (Bazer et al. 2012a, b). Similarly, placental weight increases from 0.21 to 250 g between Days 20 and 70 of gestation in pigs, while fetal weight increases from 0.06 to 313 g on Day 70, but increases another threefold to 900 g on Day 100 and about 1500 g at term (Day 114 of gestation) (Knight et al. 1977).

Pigs have a diffuse, epitheliochorial placenta and sheep have a cotyledonary, synepitheliochorial placenta with six and five (within placentomes) layers of cells, respectively, separating maternal and fetal blood. In order to overcome this significant barrier to the transport of nutrients from the uterine vasculature to the placental vasculature, blood flow to the pregnant uteri of pigs increases from about 1.25 L/min on Day 45 of gestation to 2.75 L/min by Day 110 of a 114 day gestation period and this requires considerable maternal heart work by the dam (Pere and Etienne 2000). For ewes, uterine blood flow increases from about 50 ml/min on Day 30 of gestation to 1.4 L/min on Day 140 of a 147 day period of gestation (Metcalfe et al. 1959). In the hemochorial placenta of women, the chorion is in direct contact with maternal blood and only three layers of cells separate maternal and fetal blood allowing for much more efficient transport of nutrients. Accordingly, uterine blood flow in pregnant women increases to a lesser degree than for pigs and sheep, from around 95 ml/min in early pregnancy to 342 mL/min during late gestation (Thaler et al. 1990). This evolution of placental types may reduce maternal heart work as one can appreciate from differences in uterine blood flow at the end of gestation; 2.75 L/min for pigs, 1.4 L/min for sheep and 0.342 L/min for women (Fig. 1.1).

Given the relatively inefficient placentae in sheep and pigs, allantoic fluid serves as a reservoir for a reserve of nutrients that compliments the direct transfer of nutrients across the placenta in support of growth and development of the conceptus. The placentae of sheep and pigs include the yolk sac, amnion, allantois and chorion, but only yolk sac, amnion and chorion are present in the human placenta. The yolk sac provides the initial vascular system, primordial germ cells and hematopoietic stem cells, but it regresses during the first 30–60 days of gestation, depending on species. Retention of the allantois in species with chorioallantoic and synepitheliochorial placentae provides the reservoir for the accumulation of nutrients. In pigs, allantoic fluid volume increases from Day 20 (4 ml) to Day 30 (189 ml), decreases to Day 45 (75 ml) and increases again to Day 58 (451 ml) (Bazer 1989). Thereafter, allantoic fluid volume decreases to term at Day 114 of gestation. Allantoic fluid volume in sheep conceptuses increases from Day 25 (21 ml) to Day 40 (72 ml), decreases to 32 ml on Day 70, and then increases to 450 ml on Day 140 of a 147 day period of gestation (Bazer et al. 2012a, b).

Early anatomical studies suggested that the allantoic sac and its fluid was a reservoir for fetal urine and that the mesonephric glomeruli were the "source" of allantoic fluid (see Bazer 1989). However, the urinary system does not make water, but only redistributes available water. Allantoic fluid is, therefore, of maternal origin. A comparison of concentrations of electrolytes in maternal or fetal plasma and allantoic fluid reveals that allantoic fluid is not a dialysate of plasma since the osmotic gradient favors the exchange of fluids in an allantoic-to-maternal rather than in a maternal-to-allantoic direction. Allantoic fluid and the allantoic epithelium have several key roles. First, increases in allantoic fluid volume expand the chorioallantoic membranes and force them into apposition with the maternal uterine epithelia to maximize placental surface area for nutrient and waste exchange. Second, allantoic fluid contains substantial quantities of electrolytes, water, sugars, proteins and other nutrients that are cleared by the kidney and accumulate in the allantoic sac to be reabsorbed into the fetal-placental circulation across the allantoic epithelium. Third, the allantoic epithelium is derived from the hindgut and is, therefore, an

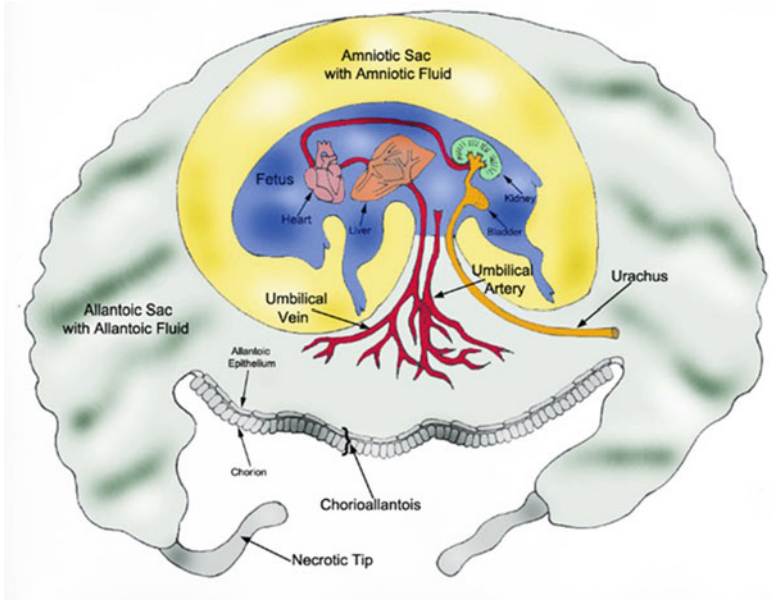

Fig. 1.1 The pig conceptus is representative of animals with epitheliochorial or synepitheliochorial placentae. The chorion is in direct contact with uterine epithelia and transports nutrients and other molecules into the vasculature of the fetal-placental tissues. The allantoic sac contains allantoic fluid that serves as a reservoir for nutrients and other molecules transported into the fetal-placental vasculature. Those nutrients and molecules support development of the conceptus; however, those not utilized are cleared through the kidney, into the bladder and then, via the urachus, transported into the allantoic sac for storage until needed. Subsequently, those nutrients and other molecules are transported across the allantoic epithelium into the fetal-placental circulation to meet metabolic or regulatory functions. This recirculation of nutrients and other molecules provides an efficient means for storage, access, and utilization of nutrients with allantoic fluid. The amnion is filled with amniotic fluid that supports the conceptus and allows it to develop symmetrically. In the latter stages of pregnancy sheep fetuses have been reported to drink amniotic fluid

epithelium capable of absorbing or actively transporting nutrients into the fetal-placental vasculature.

Rapid growth of ovine and porcine conceptuses includes extensive proliferation, remodeling and migration of trophectoderm cells, as well as growth and development of the fetus. Each of these processes consumes and depletes available oxygen and nutrients, resulting in metabolic stress for implanting conceptuses. Rapid development of conceptuses occurs in a hypoxic environment in which aerobic glycolysis provides substrates for the hexoseamine biosynthesis pathway, pentose phosphate pathway, and one-carbon metabolism, as well as production of adenosine triphosphate (ATP) required for rapid proliferation and migration of conceptus Tr cells.

A recent report on the survival of African naked mole-rats was most informative as it revealed how they tolerate hours of extreme hypoxia/anoxia and survive for 18 min under total oxygen deprivation (anoxia). Under those conditions, the Naked Mole rats switch metabolically to aerobic glycolysis fueled by fructose that was metabolized to lactate in the brain (Park et al. 2017). Global expression of the GLUT5 fructose transporter and high levels of expression of ketohexokinase (fructokinase) in tissues of naked mole rats under anoxia resulted in fructose-driven aerobic glycolysis that circumvented the normal feedback inhibition of phosphofructose kinase-dependent glycolysis. This was key to the prolonged viability of naked mole rats under hypoxic or anoxic conditions. Ketohexokinase converts fructose to fructose-1-PO_4 that is metabolized to glyceraldehyde, dihydroxyacetone phosphate and glyceraldehyde 3 phosphate. That pathway is not inhibited by pH, citrate or ATP as occurs when glucose is

metabolized via the hexokinase pathway to glucose-6-PO_4.

Trophectoderm cells of sheep and pigs in their hypoxic environment are metabolically distinct from cells of resting tissues, and reflect characteristics of cancer cells and activated lymphocytes in their ability to enhance aerobic glycolysis (Yang and Vousden 2016). There is evidence that pig trophectoderm cells express the ketohexokinase enzyme (Steinhauser et al. 2016). Utilization of the ketohexose pathway in trophectoderm cells of sheep and pigs under hypoxic conditions during the peri-implantation period of pregnancy and later stages of gestation is clearly advantageous.

L-Lactate, a major metabolic product of aerobic glycolysis, also creates an acidic environment for trophectoderm cells (Gardner 2015) and plays a role in survival of Naked Mole rats (Park et al. 2017). In mice, aerobic glycolysis also provides for a high carbon flux to fulfil biosynthetic demands, increase concentrations of lactate and lower pH around the conceptus (Gardner 2015). Lactate activates cell signaling under hypoxic conditions at implantation sites to: (1) increase expression of hypoxia inducible factor 1-α and down-stream growth factors such as bioactive vascular endothelial growth factor to increase angiogenesis; (2) modulate local immune responses to favor immune tolerance; and (3) modulate expression of enzymes that modify the extracellular matrix of the endometrium in preparation for implantation. The conversion of pyruvate into lactate via lactate dehydrogenase also regenerates NAD^+ required for glycolysis to continue. Maintenance of the NAD^+/NADH redox balance is necessary for conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate, and NADH is a cofactor for the transcriptional regulator C-terminal-binding protein involved in cell growth, differentiation, and transformation (Lunt and Vander Heiden 2011).

In cells that are not dividing and migrating, metabolism of glucose through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation is an efficient way to produce ATPs. However, as noted previously, proliferating and migrating cells are metabolically distinct from resting cells (Pearce et al. 2013; Burton et al. 2017). Cancer cells and activated lymphocytes enhance aerobic glycolysis (also known as the Warburg effect) to produce glycolytic intermediates used as substrates for metabolism via: (1) the pentose phosphate pathway (PPP) for generating a pentose sugar (i.e., ribose 5-phosphate) as a precursor for synthesis of nucleotides, and NADPH for nitric oxide synthesis an anti-oxidative reactions; (2) one-carbon metabolism for *de novo* synthesis of purines and thymidine for synthesis of nucleotides, and *S*-adenosyl methionine for methylation reactions and epigenetic modifications of genes; (3) hexosamine biosynthesis for synthesis of glycosaminoglycans (e.g., hyaluronic acid), uridine diphosphate-N-acetyl glucosamine, a cell signaling molecule, and uridine diphosphate-N-acetyl galactosamine involved in synthesis of glycolipids, glycosaminoglycans and proteoglycans; and (4) the TCA cycle for generation of NADH, FADH2 and ATP. A result of activation of the PPP and 1C metabolism is a decrease in availability of pyruvate for metabolism via the Kreb's cycle. Cancer cells overcome this metabolic restriction by utilizing glutaminolysis to convert glutamine into a TCA cycle metabolite, α-ketoglutarate (αKG), through a process known as anaplerosis. Glutaminolysis-derived αKG is converted into citrate via enzymes of the Krebs cycle, a process known as reductive glutamine metabolism, and citrate is exported into the cytosol where it is cleaved into oxaloacetate and acetyl-CoA. The latter is used for the synthesis of lipids. The active TCA cycle generates ATP that inhibits the enzyme phosphofructokinase-1 (PFK) and, therefore, glycolysis. This inhibition can be circumvented via activation of the polyol pathway to synthesize fructose from glucose, and fructose-driven glycolysis (also called fructolysis) continues to provide glycolytic intermediates. Enzymes required for the polyol pathway are expressed by conceptus trophectoderm cells of pigs and sheep. We propose that in a hypoxic environment, trophectoderm cells of pig and sheep conceptuses: (1) utilize glucose via the glycolytic biosynthetic pathway, and accumulating glycolytic intermediates are shunted into the *de novo* synthesis of nucleotides; (2) utilize glutamine

as an alternate carbon source to maintain TCA cycle flux and provide biosynthetic precursors for the synthesis of lipids; and (3) convert glucose to fructose through the polyol pathway, and fructolysis provides glycolytic intermediates from fructose-1-PO4 metabolism that is not inhibited by ATP, citrate or pH. The synthesis of nucleotides and lipids through these biosynthetic pathways is essential to support extensive proliferation and migration of conceptus Tr cells required for implantation and early placentation.

1.2 Metabolism in Trophectoderm During Peri-Implantation Period

1.2.1 Warburg Effect in a Hypoxic Environment

Implantation and early placentation in humans involves rapid growth of the conceptus that requires extensive proliferation, migration and differentiation of cells, all of which rapidly exhaust available oxygen and nutrients. In humans, the fetal heart does not start beating until the 5th week of pregnancy and an effective circulation through the placental villi is only achieved towards the end of the first trimester (Burton et al. 2017). Therefore, implantation and early placentation in humans take place in a hypoxic environment (Burton et al. 2017; Tayade et al. 2007). During the initial stages of implantation, pig and sheep conceptuses elongate and attach to the uterine LE, processes that also require extensive proliferation, migration, and differentiation of cells in a hypoxic environment. This results in hypoxia inducible factor 1-α expression by trophectoderm cells of pig conceptuses that is upstream of expression of vascular endothelial growth factor and angiopoietins required to initiate angiogenesis and transport of nutrients from the dam into the uterine lumen and or fetal-placental vascular system. Optimal utilization of multiple biosynthetic pathways is likely an essential aspect of early conceptus development for both humans and livestock species including sheep and pigs; however, little is known about the biosynthetic pathways employed by conceptuses of these species. Metabolism may occur through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation to produce ATP (O'Neill et al. 2016). However, proliferating, migrating and differentiating cells are metabolically distinct from cells of resting tissues and reflect characteristics of cancer cells and activated lymphocytes. Cancer cells and activated lymphocytes utilize aerobic glycolysis (also called the Warburg effect) (Andrejeva and Rathmell 2017; Yang et al. 2017) that generates various metabolites required to support multiple metabolic pathways. Those pathways include: (1) the PPP for generating pentoses, ribose 5-phosphate and NADPH; (2) the hexosamine biosynthesis pathway (HBP) for producing UDP-N-acetylglucosamine (UDP-GlcNAc) and glycosaminoglycans such as hyaluronic acid; (3) one-carbon metabolism that generates formate for the de novo synthesis of purine nucleotides, thymidylate, S-adenosylmethionine required for methylation reactions; and (4) generation of NADPH. These metabolic pathways require cooperation between amino acids and glucose (Wu 2018).

1.2.2 Glutaminolysis as a TCA Cycle Anaplerosis

Activation of the PPP, HBP and one-carbon metabolism decreases the generation of pyruvate as substrate for the TCA cycle. Therefore, proliferating cells such as trophectoderm cells, may utilize glutaminolysis to convert glutamine into the TCA cycle metabolite, αKG, a process known as anaplerosis (Yang et al. 2017; Jiang et al. 2016). The creatine kinase pathway is another pathway to generate ATP to support conceptus development (Brosnan and Brosnan 2016). Glutaminolysis-derived αKG can also support synthesis of fatty acids, as noted previously. Glutamine increases in the uterine lumen during the peri-implantation period of pigs and sheep that increases proliferation of porcine trophectoderm cells in vitro. All enzymes required for glutaminolysis are expressed by the Tr cells of pig conceptuses (Seo et al. 2020b).

1.2.3 Polyol Pathway and Fructolysis to Bypass Feedback Inhibition of Glycolysis

An active TCA cycle generates ATP that inhibits PFK and, therefore, glycolysis. This inhibition can be overcome by activation of the polyol pathway to synthesize fructose from glucose (see Park et al. 2017). Fructose-driven glycolysis then provides glycolytic intermediates continuously. Enzymes required for the polyol pathway are expressed by conceptus trophectoderm cells of pigs (Steinhauser et al. 2016). Again, the Naked mole rat, under conditions of anoxia or hypoxia, immediately increases the conversion of glucose to fructose and activation of ketohexokinase to generate fructose-1-PO4 for aerobic glycolysis downstream of phosphofructokinase (Park et al. 2017). The synthesis of nucleotides and lipids through these biosynthetic pathways is essential to support extensive proliferation and migration of conceptus trophectoderm cells required for implantation and placentation.

1.3 Serine as a Major Source of 1C Unit

1.3.1 Serine Biosynthesis from Glucose

Increased serine biosynthesis is one of the metabolic changes occurring in proliferating cells (see Mattaini et al. 2016; Locasale 2013; Ma et al. 2017; Yang and Vousden 2016). Serine is required for several biosynthetic pathways including the synthesis of other amino acids and the production of phospholipids, but its linkage with one-carbon metabolism is particularly relevant to populations of proliferating cells such as cancer cells and trophectoderm cells of elongating ovine and porcine conceptuses. One-carbon metabolism has been referred to as an integrator of nutrient status, an analogy often used for mTOR. Hexose sugars, particularly fructose and glucose, and amino acids enter the pathway, undergo chemical modification and then are out-sourced for diverse cellular functions. Cells can either obtain serine from the outside environment, as we propose here for serine production in uterine LE and uptake by adjacent trophectoderm, or through intracellular synthesis from hexose sugars. The reactions of serineneogenesis are catalyzed by the successive actions of the enzymes phosphoglyceride dehydrogenase (PHGDH), phosphoserine aminotransferase (PSAT), and phosphoserine phosphatase (PSPH). PHGDH converts 3-phosphoglycerate to 3-phosphohydroypyruvate which is the committed step into the pathway for serine biosynthesis. PSAT next converts 3-phosphohydroypyruvate to 3-phosphoserine (P-Ser), which is then converted to serine by PSPH. Serine can either remain in the cytosol or be transported to neighboring cells where it can enter mitochondria for incorporation into one-carbon metabolism, a network of interconnected biochemical pathways that facilitate the transfer of one-carbon units for biosynthesis. Within mitochondria, serine hydroxymethyltransferase 2 (SHMT2) catalyzes the reversible reaction of serine and tetrahydrofolate (THF) to glycine and 5,10-methylene tetrahydrofolate (mTHF). mTHF is required for synthesis of formate within the mitochondria. Formate then goes to the cytoplasm for synthesis of thymidine for DNA synthesis, purines for RNA and DNA synthesis and S-adenosyl methionine which is the primary methyl donor for methylation reactions such as those for epigenetic modifications of gene expression. The temporal and cell-specific expression of these genes in uteri and placentae of pigs and sheep indicates that glucose and fructose can be converted to serine within the uterine LE via PHGDH, PSAT1 and PSPH, while serine can also be transported into trophectoderm cells by SLC1A4 (neutral amino acid transporter A). Mechanistic target of rapamycin (MTOR) and hypoxia inducible factor 1-α can then potentially induce expression of SHMT2 and methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) in trophectoderm cells to convert serine to 1 carbon units (unpublished observations) (Fig. 1.2). Because plant porteins contain relatively low content of both serine and glycine (Hou et al. 2019; Li and Wu 2020), *de novo* synthesis of serine is of nutritional and physiological importance for successful pregnancy outcomes in ruminants and swine that typically consume plant-based diets.

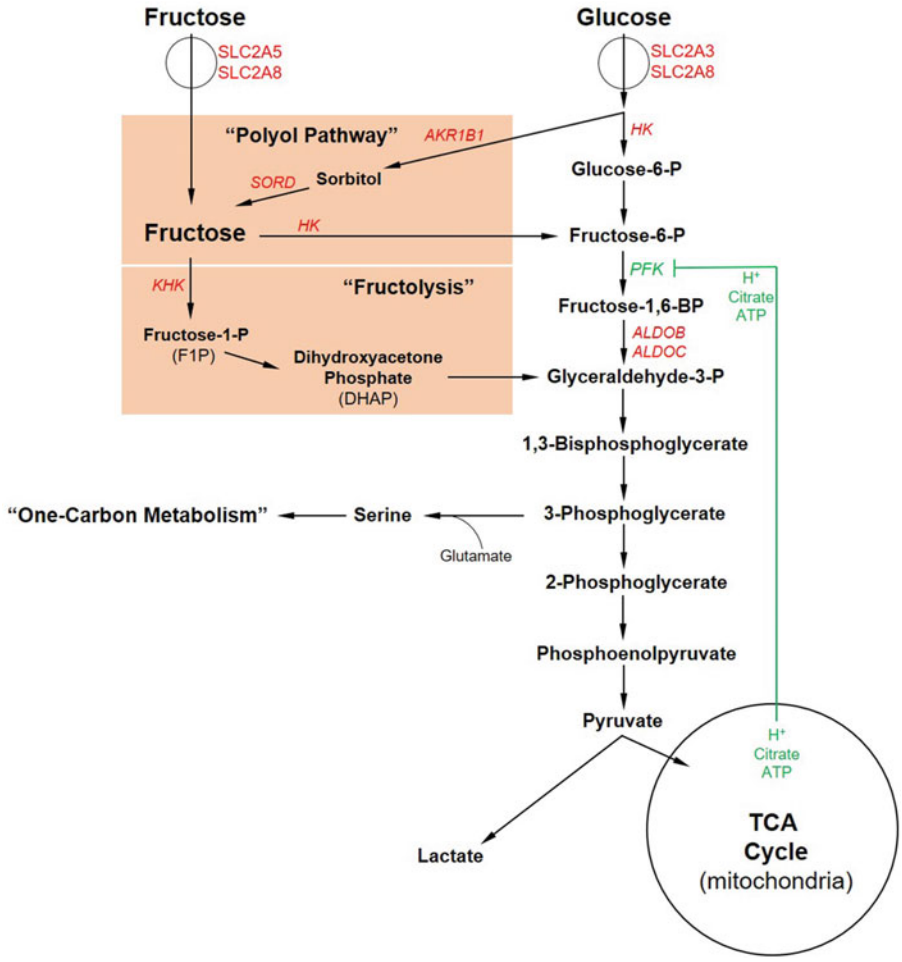

Fig. 1.2 Glucose may enter tissues via glucose transporter 1 (SLC2A1) and be phosphorylated to glucose-6-PO4 and fructose-6-PO4 by hexokinases and then PFK generates fructose-1,6 bisphoshphate that aldolases B and C convert to trioses, dihydroxyacetone phoshphate and glyceraldehyde-3-PO4 for further metabolism to pyruvate and lactate. This pathway is inhibited by increases in ATP, citrate, and decreases in pH. However, fructose produced from glucose via the aldoase pathway can enter cells via SLC2A5 and be phosphorylated by ketohexokinase to fructose-1-PO4. Aldolase B primarily the converts fructose-1-PO4 to trioses (dihydroxyacetone phoshphate and glyceraldehyde-3-PO4) that can be metabolized to pyruvate and lactate. The pathway whereby ketohexokinase yields fructose-1-PO4 at a higher efficiency than by hexokinases and fructose-1-PO4 is directly metabolized into trioses via aldolase B or aldolase C to bypass feedback inhibition by ATP, citrate and pH. However, glyceraldehyde-3-PO4 can also be metabolized via the serineogenesis pathway for one-carbon metabolism

As noted previously, there is a link between serine, one carbon metabolism and rapidly proliferating cells such as cancer cells and trophectoderm cells of elongating ovine and porcine conceptuses. In rapidly dividing cells, it is known that amino acids such as arginine, stimulate MTOR in trophectoderm cells (Bazer et al. 2015), but there is also evidence for a link between MTOR and one-carbon metabolism indicating cross-talk among metabolic pathways in such cells. It should also be noted that MTORC1 activates activating transcription factor 4 (ATF4) that stimulates expression of MTHFD2, but also PHGDH, PSAT and PSP that generates serine for one-carbon metabolism (Ben-Sahra et al. 2016). Glucose and fructose can be metabolized via the hexosamine biosynthetic pathway to activate the Akt-TSC2-MTOR signaling cascade due to glycosylation and activation of those transcription by UDP-N-acetylglucosamine, a primary product of

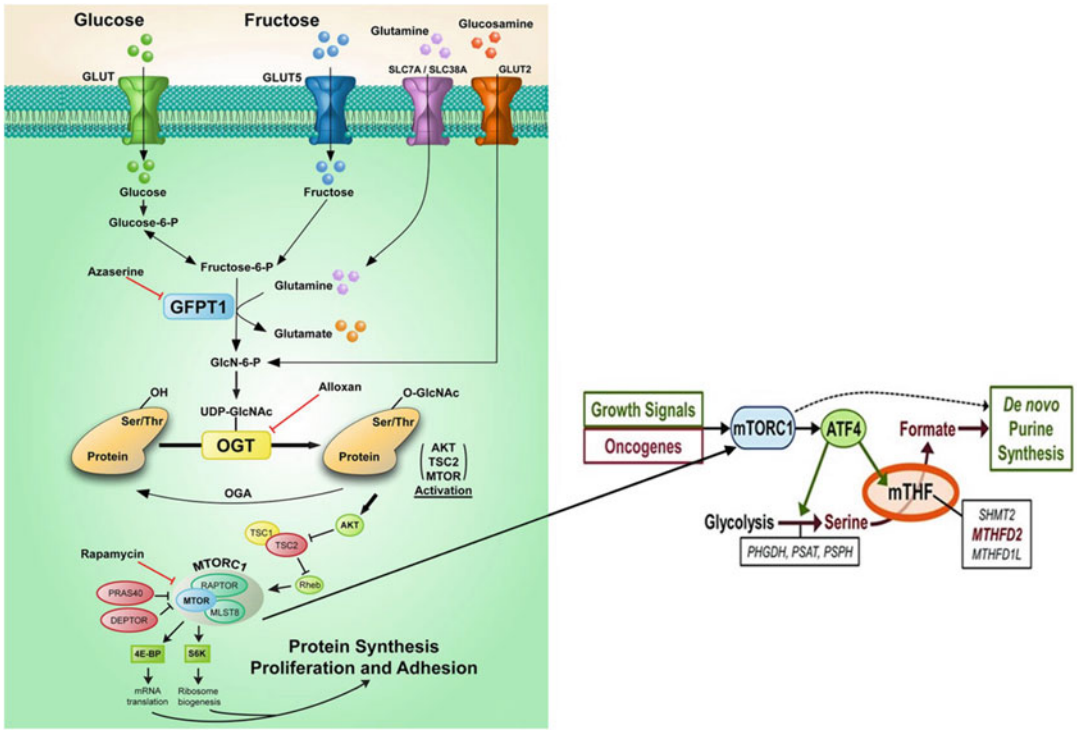

Fig. 1.3 In rapidly dividing cells, it is known that amino acids such as arginine, stimulate the mechanistic target of rapamycin cascade in that stimulates proliferation and expression of mRNAs in ovine and porcine trophectoderm cells (see Bazer et al. 2015). This figure provides evidence for cross-talk between pathways fueled by molecules involved in glycolysis. In this case, there is cross-talk between the hexosamine biosynthesis pathway and the pathway for generating molecules required for one-carbon metabolism. It should also be noted that MTORC1 activates activating transcription factor 4 (ATF4) and ATF4 then stimulates expression of phosphoglyceride dehydrogenase (PHGDH), phosphoserine aminotransferase (PSAT), and phosphoserine phosphatase (PSPH) to generate serine for one-carbon metabolism (Ben-Sahra et al. 2016). Glucose and fructose can be metabolized via the hexosamine biosynthetic pathway to activate the Akt-TSC2-MTOR signaling cascade. This results from glycosylation and activation of Akt-TSC2-MTOR transcription factors by actions of UDP-N-acetylglucosamine, a primary product of the hexosamine biosynthesis pathway and O-glycosyltransferase (OGT) (Wang et al. 2016). Thus, the mitochondrial tetrahydrofolate (mTHF) cycle is activated to generate formate for purines, thymidine and s-adenosylmethionine to support rapid proliferation of trophectoderm cells. (This figure is adapted from those published Wang et al. (2016) and Ben-Sahra et al. (2016))

the hexosamine biosynthesis pathway (Wang et al. 2016). The generation of MTOR via this pathway may then activate ATF4 to induce the pathway for generation of serine for one-carbon metabolism (Fig. 1.3).

1.3.2 Serine Biosynthesis from Fructose

Fructose is clearly the most abundant hexose sugar in allantoic fluid and fetal blood of ungulates and cetaceans (Kim et al. 2012). Fructose can be synthesized from glucose via the polyol pathway, also known as the sorbitol-aldose reductase pathway (Steinhauser et al. 2016). Glucose is converted to sorbitol by aldo-keto reductase family 1 member B (AKR1B1), then sorbitol is converted to fructose by sorbitol dehydrogenase (SORD). Cells obtain fructose from their environment through fructose transporters, particularly solute carrier family 2 member 5 (SLC2A5, also known as GLUT5) and SLC2A8 (also known as GLUT 8). Within cells, fructose is phosphorylated by ketohexokinase to fructose-1-phosphate that can be metabolized to dihydroxyacetone phosphate

and then 3-phosphoglycerate for entry into the pathway for synthesis of serine. This switch to fructose-1-PO4 by-passes the key regulatory step that limits glycolytic flux (Park et al. 2017). Glucose metabolism via hexokinases 1 and 2 yields glucose-6-PO4 that is metabolized by the pathway requiring PFK. PFK is subject to feedback inhibition by ATP, hydrogen ions, and citrate. However, fructose-1-phosphate metabolites enter glycolysis downstream of PFK which permits continued metabolic flux through aerobic glycolysis that is not inhibited by ATP, pH or citrate.

Sorbitol is present in high concentrations in porcine, ovine, bovine, and human placentae, especially during early pregnancy and there is expression of SLC2A5 and SLC2A8 by pig conceptuses (Jauniaux et al. 2005; Steinhouser et al. 2016; Bazer et al. 2012a, b). Fructose is transported into trophectoderm cells by SLC2A5 and SLC2A8, converted to fructose-1-phosphate by ketohexokinase and further metabolized via aerobic glycolysis that supports hexosamine biosynthesis, pentose phosphate pathway and one-carbon metabolism, all of which are essential to support fetal-placental development and ensure a successful outcome of pregnancy.

1.3.3 Serine in Biological Fluids During Pregnancy

Serine is abundant in the uterine histotroph of sheep (Gao et al. 2009a) and pigs (Bazer et al. 2012a, b). Concentrations of serine (nmol) in uterine flushings from ewes increase between Days 10 and 15 of pregnancy (542 ± 267 versus 1975 ± 687) and decrease, perhaps due to metabolism, by Day 16 of pregnancy (1708 ± 494). Similarly, concentrations of glycine (nmol) increase in uterine flushings from pregnant ewes between Day 10 (4214 ± 739) and 14 (8598 ± 2308) and then decrease to Day 16 (5805 ± 2004). Concentrations of serine (nmol) are much greater in litter bearing pigs. Concentrations of glycine (nmol) in uterine flushings from pigs increase between Days 10 (93,387 ± 12,435) and 15 (95,282 ± 54,745) and concentrations of glycine (nmol) are 63,8878 ± 6145 and 41,409 ± 25,858 on the same respective days. The decreases in serine and glycine in uterine flushings toward the end of the peri-implantation period of pregnancy likely reflect increased uptake and metabolism by highly active trophectoderm cells. Serine is also very abundant in allantoic fluid of sheep with mean concentrations (μmol/L) of 1636, 19,072 and 16,468 on Days 40, 100 and 140 of gestation, respectively (Kwon et al. 2003). On Day 140 of gestation in sheep, serine accounts for about 60% of total α-amino acids in allantoic fluid (Kwon et al. 2003). Glycine (μmol/L) is also very abundant in ovine allantoic fluid on Days 40 (2449), 100 (5464) and 140 (1132). Mean concentrations (μmol/L) of serine (1218) and glycine (3054) are also very abundant in allantoic fluid on Day 110 of gestation (Wu et al. 1995). Increases in abundances of serine and glycine are coincident with rapid proliferation of trophectoderm cells as the conceptus elongates and mononuclear trophectoderm cells differentiate into trophoblast giant cells that invade the uterine LE to undergo syncytialization at the uterine-placental interface (Seo et al. 2019). Also, there is rapid development of the chorioallantois and amnion during the first one-half of gestation that will support exponential growth of the fetus during the second one-half of gestation. Fetal development does not increase exponentially until after placental development is essentially complete. The trophoblast giant cells have a high level of expression of the serine transporter SLC1A4 mRNA indicating that they take up serine released by adjacent uterine LE cells. The primary membrane transporters for serine are solute carrier family 1 member 4 (SLC1A4) and SLC1A5, both of which are expressed by uterine epithelia and trophectoderm cells of ovine conceptuses during the peri-implantation period of pregnancy (Gao et al. 2009b). Patterns of expression of transporters for glycine, such as GLc6A9, by uterine epithelia and trophectoderm of sheep and pigs are unknown.

1.4 Formate as a Major Output of 1C Metabolism

Brosnan and Brosnan (2016) noted that formate is the neglected member of one-carbon metabolism because it is not linked to a tetrahydrofolate (THF) coenzyme like other molecules involved in one-carbon metabolism. They also noted that formate is more mobile than THF-linked molecules and easily provides inter-organ and inter-organelle shuttling of one-carbon groups due to its presence in blood at considerably higher concentrations than folates. Cancer cells have higher concentrations of formate than cells of healthy tissues (Wang et al. 2013) and over-expression of methylenetetrahydrofolate dehydrogenase 2 (MTHFD2), a bifunctional mitochondrial enzyme, is associated with increased proliferation of cancer cells (Gustafsson et al. 2015).

1.4.1 Neural Tube Defects

Neural tube defects occur in Mthfd1l null mice as that gene encodes for mitochondrial 10-formyl-THF synthase that catalyzes the interconversion of 10-formyl-THF to formate (Momb et al. 2013). However, neural tube defects in Mthfd1l null mice are reduced significantly when dams are provided with sodium formate in their drinking water. Dietary supplementation with sodium formate also reduced neural tube defects in Gldc (glycine dehydrogenase (decarboxylating), mitochondrial) null mice (Pai et al. 2015).

Pai et al. (2015) reported that glycine decarboxylase (Gldc) null mice are deficient in folates charged with one-carbon groups. Gldc null mice have two phenotypes. One phenotype was partially penetrant with 25–30% of mice having neural tube defects, particularly exencephaly. The second phenotype of mice exhibited nonketotic hyperglycinemia characterized by elevated concentrations of glycine in their plasma and a high incidence of hydrocephalus. Supplementing the diet with sodium formate in drinking water from Day 1 of pregnancy restored normal concentrations of folate in the plasma and eliminated the neural tube defects in the partially penetrant phenotype, but did not alleviate defects in the hyperglycinemia phenotype. Thus, the glycine cleavage system provides one-carbon groups, in the form of 5,10-methylene-tetrahydrofolate required for normal closure of the neural tube, particularly between embryonic days 8.5 and 10.5 in mice. Alternatively, glycine can be oxidized by glycine oxidase to glyoxylate, which is decarboxylated by NAD-linked glyoxylate dehydrogenase to produce formate (Wu 2013).

1.4.2 Formate During Pregnancy in Sheep and Pigs

There are limited studies in sheep and humans linking formate with the growth and development of conceptuses. For pregnant ewes at Day 120 of gestation, concentrations of format in fetal plasma and in amniotic fluid were six- and ninefold greater than those in maternal plasma, and concentrations of format in plasma from neonatal lambs remained high until around 8 weeks of life (Washburn et al. 2015). Similarly, in pregnant women, concentrations of formate, as well as its precursors (serine, glycine, tryptophan, and methionine) were greater in the plasma from cord blood than maternal plasma. However, babies with variant forms of the MTHFD1 gene (1958 G to A) and MTHFR gene (1298 A to C) had lower concentrations of formate in their blood, but infants with the more common mutation in the MTHFR gene (677 C to T) had concentrations of formate that were similar to those for normal babies.

As noted earlier, serine and glycine are abundant in uterine flushings and in fetal fluids and may be used for synthesis of one carbon groups such as formate. Cetin et al. (1992) reported significant uptake of serine by both liver and hindlimbs of fetal lambs, as well as a net uptake of serine across the placenta. They also obtained umbilical venous and maternal arterial blood from 24 normal (AGA) and 31 intrauterine growth retarded (IUGR) fetuses with 16 AGA pregnancies between 18 and 25 weeks of gestation and 8 AGA and 31 IUGR pregnancies between 27 and 39 weeks of gestation (Cetin et al. 1993). They reported no significant relationship between concentrations of amino acids in maternal arterial blood and

gestational age except for threonine, methionine, serine and glutamic acid. Further, concentrations of glycine, aspartic acid, and glutamic acid increased in umbilical vein plasma and were significantly greater in normal fetuses during the third trimester than in the second trimester, and glycine was the only amino acid in umbilical vein plasma to increase in concert with gestational age. Washburn et al. (2015) suggested that formate is synthesized in the placenta from serine and distributed to fetal tissues as a substrate for use in one-carbon metabolism since the ovine placenta exhibits high activity of mitochondrial SHMT throughout pregnancy (Narkewicz et al. 1999). Also, human placentae express an abundance of mitochondrial bifunctional protein (MTHFD2) mRNA that codes for methylenetetrahydrofolate dehydrogenase and 5,10-methenyl-THF cyclohydrolase (Prasannan et al. 2003). Each of those three enzymes is critical for the synthesis of formate in mitochondria. Thus, Washburn et al. (2015) suggest that formate is not only an intracellular metabolite in one-carbon metabolism, but an inter-organ metabolite that distributes one-carbon groups to rapidly developing tissues.

1.5 Compartmentalization of 1C Metabolism

1.5.1 Mitochondrial 1C Metabolism

The primary route for production of folates begins in mitochondria where serine, glycine, sarcosine and dimethylglycine are converted to 5,10-methylene-tetrahydrofolate (5,10 –CH_2-THF). Brosnan and Brosnan (2016) noted the following key points regarding synthesis of formate in mitochondria: (1) sarcosine is not abundant in tissues and contributes little to the synthesis of formate; (2) serine metabolism is initiated by an isoform of mitochondrial serine hydroxymethyltransferase (SHMT)-2; (3) glycine metabolism is initiated by the mitochondrial glycine cleavage system; (4) sarcosine and dimethylglycine metabolism are initiated, respectively, by sarcosine dehydrogenase and dimethylglycine dehydrogenase; (5) 5,10-methylene-THF produced from these substrates is oxidized to 10-formyl-THF by the sequential actions of the mitochondrial isoforms of 5,10-methylene-HF dehydrogenase and 5,10-methenyl-THF cyclohydrolase; (6) 5,10-methylene-THF dehydrogenase and 5,10-methenyl-THF cyclohydrolase are bifunctional proteins in mammalian mitochondria; (7) the two bifunctional mitochondrial isoforms are methylenetetrahydrofolate dehydrogenase/5,10-methenyl-THF cyclohydrolase (MTHFD2) and MTHFD2L; (8) MTHFD2 is expressed primarily in tumors and embryonic tissues; (9) 10-Formyl-THF synthase produces formate and THF from 10-formyl-THF; (10) formate is transported from the mitochondria into the cytosol by an unknown mechanism and may be incorporated into cytosolic 10-formyl-THF and then other THF-linked one-carbon intermediates; (11) limited amounts of formate may be produced from histidine catabolism to formiminoglutamate and this one-carbon group may be metabolized to yield cytosolic 5,10-methylene-THF to be oxidized to 10-formyl-THF and then to formate in the cytosol; and (12) serine is likely the most important precursor of formate as both carbons 2 and 3 of serine are incorporated into formic acid and formate.

1.5.2 Cytosolic 1C Metabolism

Three canonical functions of one-carbon metabolism are synthesis of purine nucleotides, synthesis of thymidylate, and provision of labile methyl groups to remethylate homocysteine to methionine (Brosnan and Brosnan 2016). Formate functions in the cytoplasm include actions of ATP-dependent 10-formyl-THF synthetase with 5,10-methenyl-THF cyclohydrolase and 5,10-methylene-THF dehydrogenase in the trifunctional protein MTHFD1. The 10-formyl-THF is incorporated into the 2 and 8 positions of the purine ring and may be further reduced to 5,10-methylene-THF and 5-methyl-THF, respectively, for thymidylate synthesis and remethylation of homocysteine to methionine. During folate deficiency, mammalian cells in the S phase of the cell cycle can translocate SHMT1, SHMT2α, thymidylate synthase, dihydrofolate reductase, and MTHFD1 to the nucleus to form a functional metabolon to achieve the synthesis of

thymidylate (Field et al. 2014). Those authors indicated that SHMT1 and SHMT2α function as scaffold proteins rather than as enzymes because catalytically inactive SHMT1 also enhances thymidylate synthesis. Formate produced in mitochondria enters the nucleus for conversion to 5,10-methylene-THF by the three reactions of MTHFD1. Thus, cells deficient in folate and 5,10-methylene-THF are able to achieve de novo synthesis of thymidylate at the expense of remethylation of homocysteine to methionine.

1.6 Summary

This review links morphological and functional aspects of placentae required for transport of nutrients across the placenta and into the fetal-placental vasculature for delivery to those respective tissues. Further, placentae of sheep and pigs must support rapid growth of the fetus in spite of capillaries in the placenta being separated from maternal capillaries by 5 or 6 layers of cells. Accordingly, the allantois serves as a reservoir in which nutrients in excess of metabolic needs can be stored and then reabsorbed, as needed, to compliment the on-going transfer of nutrients from maternal to fetal-placental vasculatures. The focus of the review is on the utilization of available serine and glycine by the rapidly developing placenta, as well as pathways for glucose and fructose to be used to produce 3-phosphoglycerate that can enter into the serinogenesis pathway in the presence of glutamate. It is also noteworthy that fructose in the blood and allantoic fluid of sheep and pigs is at conentrations 11–30 times those of glucose. Fructose can be phosphorylated by ketohexokinase to yield fructose-1-PO4 that is metabolized via a pathway that by-passes phosphofructokinase to assure continuous generation of metabolites via aerobic glycolysis that supports hexosamine biosynthesis, pentose phosphate pathway and one-carbon metabolism, all of which are essential to support fetal-placental development and ensure a successful outcome of pregnancy.

Acknowledgements Work in our laboratories was supported by Agriculture and Food Research Initiative Competitive Grants (No. 2016-67015-24958 and 2018-67015-28093) from the USDA National Institute of Food and Agriculture.

Conflict of Interest The authors declare no conflict of interest.

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Cell-Specific Expression of Enzymes for Serine Biosynthesis and Glutaminolysis in Farm Animals

2

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Abstract

During the peri-implantation period, conceptuses [embryo and placental membranes, particularly the trophectoderm (Tr)] of farm animals (e.g., sheep and pigs) rapidly elongate from spherical to tubular to filamentous forms. In concert with Tr outgrowth during conceptus elongation, the Tr of sheep and pig conceptuses attaches to the endometrial luminal epithelium (LE) to initiate placentation. In sheep, binucleate cells (BNCs) begin to differentiate from the mononuclear trophectoderm cells and migrate to the endometrial LE to form syncytial plaques. These events require Tr cells to expend significant amounts of energy to undergo timely and extensive proliferation, migration and fusion. It is likely essential that conceptuses optimally utilize multiple biosynthetic pathways to convert molecules such as glucose, fructose, and glutamine (components of histotroph transport by sheep and pig endometria into the uterine lumen), into ATP, amino acids, ribose, hexosamines and nucleotides required to support early conceptus development and survival. Elongating and proliferating conceptus Tr cells potentially act, in a manner similar to cancer cells, to direct carbon generated from glucose and fructose away from the TCA cycle for utilization in branching pathways of glycolysis, including the pentose phosphate pathway, one-carbon metabolism, and hexosamine biosynthesis. The result is a limited availability of pyruvate for maintaining the TCA cycle within mitochondria, and Tr cells replenish TCA cycle metabolites via a process known as anaplerosis, primarily through glutaminolysis to convert glutamine into TCA cycle intermediates. Here we describe the cell-specific expression of enzymes required for serine biosynthesis, one-carbon metabolism and glutaminolysis at the uterine-placental interface of sheep and pigs, and propose that these biosynthetic pathways are essential to support early placental development including Tr elongation, cell migration, cell fusion and implantation by ovine and porcine conceptuses.

Keywords

Enzymes · Pig/Sheep · Placentation · Glucose/Fructose · Glutamine · Glycolysis · Glutaminolysis

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Abbreviations

3-PG	3-phosphoglycerate
BNC	binucleate cells
GE	glandular epithelium

G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1285, https://doi.org/10.1007/978-3-030-54462-1_2

GLUD	glutamate dehydrogenase
LE	luminal epithelium
PHGDH	phosphoglyceride dehydrogenase
PHP	3-phosphohydoypyruvate
PSAT	phophoserine aminotransferase
PSPH	phosphoserine phosphatase
SAM	*S*-adenosylmethionine
SHMT	serine hydroxymethyltransferase
SLC	solute carrier
TCA	tricarboxylic acid
THF	tetrahydrofolate
Tr	trophectoderm
α-KG	α-ketoglutarate

2.1 Introduction

Over two-thirds of pregnancy losses in mammals occur during the peri-implantation period of pregnancy. In sheep and pigs, conceptuses [embryos and associated placental membranes, particularly the trophectoderm (Tr)] undergo dramatic morphological changes from spherical to tubular and then filamentous forms, and attach to the uterine luminal epithelium (LE) to initiate placentation (Bazer and Johnson 2014). These processes require that Tr cells expend significant amounts of energy to undergo timely and extensive proliferation and migration at a time when the conceptuses have not yet established a placental connection to the uterus, and are dependent upon limited nutrients either secreted or transported into the uterine lumen by cells of the endometrium (Perry et al. 1973; Fischer et al. 1985). Therefore, optimal utilization of multiple biosynthetic pathways to convert molecules such as glucose, fructose, and glutamine (components of histotroph secreted and/or transported by sheep and pig endometria into the uterine lumen), into ATP, amino acids, ribose, hexosamines and nucleotides is required to support early conceptus development and survival. Studies with swine have shown that glutamine is a highly abundant amino acid in porcine fetal allantoic and amniotic fluids (Wu et al. 1995, 1996), and its adequate provision in diets is critical for conceptus growth and survival (Wu et al. 2011; Zhu et al. 2018). Our previous work has also demonstrated that ovine fetal fluids contain a large amount of glutamine during gestation (e.g., about 25 mM in allantoic fluid on day 60 of gestation; Kwon et al. 2003). However, little is understood about the biosynthetic pathways employed by sheep and pig conceptuses during the peri-implantation period of pregnancy (Bazer et al. 2020).

Cell metabolism primarily occurs through the TCA cycle and oxidative phosphorylation, which is a complex, but efficient, process that requires mitochondrial biogenesis to produce ATP (Wu 2018). However, proliferating cells, such as the Tr of sheep and pigs, are metabolically distinct from cells of resting tissues, and reflect characteristics of cancer cells and activated lymphocytes (Andrejeva and Rathmell 2017). A hallmark of tumors and activated lymphocytes is their ability to enhance glycolysis, even in the presence of oxygen, a phenomenon known as the Warburg effect or aerobic glycolysis which is a classic example of the ability of proliferating cells to reprogram the activation of metabolic pathways (Yang et al. 2017; DeBerardinis and Chandel 2016). Glycolysis is a physiological response to hypoxia in normal tissues, but in the 1920s Otto Warburg observed that tumor slices and cancer cells with ascites fluid constitutively take up glucose and produce lactate regardless of the availability of oxygen, an observation now recognized in many types of cancer cells and tumors. The widely accepted theory is that cancer cells switch from using oxidative phosphorylation to using glycolysis because high glycolytic rates result in more rapid generation of ATP as compared with the oxidation of glucose and activation of the TCA cycle. The glycolytic intermediates that accumulate are then shunted into branching pathways of glycolysis for *de novo* synthesis of nucleotides, amino acids, and fatty acids to fulfill the metabolic demands of proliferating cells (Cruys et al. 2016; Rathmell et al. 2000; Wang et al. 1976; O'Neill et al. 2016).

The proliferating Tr cells of sheep and pig conceptuses appear to use a similar switch from reliance on oxidative phosphorylation to activation of glycolysis. Glucose and fructose are present in the uterine flushings from early pregnant

sheep and pigs (Zavy et al. 1982; Gao et al. 2009a). In pigs, expression of the facilitated diffusion transporter of the solute carrier family 2A1 (SLC2A1, responsible for the basal uptake of glucose into most cells) and SLC2A8, a high affinity glucose transporter that can also transport fructose, are expressed in uterine LE, whereas SLC2A3 (a high affinity and high capacity glucose transporter), and SLC2A8 are present in conceptus Tr cells. In sheep, SLC2A1 and SLC5A1 are expressed by LE while SLC2A1, SLC2A3, SLC2A4, SLC5A1, and SLC5A11 are expressed by conceptus Tr (Gao et al. 2009b). Therefore, sheep and pig conceptus Tr cells have access to glucose and fructose as energy sources during the peri-implantation period of pregnancy. These Tr cells appear to utilize the glucose for glycolytic branching pathways including the pentose phosphate pathway, serine biosynthesis, one-carbon metabolism, and hexosamine biosynthesis (Kim et al. 2012; Wang et al. 2016), because conceptus Tr cells express key enzymes required for those pathways (described in the next sections) and the pentose phosphate pathway is highly active in porcine Tr cells (Lin et al. 2013). Therefore, the Warburg effect appears to be operational in proliferating Tr cells of sheep and pig conceptuses.

Glutamine, another principal growth-supporting substrate, not only contributes carbon, but also reduces nitrogen for the de novo biosynthesis of a number of diverse nitrogen-containing compounds (Pavlova and Thompson 2016). One glutamine molecule is used in the production of uracil and thymine, while cytosine and adenine each require two glutamines, and guanine requires three molecules of glutamine for synthesis (Wu 2013). Thus, glutamine is a critical structural components in the biosynthesis of nucleotides. Accordingly, glutamine levels have been shown to be a rate-limiting factor for cell cycle progression, and glutamine shortage leads to cell proliferation arrest and S-phase accumulation in certain cellular contexts. The concentration of glutamine increases in the uterine lumen during the peri-implantation period of pregnancy, and glutamine affects proliferation of porcine Tr cells *in vitro* (Kim et al. 2013; Gao et al. 2009a). We hypothesize that the Tr cells of ovine and porcine conceptuses utilize glucose and fructose within the uterine lumen via the glycolytic biosynthetic pathway, and that accumulating glycolytic intermediates are shunted into pathways for the *de novo* synthesis of nucleotides and amino acids, and that glutamine within the uterine lumen is used as an alternate carbon source to maintain TCA cycle flux.

2.2 Overview of Serine Biosynthesis, One-Carbon Metabolism, and Glutaminolysis

Glycolysis is classically depicted as a single chain of molecular events that leads to the generation of pyruvate, but a number of glycolytic intermediates can be diverted into branching pathways, generating diverse biosynthetic precursors (Wu 2018). One of the most intensely studied growth-promoting mechanisms that shunts metabolites out of the glycolytic pathway is the use of 3-phosphoglycerate as a precursor for serine biosynthesis (Fig. 2.1). Serine is required for several biosynthetic pathways including the synthesis of other amino acids and the production of phospholipids, but its linkage with one-carbon metabolism or the folate cycle is particularly relevant to proliferating cells such as cancer cells and the Tr of elongating sheep and pig conceptuses. Cells can either obtain serine from the outside environment or through intracellular synthesis from hexose sugars. Serine biosynthesis is catalyzed by the successive actions of the enzymes phosphoglyceride dehydrogenase (PHGDH), phophoserine aminotransferase (PSAT), and phosphoserine phosphatase (PSPH). PHGDH converts 3-phosphoglycerate (3-PG) to 3-phosphohydoypyruvate (PHP) which is the committed step into the pathway for serine biosynthesis. PSAT next converts PHP to 3-phosphoserine (P-Ser), which is then converted to serine by PSPH. Increases in serine biosynthesis is one of the metabolic changes that

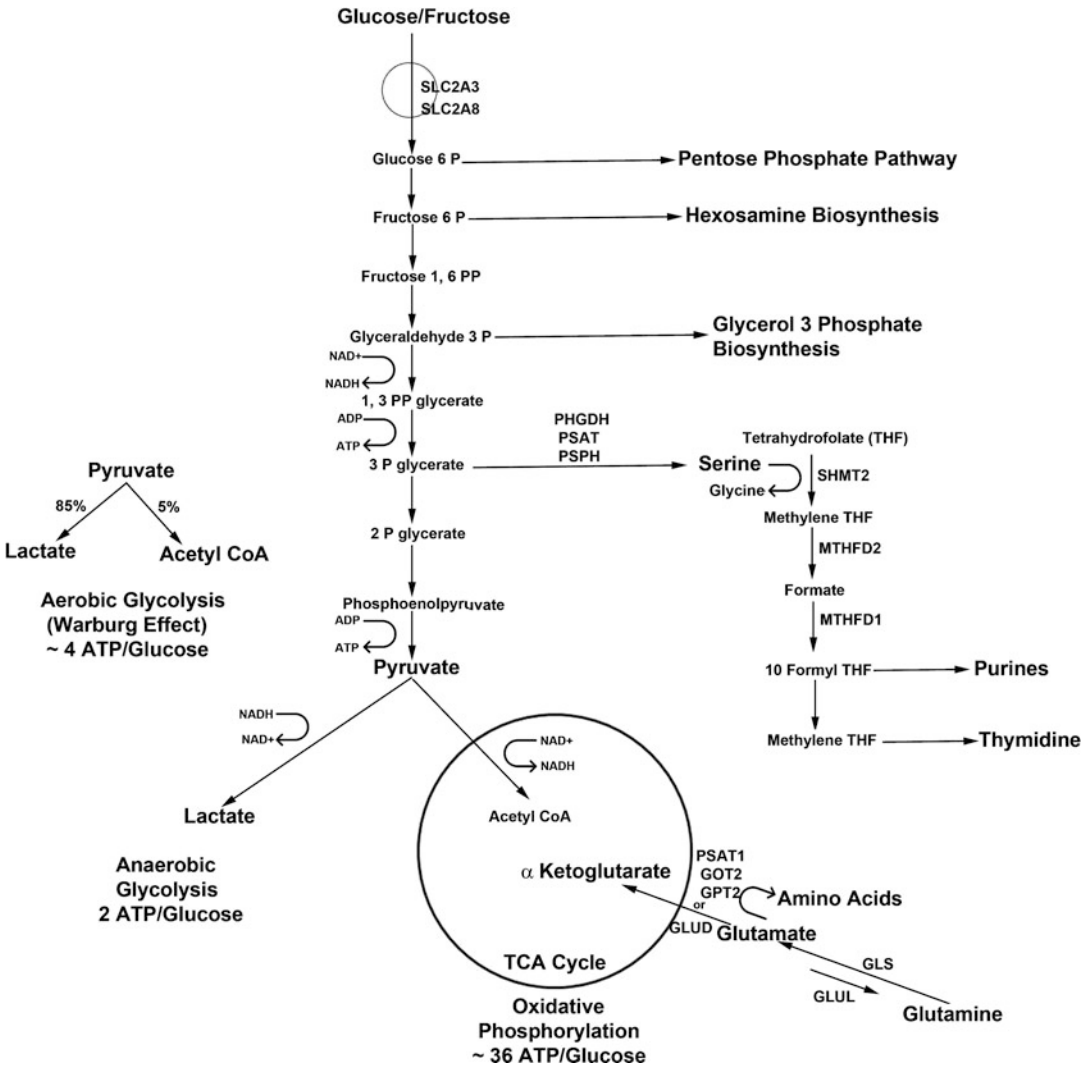

Fig. 2.1 Overview of possible utilization of glucose and fructose through anaerobic and aerobic glycolysis for serine biosynthesis and one-carbon metabolism, and utilization of glutamine via glutaminolysis. Glucose, fructose, and glutamine are abundant nutrients in the conceptuses of farm animals, and glutamine is present in food and animal proteins at relatively high content (Hou et al. 2019; Li and Wu 2020). Glucose is utilized via multiple metabolic pathways (including glycolysis in the presence or absence of oxygen). Similar pathways may exist for fructose metabolism. Phosphate-activated glutaminase (a mitochondrial enzyme in mammals) plays an important role in initiating its catabolism in conceptus, with metabolites including glutamate, aspartate, alanine, pyruvate and lactate. This pathway for partial glutamine catabolism is termed glutaminolysis analogous to glycolysis where glucose is converted into pyruvate and lactate via partial metabolism

occurs in proliferating cells. Enhanced expression of PHGDH, a rate-limiting enzyme of serine biosynthesis, occurs in breast cancer and melanoma cells (Locasale et al. 2011; Possemato et al. 2011).

The newly synthesized serine, or serine transported into the cell can be incorporated into one-carbon metabolism or the folate cycle. The carbon-3 of serine unit can be transferred to a carrier molecule, tetrahydrofolate (THF), in a enzymatic reaction catalyzed by serine hydroxymethyltransferase 2 (SHMT2) in the mitochondria, and SHMT1 in the cytosol, generating 5, 10-methylene-THF and glycine.

Then, 5,10-methylene-THF undergoes a series of oxidative-reductive reactions, generating a series of one-carbon-THF species. One-carbon-THF species are utilized as substrates for the biosynthesis of purines and thymidine, as well as production of S-adenosylmethionine (SAM) which is the primary methyl donor for methylation reactions required for epigenetic modifications of gene expression (Wu 2018). The enzymes required for one-carbon metabolism are frequently upregulated in tumors and activated T cells (Nilsson et al. 2014; Ron-Harel et al. 2016)

Glucose-derived pyruvate is a main source of carbon for the TCA cycle. However, cancer cells direct the majority of the carbon generated from glucose away from the TCA cycle, and instead use the carbon for aerobic glycolysis (Yang et al. 2017). The result is limited availability of pyruvate for maintaining the TCA cycle within mitochondria, and cancer cells replenish TCA cycle metabolites via a process known as anaplerosis. Anaplerosis is the process of replenishing metabolic pathway intermediates, and there are TCA cycle anaplerotic pathways through which TCA cycle intermediates other than acetyl-CoA can be supplied (Wu 2013). The major anaplerotic substrate in proliferating cells is glutamine.

Many cancer cells undergo metabolic reprogramming that makes them highly dependent on glutamine for survival and proliferation. Indeed, when deprived of glutamine, those cells stop growing and die (Yang et al. 2014; DeBerardinis et al. 2007). Glutamine-dependent cell lines consume glutamine as the preferred anaplerotic substrate, as is evident from their oxaloacetate pools, 90% of which are derived from glutaminolysis (DeBerardinis et al. 2007). Glutaminolysis is the process by which cells convert glutamine into aspartate, pyruvate and alanine (which can be further metabolized to form TCA cycle metabolites) through the activity of multiple enzymes (Wu 2013). Glutamine is first converted into glutamate via glutaminase (GLS/GLS2). Glutamate is then converted into alpha-ketoglutarate (α-KG) via two divergent pathways. The first is through the activity of glutamate dehydrogenase (GLUD). The second is through the activity of a group of transaminases, including glutamate–oxaloacetate transaminase, glutamate–pyruvate transaminase, and phosphoserine transaminase (PSAT). The α-KG that is generated then serves as an anaplerotic substrate for the TCA cycle. In addition to α-KG, GLUD generates ammonia and the cofactor NADH and NADPH. In contrast, aminotransferases generate α-KG, as well as other amino acids such as serine, alanine, and aspartate, which contribute to several cell functions including the biosynthesis of nucleotides. In addition to its role in TCA cycle anaplerosis, glutamine serves as a critical nitrogen donor. The deamination of glutamine into glutamate involves the donation of an amide group to enable de novo synthesis of both purines and pyrimidines. Overall, glutamine directly supports the biosynthetic needs required for growth and division of cells by directly contributing carbon and nitrogen. Whereas the carbon contributed by glutamine is used for fatty acid and amino acid synthesis, the nitrogen from glutamine contributes directly to *de novo* biosynthesis of nucleotides.

In the next section of this review, we present results indicating the cell-specific localization of enzymes that participate in serine biosynthesis, one-carbon metabolism and glutaminolysis at the uterine-placental interface of sheep and pigs during the peri-implantation period of pregnancy.

2.3 Overview of Placental and Uterine Anatomy for Sheep and Pigs

The 2- to 4-cell pig embryo moves from the oviduct into the uterus 60–72 h after onset of estrus, and reaches the blastocyst stage by day 5 (Fig. 2.2). The spherical 0.5–1 mm diameter blastocyst sheds the zona pellucida between days 6 and 7 and expands to a 2–6 mm diameter by day 10. At this stage of development pig embryos diverges dramatically from rodents or primates, and the presumptive placental membranes (Tr and endoderm) elongate rapidly to a filamentous form by day 16. Blastocysts

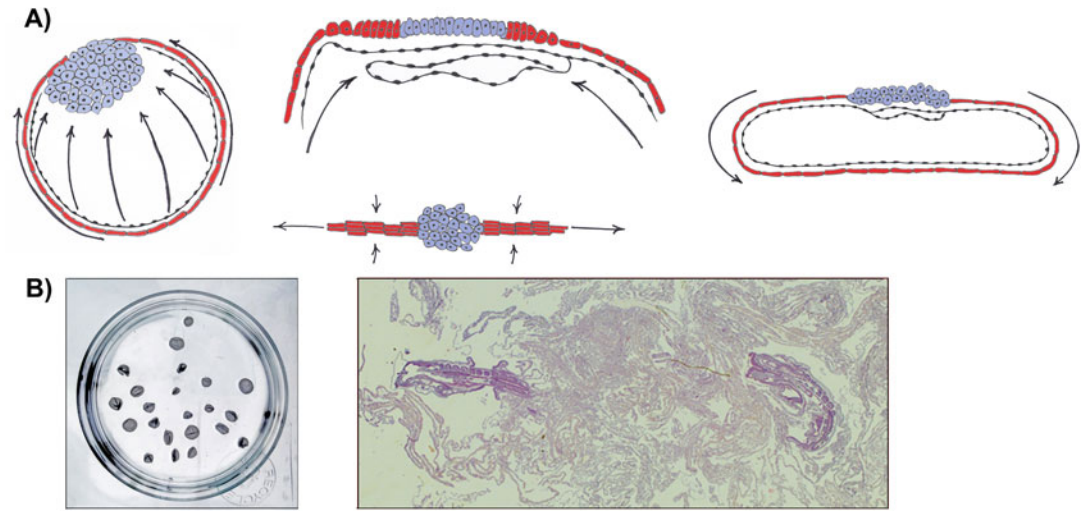

Fig. 2.2 Overview of conceptus elongation in the pig. The conceptuses of sheep undergo a similar pattern of elongation. (**a**) A cartoon depicting the Tr cells (red color) of the round blastocyst (left panel) migrating inward towards the inner cell mass to form the elongation zone (middle panels), then Tr cells migrate outwards (right panel) to form the filamentous conceptus. (**b**) Uterine flushing from Day 10 of gestation (left panel) containing 22 round blastocysts, and H&E staining of two pig conceptuses (right panel) from a uterine flushing obtained on Day 15 of gestation. (The cartoon in Panel A is adapted from that published by Geisert et al. (1982))

expand at about 0.25 mm/h from the early spherical blastocyst stage to the 4–9 mm diameter spherical blastocyst stage (Geisert et al. 1982). Then a remarkable increase in the rate of elongation to 30–45 mm/h from the 10 mm blastocyst to the 150–200 mm long filamentous conceptus occurs within a few hours. A dense band of cells (the elongation zone) composed of both endoderm and Tr extends from the inner cell mass to the tip of the ovoid blastocyst on day 10. After formation of the elongation zone, there is further rapid elongation of the 100–200 mm long conceptus to a conceptus of 800–1000 mm in length by day 16 of pregnancy mediated through alterations in microfilaments and junctional complexes of Tr cells and formation of filopodia by endodermal cells. This last period of elongation involves cellular hyperplasia and each conceptus within the litter achieves maximum surface area for contact between Tr and uterine LE to facilitate uptake of nutrients from uterine LE and uterine glandular epithelium (GE), which increase coincidentally with elongation of the conceptuses (Bazer and Johnson 2014).

Sheep share many features of early embryonic development and implantation with pigs. The morula enters the uterus on day 4 post-fertilization in sheep, and the blastocyst is formed by day 6. Before sheep blastocysts develop into a conceptus, they "hatch" from the zona pellucida on days 6–7. Sheep blastocysts are spherical on day 4 (0.14 mm) and day 10 (0.4 mm), elongate to the filamentous form between days 12 (33 mm) and 15 (150–190 mm), and extend through the uterine body into the contralateral uterine horn by days 16–17 of pregnancy. The filamentous conceptus is closely associated with the uterine LE and appears to be immobilized within the uterine lumen by day 14, although the conceptus can still be recovered intact from the uterus by lavage with only superficial damage. Apposition begins near the inner cell mass, and spreads towards the ends of the elongated conceptus, and by day 16, the Tr is firmly attached to the uterine LE with significant interdigitation between the microvilli on uterine LE and Tr cells.

The term "implantation" is somewhat of a misnomer for the pig, but nevertheless, it is used to describe the initial stages of placentation in livestock species. Pigs demonstrate true epitheliochorial placentation in which there is no displacement or invasion of the maternal tissues

and the conceptus remains within the uterine lumen throughout gestation. In epitheliochorial placentation, separation of fetal and maternal blood is always maintained by an elaborate array of endometrial and extra-embryonic fetal tissues that represent a potential barrier to hemotrophic (nutrients carried in the blood) nutrient transport from the mother to fetus. Thus, the interhaemal distance in pigs must be minimized. This is initially accomplished through degradation of much of the connective tissue separating the uterine LE and Tr/chorion (referred to as Tr throughout this paragraph) from their underlying capillary beds resulting in the blood vessels actually indenting into the basal surfaces of uterine LE and Tr cells. Surface areas of contact between the uterus and placenta increase through interdigitation of microvilli between uterine LE and Tr. However, between day 25 and day 30, extensive remodeling of the uterine-placental interface to form chorionic (placental) ridges and corresponding endometrial invaginations results in folding that further increases the area of uterine-placental association across the entire placenta, except at the openings of uterine glands in pigs (Fig. 2.3). In addition to having uterine LE closely apposed to the Tr, there are specialized chorionic epithelial cells at the openings of the mouths of uterine glands where the Tr never fuses with the uterine LE, but forms a pocket referred to as an areola (Fig. 2.4). Histotroph from the uterine GE is delivered into the areolae, absorbed and transported across the chorion by fluid phase pinocytosis for release into the placental circulation. The placenta of each piglet in a litter has about 2500 areolae and their number correlates significantly with fetal weight. The Tr cells that line the areolae and line the tops of the placental ridges of the folds are tall columnar epithelia, well suited for transport of nutrients across the epithelial barrier (Song et al. 2010).

In contrast to the pig, sheep demonstrate synepitheliochorial placentation in which limited fusion of Tr with uterine LE occurs (Fig. 2.4). Two morphologically and functionally distinct cell types, mononucleate Tr cells and binucleate Tr giant cells (BNCs), are present in the Tr of sheep placentae. The mononucleate cells constitute the majority of the Tr cells and BNCs begin to differentiate from the mononucleate Tr cells in

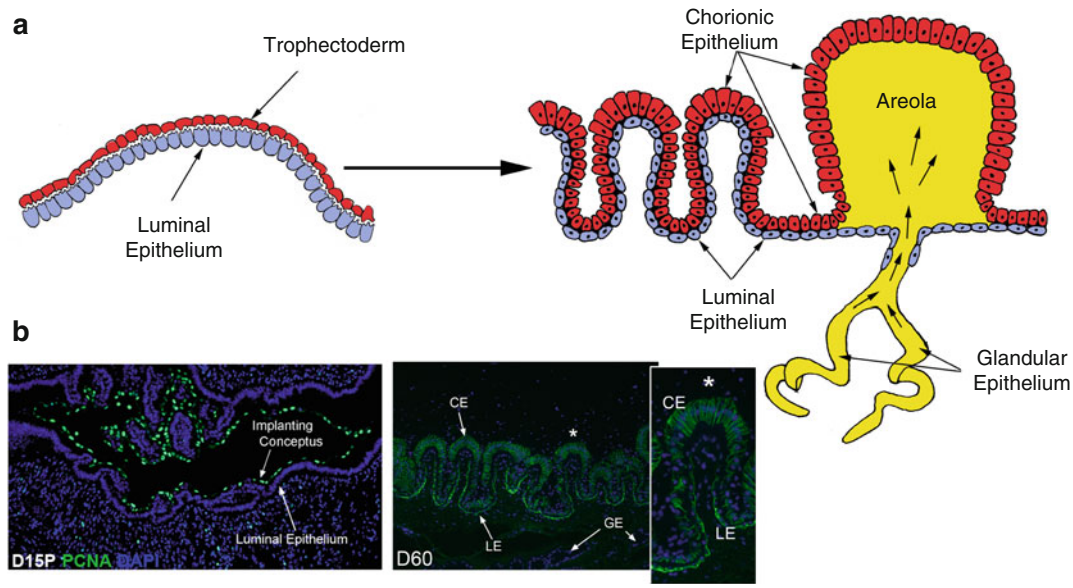

Fig. 2.3 Overview of placental development in the pig. (**a**) A cartoon depicting the uterine-placental interface during implantation (left panel) and placentation (right panel). (**b**) Immunofluorescence staining at the uterine-placental interface for PCNA (left panel) on Day 15 of pregnancy and α2β1 integrin (right panel) on Day 60 of pregnancy. The asterisk indicates a uterineplacental fold/villi

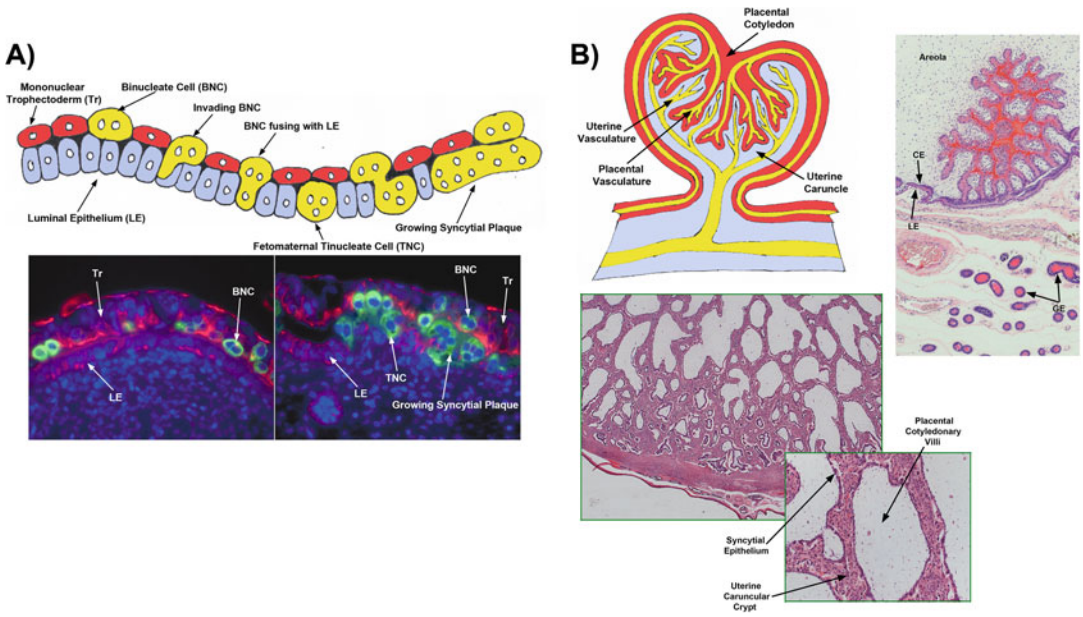

Fig. 2.4 Overview of placental development in the sheep. (**a**) A cartoon and immunostaining for pregnancy associated glycoprotein (green) and epithelial cadherin (red) depicting the current consensus for syncytia formation. (**b**) A cartoon and H&E staining depicting the structure of a placentome and an areola

concert with Tr outgrowth during conceptus elongation. BNCs first appear between days 14 and 16 of gestation in sheep conceptuses and comprise 15–20% of the Tr during the apposition and attachment phases of implantation. BNCs migrate and fuse with individual uterine LE cells to form trinucleate syncytial cells beginning about Day 16 of pregnancy in sheep, thereby assimilating uterine LE. The syncytia of sheep subsequently enlarge through continued BNC migration and fusion to form syncytial plaques. The syncytial plaques are conceptus-maternal hybrid cells composed of uterine LE and BNCs, and they eventually form the epithelial interface between uterine and placental tissues within the placentomes (described in the next paragraph). In sheep, the syncytial plaques are a consistent feature in the placentomes throughout pregnancy.

Following successful elongation of the conceptus, trophectoderm outgrowth, and implantation, the placentae of sheep organizes into placentomal and interplacentomal regions (Placentomes and areolae depicted in Fig. 2.4). During placentome development, highly branched villous placental folds, termed cotyledons, initially form by day 30 of gestation in sheep. Cotyledonary chorioallantoic villi lined by syncytial plaques then begin to protrude into crypts in the maternal endometrial caruncular tissue (aglandular areas of endometrium consisting of stroma covered by a single layer of uterine LE), resulting in extensive interdigitation of endometrial and placental tissues by day 40 of gestation. Placentomes provide a conduit for hemotrophic nutrition to the fetus wherein maternal and placental blood vessels are in close proximity for exchanging oxygen and micronutrients, and there is a close correlation between placentomal mass and birth weight of the fetus. In contrast, interplacentomal areas exhibit epitheliochorial attachment of uterine LE to trophectoderm, and contain areolae that take up histotroph secreted by the uterine GE for transport to placental vasculature that rings the areola (Wooding and Burton 2008).

2.4 Enzymes for Serine Biosynthesis, One-Carbon Metabolism, and Glutaminolysis Are Expressed in a Cell-Specific Manner

Serine biosynthesis is catalyzed by the successive actions of the enzymes PHGDH, PSAT and PSPH. PHGDH converts 3-PG to PHP which is the committed step in serine biosynthesis. PSAT next converts PHP to P-Ser and PSPH removes the phosphate group to yield serine (Wu 2013). We performed immunofluorescence microscopy on cells at the uterine-placental interface of Day 18 pregnant sheep and Days 15, 20 and 30 pregnant pigs using antibodies to PHGDH and PSPH, the first and last enzymes in the conversion of hexose sugars to serine. Both PHGDH and PSPH are expressed by the uterine LE of sheep on Day 18 of gestation. Similar to sheep, the uterine LE, but not Tr, of pigs express PHGDH, PSAT1 and PSPH on Days 15 and 20 of gestation, and expression begins to decrease in uterine LE between Days 20 and 30 of pregnancy (Table 2.1).

Within mitochondria, SHMT2 catalyzes the reversible reaction of serine and tetrahydrofolate (THF) to glycine and 5,10-methylene tetrahydrofolate (mTHF). Mitochondrial MTHFD2 converts mTHF to formate, which is transported to the cytoplasm for synthesis of purines, thymidine and SAM which is the primary methyl donor for cellular methylation. In sheep, SHMT2 and MTHFD2 are expressed by BNCs of the placenta on Day 18 of gestation. These SHMT2-expressing cells fuse together to form BNCs that actively invade into the uterine LE to begin formation of the synepitheliochorial placenta characteristic of sheep. The Tr cells of pigs expresses high levels of SHMT2 and MTHFD2 on Days 15 and 20 of gestation. In summary, uterine LE expresses enzymes of serine biosynthesis including PHGDH, PSAT, PSPH in sheep and pigs, and SHMT2 and MTHFD2, enzymes for one-carbon metabolism, are highly expressed by the conceptus Tr of sheep and pigs during the peri-implantation period. Taken together, our observations suggest that glucose and fructose can be converted to serine within the uterine LE, serine can be transported into conceptus Tr to be utilized as a substrate of one-carbon metabolism in conceptus Tr to support proliferation during the peri-implantation period of pregnancy (Fig. 2.5).

Different cancer subtypes have distinct patterns of glutamine metabolism depending on whether they reside in a glutamine rich or glutamine-poor environment. In the presence of glutamine, cancer cells convert glutamine to glutamate through the action of GLS, glutamine anaplerosis, and this metabolic pathway maintains the flow of substrates that drive the TCA cycle. However, in the absence of glutamine, or in a glutamine–poor environment, some cancer cells use glutamine synthetase (GLUL) to synthesize glutamine from glutamate and this allows the cells to survive (Cluntun et al. 2017). Luminal breast cancer cells frequently exhibit

Table 2.1 Major cell types that express enzymes for serine biosynthesis, one-carbon metabolism, and glutaminolysis during peri-implantation period of pigs and sheep (between Days 15–20 of pregnancy in pigs and between Days 18–20 in sheep)

		Pig		Sheep	
Metabolic pathways	Enzymes	Endometrium	Conceptus	Endometrium	Conceptus
Serine biosynthesis	PHGDH	LE	En	LE	Tr
	PSAT1	LE	Tr	n.a.	n.a.
	PSPH	LE	–	LE	–
One-carbon metabolism	SHMT2	–	Tr	–	BNC
	MTHFD2	–	Tr	–	BNC
Glutaminolysis	GLUL	–	En	LE	–
	GLS	–	Tr	n.a.	n.a.
	PSAT1	LE	Tr	n.a.	n.a.

n.a. not analyzed

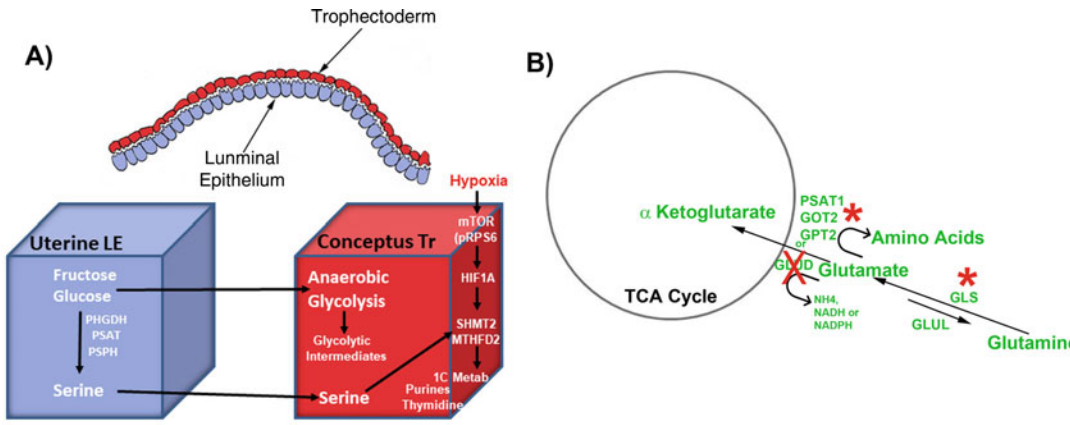

Fig. 2.5 (**a**) The proposed utilization of glucose and fructose during early placental development in sheep and pigs. (**b**) The proposed utilization of glutamine by trophectoderm cells during early placental development in pigs

high GLUL and low GLS expression, whereas the opposite is true for basal breast cancer cells. Matching these expression patterns, most luminal breast cancer cells can be cultured in glutamine-free media, whereas basal cells are highly sensitive to glutamine withdrawal and to inhibition of GLS, both in cell culture and when grown as xenograft tumors *in vivo* (Gross et al. 2014; Kung et al. 2011). The expression of GLS and GLUL in porcine conceptuses is cell type-specific during the peri-implantation period. GLS protein is localized to the conceptus Tr on Day 15 of pregnancy, and GLUL protein is localized to conceptus endoderm. This indicates heterogeneity in glutamine metabolism in the conceptus during the peri-implantation period and suggests that conceptus endoderm is able to synthesize glutamine, and conceptus Tr is able to consume glutamine for glutaminolysis.

Glutamate is converted into α-KG via two divergent pathways. There are some key differences in the two pathways for the conversion of glutamate into α-KG. Both pathways generate α-KG, but they have different by-products (Altman et al. 2016). GLUD generates ammonium and the cofactors NADH and NADPH as by-products. In contrast, aminotransferases generate other amino acids such as serine, alanine, and aspartate, which contribute to the biosynthesis of nucleotides (Wu 2013). PSAT1 protein is highly abundant in conceptus Tr cells on Day 15 of the peri-implantation period, whereas GLUD1/2 are not detectable in those same cells. Therefore, glutaminolysis likely occurs in conceptus Tr through the GLS-aminotransferase metabolic pathway. Furthermore, increased expression of aminotransferase suggest that proliferating conceptuses utilize aminotransferases to support rapidly growing conceptuses possibly by providing other amino acids (Fig. 2.5).

2.5 Summary

Conceptus elongation, implantation and early placental development in sheep and pigs are complex events that require significant energy, the substrates for which are primarily supplied from histotroph within the uterine lumen. Glucose, fructose and glutamine are major components of histotroph. Embryonic mortality during this complex, energy consumptive, peri-implantation period of pregnancy remains a major constraint to improving reproductive efficiency and profitability in livestock enterprises, and is a major source of difficulty to women trying to maintain successful pregnancies. We have presented results indicating cell-specific localization of enzymes that participate in serine biosynthesis, one-carbon metabolism, and glutaminolysis at the uterine-placental interface of sheep and pigs during the peri-

implantation period of pregnancy. PHGDH and PSPH, enzymes for serine biosynthesis, are expressed by the endometrial LE of both sheep and pigs. SHMT2 and MTHFD2, enzymes for one-carbon metabolism, are expressed by the proliferating Tr of pig conceptuses and expressed by the migrating BNCs of sheep conceptuses. We also showed cell type-specific expression of GLS in the Tr and GLUL in the endoderm of conceptuses, suggesting that endoderm synthesizes glutamine, and Tr converts glutamine into glutamate. The aminotransferase PSAT1 is preferentially expressed in Tr suggesting generation of α-KG and amino acids which support rapidly growing conceptuses. The temporal and cell-specific expression of these enzymes illustrate that glucose and fructose can be used for serine biosynthesis followed by one-carbon metabolism, and that glutamine can be converted to α-KG within the conceptus Tr, and glutaminolysis-derived α-KG enters the TCA cycle for synthesis of nucleotides. These biosynthetic pathways are essential to support elongation, migration, implantation and early placental development of sheep and pig conceptuses. As shown in Fig. 2.1 of this review, glycolysis provides a means whereby substrates are available for conceptuses to optimally utilize multiple biosynthetic pathways to use molecules such as glucose, fructose, and glutamine in the uterine lumen in early pregnancy or in the fetal-placental vasculature in later pregnancy to synthesize ATPs, amino acids, ribose sugars, hexosamines and nucleotides required for growth, development and survival of conceptuses.

Acknowledgements Work in our laboratories was supported by Agriculture and Food Research Initiative Competitive Grants (No. 2016-67015-24958 and 2018-67015-28093) from the USDA National Institute of Food and Agriculture.

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Amino Acids in Beef Cattle Nutrition and Production

3

Werner G. Bergen

Abstract

Proteins have been recognized for a long time as an important dietary nutritional component for all animals. Most amino acids were isolated and characterized in the late nineteenth and early twentieth century. Initially dietary proteins were ranked high to low quality by growth and N balance studies. By the 1950s interest had shifted to studying the roles of individual amino acids in amino acid requirements by feeding studies with non-ruminants as rodents, poultry and pigs. The direct protein feeding approaches followed by measurements of nutritional outcomes were not possible however in ruminants (cattle and sheep). The development of measuring free amino acids by ion exchange chromatography enabled plasma amino acid analysis. It was thought that plasma amino acid profiles were useful in nutritional studies on proteins and amino acids. With non-ruminants, nutritional interpretations of plasma amino acid studies were possible. Unfortunately with beef cattle, protein/amino acid nutritional adequacy or requirements could not be routinely determined with plasma amino acid studies. In dairy cows, however, much valuable understanding was gained from amino acid studies. Concurrently, others studied amino acid transport in ruminant small intestines, the role of peptides in ruminant N metabolism, amino acid catabolism (in the animal) with emphasis on branched-chain amino acid catabolism. In addition, workable methodologies for studying protein turnover in ruminants were developed. By the 1990s, nutritionists could still not determine amino acid requirements with empirical experimental studies in beef cattle. Instead, computer software (expert systems) based on the accumulated knowledge in animal and ruminal amino acids, energy metabolism and protein production were realized and revised frequently. With these tools, the amino acid requirements, daily energy needs, ruminal and total gastrointestinal tract digestion and performance of growing beef cattle could be predicted.

Keywords

Cattle · Metabolism · Protein synthesis and turnover · Amino acid requirements

Abbreviations

BCAA	branched-chain amino acids
BCKDH	branched-chain α-ketoacid dehydrogenases
CNCPS	Cornell Net Carbohydrate and Protein System

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1285, https://doi.org/10.1007/978-3-030-54462-1_3

IAA	indispensable amino acids
MP	metabolizable protein
NRC	national research council
PAA	plasma free amino acid profiles
RDP	rumen degradable protein
RUP	rumen undegradable protein
SCFA	short-chain fatty acids

3.1 Introduction

The overall role of amino acids as required precursors for protein synthesis and growth was well appreciated by physiology and nutrition researchers by the 1950s. However, concentration profiles of 20 amino acids (aminograms) in blood/serum or plasma are much more difficult to relate to physiological processes than for example plasma glucose or thyroxine concentrations. During the middle of the twentieth century, Rose (1957) established which amino acids were indispensable and dispensable (essential/nonessential) from the diet for human subjects. Rose and coworkers principally used the nitrogen balance technique as a response criterion in such work. Empirical experimental studies, based on nitrogen balance and growth responses were also being conducted with poultry and swine to delineate qualitative and quantitative aspects of amino acid (protein) nutrition (Almquist 1954; Baker 2009; Baker et al. 1966; Klain et al. 1960). Amino acid nutrition was much more enigmatic in ruminants. During the 1940s and 1950s recognition grew that rumen bacteria (a likely source for amino acids to ruminants; Owens and Bergen 1983) possessed all amino acids found in meat proteins (Weller 1957). Experiments utilizing proteins of known amino acid composition or amino acids directly for protein nutrition studies using the feeding approach could not be done in cattle and sheep as the rumen microbiota ferments, catabolizes or remodels dietary proteins into microbial proteins before digestion and amino acid absorption in the small intestine. Thus, for many years, quantitative aspects of ruminant protein/nitrogen metabolism were assessed using nitrogen balance and growth responses (Perry et al. 1967; Burroughs et al. 1971).

3.2 Emerging Issues on the Utilization of Plasma Amino Acid Analysis in Nutrition Studies

The prospect of employing quantitative free amino analysis in body fluids to enhance the understanding of amino acid nutrition in humans and animals (Snyderman et al. 1968) became a reality with the commercial application of the Moore and Stein (1954) ion-exchange, post column ninhydrin detection amino acid chromatography technology in the late 1960s. The then concept of a special role of the limiting amino acid in nutrition furthered the movement toward plasma/serum amino acid analysis in clinical studies to assess amino acid status. It was thought that identifying limiting amino acids in diets would allow optimizing protein nutrition and health in particular in children in "so called" underdeveloped countries (Allison 1955, 1961; Snyderman et al. 1968). Likewise, for nutrition research in ruminants, obtaining a blood sample followed by an amino acid analysis was deemed to expedite the understanding of protein/amino acid nutrition without the necessity of nitrogen balance determinations. Workers in human nutrition, pediatrics and animal nutrition pursued this research paradigm with exceeding gusto, but unfortunately immediate amino acid results were not too interpretable. In time, the whole field of plasma amino acid analyses and nutrition research matured and data became interpretable (Bergen 2007).

For beef cattle and sheep, approaches utilizing plasma amino acid analyses for amino acid requirement studies were conducted in some laboratories (Fenderson and Bergen 1975; Shelling et al. 1967). Unfortunately, beef cattle are seldom growing fast enough and the supply of amino acids from the rumen outflow may just meet the animal's amino acid/metabolic protein requirements for that rate of growth. Thus, amino acid infusions, broken line PAA response curves (or the current marker amino acid or direct amino oxidation studies; Bergen 2007) will not show that a stimulation in amino acid utilization has occurred. Using plasma amino acids to study

amino acid utilization in high producing dairy cows is much more satisfactory as the protein needed for digestion in the small intestine and mammary protein synthesis exceeds the microbial protein production capacity for indispensable amino acids. In amino acid infusion studies, the roles of dispensable amino acids was not evaluated (Schwab and Broderick 2017; Clark et al. 1978; King et al. 1981, 1990; Kung et al. 1984; Huber et al. 1984).

The rumen microbiota eco-system and the host animal are intertwined in a symbiotic relationship. In essence, the rumen produces short-chain fatty acids (SCFA) and microbial cells during feed fermentation while the animal provides buffers to stabilize rumen pH, absorbs SCFA, and gastrointestinal mobility moves digesta out of the rumen-reticulum to the abomasum and small intestine. This review will not include the role of the rumen microbial ecosystem on protein nutrition and metabolism in ruminants. Readers are referred to recent reviews instead (Gilbreath et al. 2020; NRC 2016; McCann et al. 2014). Currently, traditional rumen research work on N/urea cycling, quantitative aspects of ruminal microbial growth, fermentation and SCFA production, dietary protein degradation, and metabolism of isolated ruminal anaerobes, has been replaced by and large by molecular biology approaches in the rumen (McCann et al. 2014; Firkins and Yu 2015) particularly in the identification of bacteria and other organisms in the rumen. These studies are providing us with new insights; however, the myriads of the organisms identified in the rumen will be difficult to isolate, culture to characterize all aspects of fermentation of fibrous or highly digestible carbohydrates (Seshadri et al. 2018).

Performance response data in ruminants to dietary amino acids has almost no interpretable outcome unless the amount of amino acids reaching the abomasum is quantified. To obtain such data on every feedstuff and combination of feeds by direct experimentation would be an impossible task. Expert system such as the *Cornell model* (Fox et al. 1992) have the ability to predict protein flows to the small intestine and allow workers to estimate quantitative amino acid absorption. It is not yet clear how molecular level characterization of the rumen microbiome will eventually enhance our ability to utilize the rumen fermentation with less energy wastage including methane generation. In any case, it is clear that the rumen-produced microbial protein is the most important source of amino acids for many domestic and wild ruminants. Likewise, differential gene expression (DE) in the liver, mammary gland, skeletal muscle, adipose tissue and enterocytes have been measured in varying degrees in dairy and beef cattle to study amino acid metabolism.

3.3 Plasma Amino Acid Responses to Dietary Manipulations

The essence of determining amino acid requirements in farm species is to improve performance, enterprise sustainability and depress environmental impact of protein feeding to animals. Thus, the National Research Council-Animal Nutrition expert committees (NRC 2016) over years have evaluated and summarized innumerable studies on dietary protein content and performance to better define protein requirements in beef cattle. NRC committees have had difficulty to evaluate and provide quantitative amino acid requirements in beef cattle in their reports. There have been very few direct amino acid nutrition studies in beef cattle under typical production systems; hence NRC committees have estimated amino acid requirements of beef cattle by indirect methods based on product (i.e., amino acid) content and the efficiency of amino acid utilization from ingestion to skeletal muscle protein synthesis.

As plasma amino acid analysis became routine, workers began to explore the effect of protein sources and time after feeding on arterial (general systemic) and portal vein plasma free amino acid profiles (PAA). This work was complemented by tissue free amino acid analysis. In all cases, the emphasis was on comparing the amino acid profiles of dietary proteins with PAA. Further, identifying indispensable amino acids (IAA) requirements in human (or farm animals) using nitrogen balance studies (Rose 1957) was not an efficient procedure. In addition, the general

notion was advanced that the limiting amino acid of most individual dietary protein could likely be identified by PAA studies (Allison 1961; Snyderman et al. 1968). For non-ruminants studies of PAA profiles from given protein sources (often accompanied with a high protein quality control group) could identify many limiting dietary amino acids (McLaughlan 1974). Plasma AA were also studied in protein malnutrition as well as for the development of diagnostic tools for such maladies. A consensus (see Fig. 3.1) emerged after many such studies that PAA would rise after feeding high quality proteins, but poor quality proteins resulted often in depressed plasma IAA. PAA responses to various dietary and other experimental conditions formed basic principles for the interpretation of such data (Bergen 2007). These principles were adapted to studying amino acid requirements by direct and indirect oxidation utilizing stable isotope labelled amino acids in experimental animals and humans (Ball and Bayley 1986; Kim et al. 1983a, b; Pencharz and Ball 2003; Elango et al. 2008; Kurpad et al. 1998). There are those however, that have vigorously challenged amino acid oxidation studies as a procedure to determine human amino acid requirements and insists that all such studies should be based on nitrogen balance procedures (Millward 2004). In poultry use of empirical feeding approaches for amino acid nutrition where growth/performance is the response criterion is faster and less controversial (Dozer et al. 2011).

In ruminants, the adoption of PAA studies were also proposed as a method to determine amino acid requirements. Unfortunately, PAA behaved differently in ruminants than in non-ruminants. Often PAA show a postprandial decline in ruminants rather than elevations noted in non-rumonants after feeding (Bergen 1978). For most situations, in ruminants (microbial protein and a some rumen undegradable protein (RUP) will satisfy AA requirements (Bergen 1986) or AA needs of most ruminants are at or below the total digesta AA flow. This scenario makes direct studies of amino acid requirements using PAA profile changes less satisfying in beef cattle. Dairy cows are much more responsive to small intestinal AA availability since the rumen microbiota and some RUP will not satisfy amino acid needs for high mammary protein synthesis (King et al. 1990; Schwab and Broderick 2017).

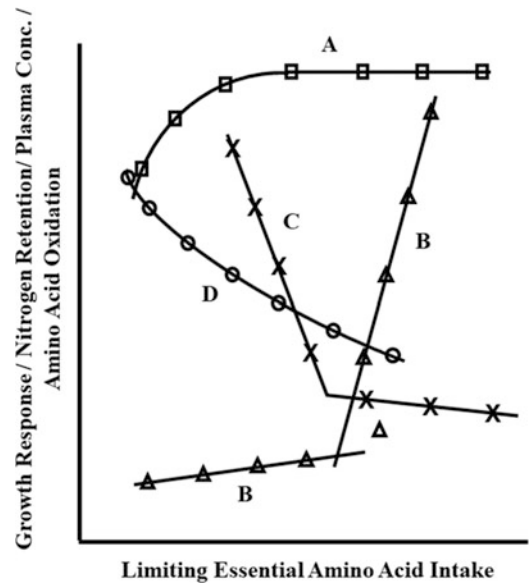

Fig. 3.1 A template for interpretation of plasma amino acid responses to increasing intake of the first limiting amino acid (from Bergen 2007). (**a**) Effect of increasing limiting amino acid intake on weight gain or nitrogen balance, (**b**) Plasma concentrations of limiting amino acid stay constant and then increase rapidly with increasing intake. The intersection of the two straight **b** lines indicates the limiting amino acid requirement under the given experimental circumstances. This experiment can also be done with amino acid heavy isotopes. As the limiting amino acid requirement is reached, catabolism of the limiting amino acid increases rapidly (Also called direct oxidation method). (**c**) A representation of the oxidation of an indicator amino acid during limiting amino acid requirement studies; the oxidation will decline as required limiting amino acid reaches requirements. (**d**) Plasma concentrations of the sum of non-limiting essential amino acids. The concentrations decline as upon addition of the limiting amino acid, protein synthesis increases lowering circulating concentrations of other essential amino acids

Because of the different responses of circulating amino acids to protein intake plus time after feeding for ruminants, the questions arose whether ruminants can rank the quality of various proteins the same as nonruminants? Further, can the same limiting amino acids be identified in various pure protein sources in both

ruminants as identified in non ruminants? These questions required a direct study with an sheep animal model (Potter et al. 1972; Potter and Bergen 1974). The sheep were prepared with a duodenal cannula and all rumen outflow was discarded. The fermentation of dietary carbohydrates (SCFA production) in the rumen was not affected here. The ruminal outflow was replaced with an artificial chyme containing various purified proteins, minerals and vitamins which were pumped directly into the small intestine and bypassed rumen metabolism entirely (Potter et al. 1972). A group of rats were fed each the identical protein sources for a nonruminant control comparison. Under these experimental conditions, the sheep responded with PAA profiles similar to non-ruminants and the correct limiting amino acids could be identified (Potter et al. 1972).

3.4 Interlude

While AA requirements and metabolism research has continued in agriculturally important animals, in humans research protocols shifted to the utilization of stable isotopes (^{13}C; ^{18}O) of amino acids for oxidation or marker amino acids procedures to study amino acid requirements (Kim et al. 1983a, b; Ball and Bailey 1986; Kurpad et al. 1998; Pencharz and Ball 2003; Elango et al. 2008). Such approaches have not been adopted in beef cattle protein/amino acids needs research. In cattle, there was a shift away from amino acid metabolism/requirement work to factors affecting bovine muscle growth and protein turnover (Owens and Bergen 1983). Then emerged molecular biology/gene expression procedures and subsequent bioinformatics approaches and most work with profiling PAA for amino acid metabolism and requirements ceased. Instead, epithelium transporters gene expression in gut tissues and mammary glands became a focus. More recently metabolomics have been added to study protein nutrition status in tissue fluids. Meanwhile the *Cornell model* (Fox et al. 1992) and NCR (2016) calculations of AA requirements are using tissue/muscle protein AA profiles as a starting point. Here several assumption must be used to translate qualitative understanding to a quantitative basis. It should also be noted that according to Lapierre et al. (2006) single efficiency constants in converting from actual data to derived values can be fraught with problems and should be approached with caution. The reason for all this research was to accurately describe the amino acid needs of beef cattle. Secondarily, data could be applied to trait selection and metabolic issues.

3.5 Small Intestine Amino Acid and Peptide Absorption and Metabolism

Perusing previous reviews and publications on the subject, the overall digestive physiology in the abomasum and small intestine of ruminants closely resembles digestion/absorption processes as observed in rodents and humans with a few exceptions (Bergen 1978). As the ruminal digesta flows from the abomasum to the lower gut (small intestine) for some time the digesta pH will remain acidic. In a large measure this is because ruminants appear to have less copious flow/sodium bicarbonate buffering than noted in rodents or humans (Bergen 1978; Taylor 1962). This would certainly modify the dynamics of intestinal/pancreatic digestive proteases such as trypsin and chymotrypsin whose pH optima are in the slightly basic range. A putative quantitative effect on digestion/absorption of amino acids and proteins in the small intestine of ruminants in comparison to non-ruminants has not been assessed. While the number of characterized enterocyte solute/amino acid transporters has expanded over the years (Wu 2013), there is no compelling evidence that ruminants possess different intestinal amino acid transporters than other species.

The digesta in ruminants arriving at the small intestine contains considerable amounts of nucleic acids due to its rich microbial organic matter content. The breakdown of these nucleic acids is achieved via DNase and RNase phosphodiesterases and phosphomonoesterases. According to Barnard (1969), the pancreatic flow in ruminants has a 1000–2000 times greater RNase activity than in primates, dogs, cats or

rabbits and a fourfold greater activity than that of rats. Smith et al. (1970) reported apparent digestibilities of 85% and 75% for RNA and DNA respectively in calves. The traditional view about the disposition of absorbed nucleotides has been that they may be utilized in salvage pathways for nucleic acid synthesis or dephosphorylated and the resulting nitrogenous bases are metabolized or excreted.

Early phases of amino acid transport explorations were conducted with rodents and cell cultures. Initially there was discovery of transport systems and substrate-amino acid overlap/competition. During these studies the A and L transporter systems of amino acids were identified (Christensen 1962; Wiseman 1968). Next followed extensive kinetic studies to determine substrate (amino acid) affinity, role of cations and pH effects, to identify passive, facilitated, active transporters and capacity of such transporters. Very little effort was directed toward ruminants as such experiments involve high animal costs (Hume et al. 1972; Williams 1969). In the early 1970s, Johns and Bergen (1973) measured amino acid transport in sheep. Small intestines were harvested from sheep and amino uptake was determined in enteric epithelial/mucosal cell and preparations utilizing documented in vitro procedures. Uptake of methionine, glycine, lysine and leucine –lysine competion were assessed. They showed that AA transport measured here was dependent on respiratory ATP production (active transport) and L-lysine absorption was maximized in the ileum. The relationships between the rate of amino acid uptake in small intestinal rings (V) and substrate concentrations (S) resulted in hyperbolic curves. Thus Vm and Km (affinity) were determined from these data using the Lineweaver-Burk (1934) plot. Glycine had the lowest Km but the highest Vm (data not given). Methionine had K_M and V_{max} of 2.43 mM and 1.52 μmol/100 mg wet tissue per 0.5 h, respectively, whereas lysine had K_M of 5.80 mM and V_{max} of 0.87 μmol/100 mg wet tissue per 0.5 h, respectively. These results showed that carrier affinities were strong for both methionine and lysine while their transport capacity was modest. Simultaneous incubation of leucine and lysine in the small intestinal rings inhibited lysine uptake from 25% to 48%. Finally, the role of enteric amino acid catabolism as related to first pass metabolism (Stoll et al. 1998) has not been evaluated in cattle.

By the 1980s, numerous brush border amino acid transport systems had been identified and characterized. These included: alanine, serine, cysteine and threonine transporters (ASCT1); Na^+ and Cl^- depended neutral and cationic amino acid transporters ($ATB^{o,+}$); Na^+ dependent neutral amino acid transporter (B^o AT); Na^+ independent cationic and zwitter-ionic amino acid transporters ($b^{o,+}$ AT); Na^+ independent cationic amino acid transporter 1 (CAT1); Na^+ independent cationic amino acid transporter 2; Na^+ independent cationic and Na^+-dependent neutral amino acid transporters (y^+LAT1 and 2) and L type amino acid transporter-1 (LAT1). These transporters are located either in the apical or basolateral membranes of the small intestinal epithelia (Liao et al. 2008).

During this time period, the putative role of peptide transporters in amino acid absorption from the small intestine was examined. Indeed, peptides can be taken up by enterocytes and this presents a whole new viewpoint on intestinal absorption (Gilbert et al. 2008). It is not yet clear that peptide transport in a time course manner will change the pattern of amino acid uptake from the gut. In as much as peptides are not directly utilized for protein synthesis since obviously there are no tRNA for peptides, clearly cellular peptidases must be involved after absorption (Munk 1976). It may be speculated that amino acid utilization efficiency may be enhanced or depressed through peptide transport. As yet, such downstream ideas about the consequences of an intermediate pool of peptides during digestion and absorption have not been explored. Research on intestinal, mammary and colonic solute transporters has continued in a new direction in the twenty-first century. Instead of measuring functional kinetic aspects of amino acid transporters, gene expression and molecular regulation in response to nutritional regimen and phenotypic changes are now at the forefront of intestinal solute (amino acid) transporters research in ruminants (Liao et al. 2008; Foote et al. 2017).

3.6 Metabolism of Amino Acids in Ruminants

Beyond quantitative needs of amino acids for protein synthesis, the carbon chains of amino acids are also metabolites/intermediates important in a variety of physiological processes (Wu 2018). Amino acid catabolism was studied extensively in prokaryotes and later eukaryotic organisms. Two major findings in the past were that most amino acids are transaminated/deaminated in the liver before further catabolism of the carbon skeleton, while for branched-chain amino acids (BCAA, leucine, valine, isoleucine) catabolism occurs in extra hepatic tissues (Harper et al. 1984). These amino acids are highly abundant in both plant and animal proteins (Hou et al. 2019; Li and Wu 2020). Activity of BCAA amino transferases and branched-chain α-ketoacid dehydrogenases (BCKDH) are highly regulated by covalent modification (phosphorylation). In addition, BCAA are highly non-polar and integrally involved in protein folding phenomena and in the membrane domain of transmembrane receptors. Amino acid synthesis and catabolism pathways have recently been reviewed and summarized by Bender (2012).

In the 1980s, little data were available on the dicodomy of BCAA and all other AA metabolism in ruminants. Working with an perfused, isolated sheep diaphragm preparation, Coward and Buttery (1979) detected only minimal BCAA catabolism in these preparations and concluded that muscle is not a major site for BCAA catabolism; however the BCAA aminotransferase and BCKDH may have been highly down regulated in the diaphragm preparation. Later, Bergen et al. (1988) studied the relative activities of BCAA amino transferase and BC keto acids dehydrogenase in sheep liver, adipose tissue and skeletal muscle. Enzymes were assayed immediately after post-slaughter tissue harvest. Their results indicated that in ruminants, BCAA catabolism is centered in extra-hepatic tissues as also reported by others (Suryawan et al. 1998; Webb et al. 1992). In general, adipose tissues had the highest activities in sheep (Bergen et al. 1988). Unfortunately this work was also done before the strong covalent regulatory modifications of BCAA aminotransferase and BCKDH were appreciated. While these data of Bergen et al. (1988) from non-lactating ruminants would agree with a extra-hepatic/hepatic dicodomy of BCAA vs. other essential amino acids in sites of catabolism as found in rodents, these experiments were not designed to address the phosphorylation (inactive)/dephosphorylation (active) issue (Lu et al. 2009; Shimomura et al. 1990; Crowell et al. 1990). More recent work measuring tissue fluxes (labelled amino acids), enzyme protein abundance, enzyme expression and enzyme activity in tissue preparations showed considerable differences between BCAA tissue fluxes as related to catabolizing enzymes activity, protein abundance and gene expression (Webb 1986; Webb et al. 2019). From flux data in ruminants, liver oxidation of BCKA was not too high; maybe enzymes were allosterically down regulated in vivo, while typical tissue enzyme preparations and assays of BCKDH may have been less downregulated, resulting in higher activity values but not necessarily reflecting the in vivo physiological milieu.

By the 1950s, workers had realized that both propionic acid and amino acids can serve as carbon precursors for gluconeogenesis. This finding was of particular interest to ruminant nutrition researcher working with animals on typical forage plus some concentrate diets. Cattle and other ruminants absorb relatively small amounts of glucose from the small intestine (Huntington and Reynolds 1986). It was previously hypothesized that propionic acid could supply all the carbon intermediates for obligatory gluconeogenesis (Leng et al. 1967). At the same time others showed that amino acid carbon may also be a precursor for gluconeogenesis in ruminants (Annison and Lindsay 1962). Since lactating ruminants (especially high producing dairy cows) have to synthesize glucose, amino acids used in gluconeogenesis, which are often not in excess, supply may become limiting for milk production. Thus, determining the contributions of propionic acid and/or amino acids to gluconeogenesis became a major research priority. This was further compounded by

occurrences of ketosis, a metabolic disorder, when highly producing ruminants often were unable to supply adequate glucose and amino acids for optimal milk production. An up to 50% of propionate and about a 30% contribution from amino acids to whole body and individual organ fluxes of glucose were determined by Wolff and Bergman (1972) and Bergman and Heitman (1978) using flux and tissue A-V differences. They found that alanine was the highest contributor to hepatic gluconeogenesis (Wolff and Bergman 1972; Bergeman and Heitman 1978). Alanine is also an end product of mostly deamination in skeletal muscle from BCAA (Felig 1975). Thus, if in ruminants BCAA are not deaminated in muscle, the contribution of alanine from the muscle-liver glucose alanine cycle to hepatic gluconeogenesis would likely be minor. However, Ahmed et al. (1983) noted a net efflux of alanine and dispensable amino acids from steer hind limbs. Estimation of glucose production from amino acids can be achieved from urea-N excretion where it is assumed that urea was not recycled and all urea was quantitatively excreted in the urine (Bergen and Wu 2009). This experimental approach, however, is much less feasible in ruminants because of urea recycling (Packett and Groves 1965; Reynolds and Kristensen 2008; Bergen and Wu 2009). Today we know that urea can also be made in enterocytes (Wu 1995) and the assumptions about estimates of amino acid use for glucose synthesis based on data on urinary urea excretion are prone to errors even in non-ruminants (Bergen and Wu 2009). An important issue in assessments of quantitative amino acid metabolism is that our contemporary cattle and sheep have undergone many years of genetic selection and differ for instance with higher lean deposition, lower fat deposition and also size. The argument can be made that certain studies need to be redone to evaluate the metabolism in our contemporary farm species.

Time-course studies of time after feeding on plasma amino acids concentrations have been conducted in many species. In ruminant animals, as noted above, after feeding, plasma AA often decline initially while in non-ruminants plasma AA increase (Bergen 1978). This appears to be related to the relative absorption of energy substrates and amino acids. In ruminants, commencing immediately after feeding the rumen starts to produce SCFA which are readily absorbed and are the main energy substate to the animal. This rapid energy availability could stimulate muscle protein synthesis and hence cause the decline in plasma AA concentrations; however in non-ruminants amino acids and energy substrates are absorbed simultaneously and PAA initially increase as related to protein intake (Bergen 1978).

The process of ammonia and urea recycling between the rumen ecosystem and the host animal has been well appreciated for years (Reynolds and Kristensen 2008). This unique symbiotic interaction favors ruminant survival on low protein feedstuffs when compared to non ruminants (Reynolds and Kristensen 2008). Batista et al. (2016) conducted a meta-analysis of urea kinetics and microbial N assimilation of recycled urea by ruminants as related to feed intake and dietary protein concentrations. The beta analysis data base originated from 25 experiments (reported between 2001 and 2016) with 107 treatment means (Batista et al. 2016). Major meta analyses conclusions were that rumen ammonia concentrations increased and the fraction of recycled urea that is assimilated into microbial protein decreases with increasing dietary protein intake from 53% (low N intake) to 21% respectively (Batista et al. 2016). These results are also in concert with the findings of Satter and Slyter (1974) who found that rumen ammonia concentration in the rumen in excess of what a 12–13% protein diet usually can provide will not support increased assimilation of ammonia nitrogen into microbial protein. Since energy availability and rumen outlow rate will set a threshold of microbial proteins synthesis (Bergen 1982), ruminants provided high RDP diets will waste considerable N as urinary –N excretion. Thus with low protein diets ruminants are very efficient in conserving N, while at higher protein intakes (often from high quality forages) the symbiotic system will waste nitrogen.

3.7 Protein Synthesis Concepts in Ruminants

The translational process in eukaryotic cells is highly conserved evolutionarily across organisms; thus the roles of tRNA, ribosomes,

amino acid activation, mRNA and genetic code, eukaryotic initiation factors, eukaryotic elongation factors and the final release factors are believed to be the same across eukaryotic cells. Thus, it generally assumed that the protein synthesis process in ruminants is similar to all other animals. In the past, Beecher (1974) developed some in situ and in vitro methods to study protein synthesis in rodent and then apply these methods to ruminant skeletal muscle. These procedures were based on previous work by Florini (1962) and others who developed functional cell free protein synthesis systems from rodents. There was no indication that the translation process in ruminants differed from other animals (Beecher 1974; Bergen 1974). From a more contemporary perspective, little research has been done for example on the role of mTOR in ruminant muscle protein synthesis/translation, effect of leucine on translation, the role in nutrient sensing and in insulin mediated processes. The role of mTOR in ruminant has been studied in brown adipose cells and ovine trophectoderm cells (Ma et al. 2017; Wang et al. 2015a, b, 2016a, b).

Much of either tissue or whole body protein synthesis has been pursued in cattle with in vivo studies. This is because in beef cattle, the interest is in lean deposition rates and extent during various experimental conditions. Well established procedure utilizing labelled amino acid infusions or labelled amino acid flooding dose procedures (Fern and Garlick 1973; Garlick and Millward 1972; McNurlin et al. 1979; Garlick et al. 1989) are not very cost effective in large cattle and have not been tested. Protein deposition in the whole carcass can be done by differential slaughter, dissection and protein (Kjeldahl) analysis procedures. The basic drawback here is estimating the initial organ or total body protein in cattle before protein deposition studies were started (Anderson et al. 1988). The use of amino acid isotope (such as ^{15}N, ^{14}C, ^{13}C, and ^{18}O) infusion methods first outlined by Picou and Taylor-Roberts (1969) are not cost effective in beef cattle (Bergen et al. 1987). An alternate procedure using quantitative collection (via urine) of a post translation modified amino acid that arises from protein degradation, N-Tau-Methyl histidine, emerged as a tool to study quantitative protein degradation in skeletal muscle proteins in beef cattle (Rathmacher and Nissen 1998; McCarthy et al. 1983; Bergen et al. 1987; Bergen 2007, 2008). Actual accumulation of muscle protein during a trial was determined by initial and final slaughter based differences in muscle size and composition from a very uniform pool of beef cattle (same breed, age, bodyweight and background genetics); the value obtained was referred to as net accretion. Protein degradation was estimated from urinary N-T MeHis excretion from the same animals. Since net protein (lean) synthesis equals total protein synthesis minus protein degradation, total carcass protein synthesis could estimated by: protein synthesis = net protein accretion plus protein degradation. It is, however, necessary here to know the total body weight of animals. The above equation can also be expressed on a fractional basis (activity expressed per unit tissue rather than the whole animal) where fractional accretion rate = fractional protein synthesis rate minus fractional protein degradation rate (Waterlow et al. 1978). Nissen and co-workers (Rathmacher and Nissen 1998), McCarthy et al. (1983), and Bergen et al. (1987) utilized these procedures.

Amino acid metabolism in ruminants has since evolved into multiple directions. Such work includes fetal sheep amino acid metabolism, tissue and splanchnic amino acid metabolism during disease states and mammary amino acid metabolism using amino acid infusions or evaluating the expression of genes critical for casein synthesis. Inflammations or infections result in marked changes in amino acid and protein metabolism in sheep (McNeil et al. 2016). Such responses may result in increased amino acid demands by the liver for increased acute-phase proteins synthesis and hepatic gluconeogenesis during major stress states (Bruins et al. 2002a, b; Shaw and Wolfe 1986).

Much of the past work on amino acid metabolism in ruminants has ultimately focused on amino acid needs for muscle growth and milk production. Particularly in dairy cattle, in recent times there has been tremendous focus on mammary amino acid utilization; but as far back as the 1970s, workers in the US, UK and New Zealand were already engaged in studying mammary amino acid utilization (Bickerstaffe et al. 1974;

Clark et al. 1978; Schwab et al. 1976; Vik-Mo et al. 1974).

3.8 Standard Estimates of Amino Acid Requirements in Beef Cattle

When an amino acid requirement has to be established by direct experimental procedures in ruminants, total digesta amino acid flow to the duodenum has to be determined, amino acids will have to be infused into the abomasum/duodenum at incremental amounts and blood samples have to be obtained followed by PAA analyses (Fenderson and Bergen 1975). A much more useful system in beef cattle is to first determine net amino acid requirements from growth/muscle lean accretion data coupled with the amino acid composition data of products, here skeletal muscle (NRC 2016). This can be followed by predicting rumen microbial protein yield and rumen escape protein (RDP; RUP- corrected for indigestible protein) and total amino acid flow to the duodenum. The total digestible/absorbable amino acids can then be compared to the amino acid requirements based on the amino acid needs from step one above. The fundamental underlying assumptions for the production of microbial protein, the rumen degradability of dietary proteins, utilization of energy feedstuffs, amino acid flow to the duodenum, amino acid deposition into muscle (or milk) proteins were modelled by Cornell workers to predict net energy content, gain, absorbable amino acids and RDP and RUP by constructing the Cornell Net Carbohydrate and Protein System (CNCPS; Russel et al. 1992; Sniffen et al. 1992; Fox et al. 1992; O'Connor et al. 1993). The CNCPS model will then estimate net energy, absorbed protein (metabolizable protein) and amino acid supply and predict amino acid requirements for a given bovine fed a given diet. Cornell workers then used data from 25 feeding trials (Holstein steers) to conduct validation experiments (Ainslie et al. 1993). The CNCPS system predicted metabolizable protein (MP) and EAA allowable gains with solid statistical confidence (Ainsle et al. 1993). In more recent version of the Cornell system software, an adjustment for microbial protein flow or efficiency of microbial protein synthesis (according to trade journals) has improved the system as previously suggested by Bergen (1982). An approach similar to the Cornell work has been promulgated by the NRC Committee on Nutrients Requirements for Beef Cattle (2016) to also developed models and software to predict amino acid requirements. The approach here is based on MP requirements, the efficiency to convert MP to net protein, and the amino acid content of tissue (e.g. carcass protein). NRC provides software to calculate amino acid requirements based on MP requirement (NRC 2016). A comparison between estimates of daily essential amino acid requirements in steers from NRC (2016) and Fenderson and Bergen (1975) is shown in Table 3.1. The values of Fenderson and Bergen (1975) are generally lower than NRC (2016), but

Table 3.1 Essential amino acid requirements of steers estimated by National Research Council (NRC 2016) and from a direct empirical experimental amino acid infusion approach[a]

Amino acid	NRC (2016)[b] g/day	Fenderson and Bergen (1975)[c] g/day
Arginine	21.92	9.0
Histidine	16.61	8.7
Isoleucine	18.60	22.4
Lysine	44.51	31.0
Methionine	13.29	9.9
Phenylalanine	23.25	22.9
Threonine	25.91	20.1
Valine	26.37	22.4
Leucine	47.52	26.7

[a]Adapted from Fenderson and Bergen (1975)
[b]Steer weights 300–500 kg; fed a alfalfa hay, steam flaked corn meal and soybean meal, minerals and micronutrients
[c]Steer weight 250 kg; fed ground oats wheat bran, ground corn, soybean meal, sugarcane, urea, minerals and vitamins

the steers used were lighter. The profiles of amino acid requirements resemble each other as Ile, Lys, Phe, Thr, Val, and Leu are higher (g/day) while His, Met and Arg are in the lower group.

Acknowledgements Research presented in this review from the author's laboratory and fellow faculty in the dairy sciences at Michigan State University was supported by the Michigan Agricultural Experiment Station (now Ag Bio Research).

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Amino Acid Nutrition and Reproductive Performance in Ruminants

4

Kyler R. Gilbreath, Fuller W. Bazer, M. Carey Satterfield, and Guoyao Wu

Abstract

Amino acids (AAs) are essential for the survival, growth and development of ruminant conceptuses. Most of the dietary AAs (including L-arginine, L-lysine, L-methionine and L-glutamine) are extensively catabolized by the ruminal microbes of ruminants to synthesize AAs and microbial proteins (the major source of AAs utilized by cells in ruminant species) in the presence of sufficient carbohydrates (mainly cellulose and hemicellulose), nitrogen, and sulfur. Results of recent studies indicate that the ruminal microbes of adult steers and sheep do not degrade extracellular L-citrulline and have a limited ability to metabolize extracellular L-glutamate due to little or no uptake by the cells. Although traditional research in ruminant protein nutrition has focused on AAs (e.g., lysine and methionine for lactating cows) that are not synthesized by eukaryotic cells, there is growing interest in the nutritional and physiological roles of AAs (e.g., L-arginine, L-citrulline, L-glutamine and L-glutamate) in gestating ruminants (e.g., cattle, sheep and goats) and lactating dairy cows. Results of recent studies show that intravenous administration of L-arginine to underfed, overweight or prolific ewes enhances fetal growth, the development of brown fat in fetuses, and the survival of neonatal lambs. Likewise, dietary supplementation with either rumen-protected L-arginine or unprotected L-citrulline to gestating sheep or beef cattle improved embryonic survival. Because dietary L-citrulline and L-glutamate are not degraded by ruminal microbes, addition of these two amino acids may be a new useful, cost-effective method for improving the reproductive efficiency of ruminants.

Keywords

Amino acids · Nutrition · Reproduction · Lactation · Ruminants

Abbreviations

AA	amino acid
DAPA	2,6-diaminopimelic acid
DDG	dried distillers grain
DIP	digested intake protein
EAA	nutritionally essential amino acid
GnRH	gonadotropin-releasing hormone
IUGR	intrauterine growth restriction
NEAA	nutritionally nonessential amino acid
NO	nitric oxide
NPN	non-protein nitrogen
RPAA	rumen-protected amino acid
RUAA	rumen-unprotected amino acid

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1285, https://doi.org/10.1007/978-3-030-54462-1_4

RUP ruminally undegraded protein
UIP undegraded intake protein

4.1 Introduction

Human consumption of animal protein is projected to increase with the expected increase in the world population. Currently, for the global average, animal-derived protein is about 30% of total protein consumption by humans and this percentage will be greater in the near future due to the growing middle class of people in many developing countries (Wu et al. 2020). To supply adequate amounts of animal protein, increases in the number of animals and productivity across animal industries will be required. In terms of the cattle, sheep and goat industries, the enhancement of reproductive efficiency is an attractive approach to increasing both beef cow inventory and the beef industry's overall productivity (Bazer et al. 2020; Dahlen et al. 2014).

Among the most important determinants of the financial viability and sustainability of ruminant production systems is reproductive performance. Quantification of infertility is challenging when considering the numerous methods of management and potential environmental factors that can play a part in altering reproductive performance in beef cattle. Bellows et al. (2002) estimated that $441 to $502 million in losses of yearly income occurs in the beef cattle industry due to reproductive diseases and conditions favoring reductions in fertility. Three-fourths of this cost was associated with the infertility of females and their inability to produce a healthy calf that survived beyond 24 h of life (Looney et al. 2006). This estimated loss was approximately 3.6% of the total value of production by the beef industry in the same year. Reproductive failure creates losses from a decline in production stemming from delayed conception and increases in financial inputs in the form of treatment and cost of preventative measures (Bellows et al. 2002; Thatcher et al. 2001). Due to reproduction being the primary factor influencing profitability, new strategies that enhance fertility and promote the efficiency of reproduction within the beef cattle industry should be studied and implemented. This is also a strategy to enhance milk production and fertility of dairy cows, as well as other ruminant species (e.g., sheep and goats).

There has been considerable progress in our understanding of amino acid (AA) nutrition and metabolism in ruminants over the past century, from the concept of crude protein to metabolizable protein (Schwab and Broderick 2017) and functional AAs (Wu 2018). The major objective of this article is to highlight results of recent studies of reproductive efficiency in beef cattle and sheep, AA metabolism and nutrition in ruminants, and the use of arginine or citrulline to improve embryonic/fetal survival in ruminants.

4.2 Reproductive Efficiency in Beef Cattle

In terms of increasing the net calf crop, the primary factor is the ability of the heifer or cow to become pregnant and maintain their pregnancy to term (Bazer et al. 2015; Santos et al. 2004). Prior research has shown that embryonic death is the primary determinant of reproductive failure and ultimately the major source of inefficiency in livestock reproduction. Approximately 30% of all potential neonates in beef cattle are lost between the events of the initial cleavage stages of embryos, and parturition and embryonic death usually begin by day 16 of gestation (Maurer and Chenault 1983). This was further confirmed by findings of higher embryonic loss (~30%) by day 7 of pregnancy in sub-fertile cows (e.g., repeat breeders) (Maurer and Chenault 1983; Gustafsson 1985), whereas in cows with improved fertility, embryonic losses (~40%) were observed to occur more gradually between days 8 and 17 of gestation (Diskin and Sreenan 1980; Roche et al. 1981). Of interest, pregnancy loss can be as high as 80% in some heifers due to genetic and environmental factors (Moraes et al. 2018; Diskin et al. 2011). Bellows et al. (1979) recorded the various factors accounting for reductions in net calf crops over 14 years in a naturally bred group

of beef cattle. A calf crop of 71% was reported as a result of the failure of females to conceive or early embryonic death (17.4%), fetal deaths during gestation (2.9%), perinatal calf deaths (6.4%), and calves dying between birth and weaning (2.9%). Thus, the greatest limitation to reproductive efficiency across mammalian livestock species (including ruminants) is the high rate of embryonic mortality (Bazer et al. 2015).

Several different factors can influence the prevalence and specific timing of embryonic death, such as parity, nutritional status, semen quality of the bull, environmental factors (e.g., temperatures), hormonal influences, genetics, and disease (Amundson et al. 2006; Thatcher et al. 1994). An experiment exposing beef cows to heat stress during early pregnancy showed potential negative impacts, such as decreased pregnancy rates and lower conceptus weights (Biggers et al. 1987; Putney et al. 1989). Heat stress results in an increase in uterine temperature, which is believed to alter the metabolic rate of the developing conceptus and create a sub-optimal environment that limits embryonic, placental and fetal growth (Biggers et al. 1987). By changing the metabolic rate, the uptake of nutrients by the conceptus is also affected and was thought to be the primary factor behind decreased pregnancy rates and decreased conceptus weights (Biggers et al. 1987). Another potential reason for higher embryonic loss is the increase in temperature causing chromosomal abnormalities as the oocyte is released from its first meiotic arrest and resumes meiosis (Thatcher et al. 1994).

Dystocia poses a significant threat to cow calf producers, due to the potential loss of both cow and calf. This not only affects the likelihood of a successful birth, but the future production of both cow and calf (Bellows et al. 1988). The occurrence of dystocia-related fatalities was estimated to be 45.9% of all preweaning deaths (Patterson et al. 1987). Laster and Gregory (1973) studied the factors related to early postnatal mortality over a 5-year period involving 5064 cows for which all parturitions were evaluated and scored based on calving difficulty. Calf mortality for cows experiencing dystocia was recorded at 20.4%, while only 5.0% of neonatal mortality occurred when parturition occurred without assistance (Laster and Gregory 1973).

Nutritional strategies to enhance embryonic survival has drawn increased attention due to the livestock producers' ability to control nutritional inputs (Dunn and Moss 1992). Oocyte development, ovulation, fertilization, embryonic survival, and establishment of pregnancy are all directly influenced by nutrition through the supply of specific nutrients, whereas nutrition also indirectly affects fertility through circulating concentrations of hormones and metabolites in blood (Robinson et al. 2006). Diets that are lacking in either energy or protein put animals in a negative energy balance, and the animal's energy demands for maintenance, reproduction, and lactation exceed the energy intake. For this reason, body weight and body condition scoring have been used by producers and researchers as indicators of energy status in cattle and potential rebreeding performance post-calving (Randel 1990). Body condition or body nutrient stores has a strong influence on pregnancy rates in beef cows and heifers. One potential mechanism whereby nutritional deficiencies can affect embryonic survival is an impairment of embryonic growth and development. Ewes that were fed 25% of their maintenance energy requirements showed no differences in embryonic survival, but ewes that were nutrient-restricted on days 11–21 post-mating had fetuses that were less well developed and had shorter crown-rump lengths (Parr et al. 1982).

Quality of nutrition also alters normal hormonal cycles that accompany stages of the cow's normal estrous cycles. Increased sensitivity to the negative feedback effects of estradiol was observed in cows that were nutritionally deficient (Keisler and Lucy 1996; Wettemann et al. 2003) and this resulted in the animal being anestrus for up to 100 days or longer (Williams 1990). This hypersensitivity to estradiol prolongs the postpartum anestrus period due to a decrease in the release of gonadotropin releasing hormone (GnRH), and this delay in return to cyclicity reduces a cow's reproductive efficiency. Poor nutrition (e.g., 25% of the maintenance energy requirement) was also shown to be correlated

with higher levels of circulating progesterone in sheep during early gestation (Parr et al. 1982). This relationship was reported to occur whether the progesterone source was endogenous (ovary) or administered exogenously to ovariectomized ewes (Parr et al. 1982). Underfed ewes (50% of the maintenance energy requirement) had concentrations of progesterone in blood that increased more rapidly between days 2 and 10 after mating, when compared to ewes consuming diets with adequate energy (Rhind et al. 1989).

4.3 Overall AA Nutrition in Ruminants

Continuous fermentation within the rumen environment is possible due to its wide array of bacteria, archaea, protozoa, and fungi (Firkins et al. 2007). The utilization of both dietary protein and non-protein nitrogen (NPN) by ruminants involves the rumen, abomasum, and small intestine (Bergen 2020; Firkins et al. 2007). For the ruminant to achieve maximum feed intake, nutrient digestion, and ruminal health, sufficient amounts of rumen-degraded crude protein (RDP) must be provided (Wu 2018). As research on protein nutrition has continued, more emphasis has been put on the AA profile in ruminal fluid available to be absorbed after microbial and enzymatic digestion of microbial proteins. The breakdown of dietary protein and the resulting synthesis of microbial proteins in the rumen results in a lack of correlation between the AA profile of the diet and the AA profile in the blood of ruminants (Bergen 1979).

The microbiome within the rumen allows ruminants to receive higher-quality microbial protein than the protein provided in lower quality feedstuffs. Research by Sok et al. (2017) attempted to show the different AA profiles that existed between ruminal fluid-associated bacteria and particle-based bacteria within the rumen and how this could affect the AA composition of the microbial protein flow. Optimizing protein nutrition in ruminants has long been pursued by researchers as a strategy to increase overall production without sacrificing profitability. The pursuit of optimizing the amount of protein provided to ruminant species requires reliable estimates of quantities pertaining to: (1) the AA profile of protein flow, (2) dietary AAs entering and being absorbed by the small intestine, and (3) AA requirements for maintenance and production (Merchen and Titgemeyer 1992).

The 20 proteinogenic AAs in animals were traditionally classified as nutritionally essential (EAA) or non-essential AA (NEAA) (Hou et al. 2015). However, this classification has now been modified to indicate that arginine, glutamine, glycine, and proline are conditionally EAAs because they are not synthesized in the body in sufficient amounts to meet requirements for optimum production under certain physiological conditions such as pregnancy and lactation (Wu 2013). Early studies involving isotopic tracers in dairy cattle and sheep led to the conclusion that the classification of EAAs in ruminants was similar to that for EAAs in non-ruminants (Black et al. 1957; Downes 1961). This view should be reconsidered in light of recent advances in AA nutrition and metabolism. There is little evidence for a sufficient synthesis of cysteine from methionine or of tyrosine from phenylalanine in ruminants. It is noteworthy that glycine is the most abundant free AA in the plasma of adult cattle and sheep (Table 4.1). This is consistent with a high rate of the synthesis of creatine (an abundant metabolite participating in energy metabolism and antioxidative reactions in skeletal muscle, heart, and brain) from glycine, arginine and methionine in ruminants, whose plant-based diets lack creatine (Hou et al. 2019; Li and Wu 2020). Likewise, cysteine is used for the production of taurine (an abundant antioxidant in skeletal muscle, heart and brain but is absent from plant-source feedstuffs) in ruminants.

AAs serve as precursors for protein synthesis and other nitrogen containing metabolites involved in gluconeogenesis, and as metabolic energy when they are oxidized to CO_2 (Wallace and Chesson 1995). Ruminal bacteria can synthesize all EAAs, assuming that the supplies of ammonia, carbohydrates, and sulfur are readily available. Some of the bacteria are engulfed by

Table 4.1 Composition of amino acids (AAs) in the feeds, ruminal bacterial protein, plasma, and skeletal muscle proteins of adult sheep and cattle[a]

	AAs in feeds (g/100 g AAs)		AAs in ruminal bacterial proteins[b] (g/100 g AAs)		Free AAs in plasma (μmol/L)		AAs in skeletal muscle proteins[c] (g/100 AAs)	
AAs	Sheep[d]	Cattle[e]	Sheep	Cattle	Sheep[d]	Cattle[e]	Sheep	Cattle
Ala	6.53	8.02	6.74 ± 0.24	6.72 ± 0.29	182	181	5.52 ± 0.19	5.55
Arg	5.91	5.18	5.03 ± 0.21	5.01 ± 0.25	190	121	6.58 ± 0.31	6.57
Asn	5.13	4.71	5.34 ± 0.26	5.36 ± 0.31	33	31	4.16 ± 0.22	4.18
Asp	5.83	6.58	6.74 ± 0.33	6.75 ± 0.36	11	5.4	5.15 ± 0.28	5.16
Cys[f]	1.87	1.61	1.48 ± 0.06	1.49 ± 0.07	114	132	1.38 ± 0.06	1.35
Gln	9.02	5.95	5.11 ± 0.30	5.13 ± 0.34	372	286	5.66 ± 0.33	5.64
Glu	7.85	10.8	8.02 ± 0.47	7.99 ± 0.55	61	52	9.35 ± 0.47	9.32
Gly	4.90	4.93	5.06 ± 0.19	5.07 ± 0.23	511	347	4.17 ± 0.15	4.18
His	2.18	2.28	2.05 ± 0.06	2.07 ± 0.07	62	67	3.94 ± 0.13	3.95
Ile	4.20	4.47	5.53 ± 0.22	5.51 ± 0.26	62	100	5.13 ± 0.26	5.15
Leu	8.32	8.67	7.67 ± 0.31	7.66 ± 0.36	107	148	8.34 ± 0.34	8.33
Lys	4.98	4.66	7.70 ± 0.34	7.70 ± 0.41	94	104	9.02 ± 0.37	9.03
Met	1.63	1.79	2.42 ± 0.09	2.40 ± 0.12	24	27	3.18 ± 0.12	3.17
Phe	4.90	5.30	5.13 ± 0.17	5.16 ± 0.19	36	51	4.19 ± 0.10	4.18
Pro	7.93	4.90	3.67 ± 0.20	3.66 ± 0.24	156	184	4.06 ± 0.25	4.08
Ser	5.05	4.56	4.65 ± 0.23	4.62 ± 0.27	75	67	4.38 ± 0.18	4.41
Thr	3.81	4.78	5.52 ± 0.28	5.57 ± 0.34	60	62	4.61 ± 0.20	4.59
Trp	1.24	1.53	1.39 ± 0.08	1.38 ± 0.09	39	49	1.26 ± 0.04	1.25
Tyr	3.73	3.42	4.65 ± 0.15	4.63 ± 0.18	61	70	3.76 ± 0.11	3.75
Val	4.98	5.91	6.08 ± 0.27	6.11 ± 0.32	128	224	5.96 ± 0.21	5.94
Hyp	ND	ND	ND	ND	41	45	0.20 ± 0.01	0.21

Hyp, 4-hydroxyproline; *ND*, not detected

[a]Adult Sulfolk female sheep (60–65 kg) were fed a soybean hulls-, wheat middlings-, and corn-based diet (Gilbreath et al. 2020b), whereas adult Angus × Hereford steers (mean body weight of 538 kg) fitted with a ruminal cannula consumed daily 14.02 kg (dry matter) of Bermudagrass hay and 0.506 kg (dry matter) of dried-distillers' grains with solubles. These diets contained no taurine. Fresh rumen-fluid was collected from sheep and steers that had been deprived of food for 16 h. The amounts of AAs in the feeds, ruminal bacterial proteins, and skeletal muscle (longissimus lumborum muscle; loin muscle) proteins were calculated on the basis of their intact molecular weights. Values are either means ± SEM, n = 10 for ruminal bacterial proteins, ruminal bacterial 2,6-diaminopimelic acid (DAPA), and sheep skeletal muscle proteins, or means taken from our published studies. The content of DAPA in the ruminal bacteria of sheep and cattle was 0.865 ± 0.033 and 0.872 ± 0.041 g/100 g of bacterial protein, respectively

[b]Protein-bound AAs and DAPA were analyzed by high-performance liquid chromatography after acid and alkaline hydrolyses as previously described (Wu and Meininger 2008; Hou et al. 2019). Calculations were based on the molecular weights of intact AA.

[c]Protein-bound AAs were analyzed by high-performance liquid chromatography after acid and alkaline hydrolyses as previously described (Dai et al. 2010; Hou et al. 2019). Values for cattle were taken from Wu et al. (2016). Calculations were based on the molecular weights of intact AA.

[d]Taken from Gilbreath et al. (2020b)

[e]Taken from Gilbreath et al. (2020a)

[f]Total cysteine (cysteine plus ½ cystine)

the ruminal protozoa to generate protozoal proteins, with bacterial and protozoal proteins entering the abomasum and the small intestine of ruminants for digestion (Wu 2018). A marker for bacterial protein is 2,6-diaminopimelic acid (DAPA) present in the peptide component of peptidoglycans in the cell wall of Gram-negative bacteria (Kung and Rode 1996). Of note, the content of DAPA in ruminal bacteria (g/100 g of bacterial protein) is similar between sheep and cattle (Table 4.1). Although the synthesis of these AAs in the rumen is a continuous biochemical process, it may not be provide adequate amounts of EAAs and NEAAs to meet the

requirement of a high-producing animal (Lapierre et al. 2006; Satterfield et al. 2012, 2013). Thus, there is growing interest in the nutritional and physiological roles of NEAAs in gestating ruminants. For example, intravenous administration of L-glutamate (7 mg/kg body weight) twice weekly (Monday and Friday) between mid-June and late September in northern Mexico enhanced the onset of puberty in female goats without affecting body condition scores or concentrations of insulin, urea and glucose in plasma (Torres-Moreno et al. 2009). Furthermore, dietary supplementation with 5, 10 and 15% monosodium glutamate by-product (providing 0.24%, 0.48% and 0.72% supplemental glutamate in the diet) increased the milk production profit by 15%, 22% and 33%, respectively (Padunglerk et al. 2017). Similarly, dietary supplementation with citrulline plus glutamine to cows during early lactation reduced the coefficient of variation in milk yield change over a 7-day period by 50% (Keith et al. 2018).

In non-ruminants, dietary intake reflects the supply of nutrients. This makes correcting dietary deficiencies relatively easy to manage. Deficiencies of AAs in these animals can be corrected by simply adding the deficient AAs directly into the diet (Wu 2018). However, AA deficiencies in ruminants must be overcome using other strategies to fulfill AA requirements because of difficulties in the control of metabolic pathways in ruminal microbes. Simply adding any AA into the diet is not an efficient option to increase AA flow within the duodenum in cattle (Lapierre et al. 2006). There is a limited amount of data regarding the AA content in ruminal fluid, endogenous protein sources, and undegraded intake protein (UIP) fractions of consumed feedstuffs (Clark et al. 1992). This protein is hydrolyzed by proteases in the small intestine to free AAs, dipeptides, and tripeptides. Researchers have attempted to predict this supply of protein with estimates derived from experiments with ruminants cannulated either at the level of the abomasum or small intestine (Hvelplund 1986). Results from several experiments have been called into question because the location of the cannula in relation to the digestive tract can cause wide variations in the measurements of AA flow. The interpretation of the data becomes difficult because the non-ammonia nitrogen supply that reaches the small intestine consists of free AAs, peptides, UIP, microbial protein, and endogenous protein. This creates more difficulty in making inferences on any one of the individual sources of the microbial and endogenous proteins.

An assumption existed that the AA composition of the RUP was identical to that of the original feedstuff. However, this assumption is not valid for plant-based diets (Bergen 1979). Further, the AA profile of the undegradable fraction of protein leaving the rumen is different from the dietary AA profile following ruminal fermentation (Schwab and Broderick 2017). However, others have reported that the profiles of some AAs in both the RUP and original dietary source are similar (Ganev et al. 1979). There have been attempts to use concentrations of individual AAs in a wide variety of feeds (Hvelplund 1986) and silages (Von Keyserlinkgk 1998) to identify relationships between the flow of individual AAs from feed to the duodenum, but no strong relationship has been established. We noted that the profiles of most AAs in the diets of ruminants (e.g., adult sheep and cattle) differ substantially from those in ruminal microbes (Table 4.1) due to microbial protein synthesis from ammonia, as well as extracellular AAs and small peptides (Wu 2018). However, the composition of alanine, asparagine, glutamate and glycine in the diet of adult sheep fed a soybean hulls-, wheat middlings-, and corn-based diet is similar to that in their ruminal microbes, whereas the composition of arginine, aspartate, glycine, phenylalanine, serine and valine in the diet of adult steers fed a Bermudagrass hay-based diet is similar to that in their ruminal microbes (Table 4.1). Because the sheep and steers were fed different diets but the composition of AAs in their ruminal bacterial proteins was similar (Table 4.1), we conclude that diets that provide adequate nutrients (including protein, fiber, and minerals) have little effect on the composition of AAs in ruminal microbial proteins.

There is limited data on the contributions of the endogenous protein supply (the protein

secreted into the lumen of the gastrointestinal tract plus the protein of epithelial cells sloughed into the lumen) to total protein flow in the small intestine. Likewise, little is known about the factors that affect the overall impact of those proteins. Most attention is directed towards the other two sources of protein, although estimates of total protein flow have varied from 16% (Lammers-Weinhoven et al. 1998) to 56% (Hannah et al. 1991). This endogenous source of protein ultimately provides AAs to the body although the protein is originally derived from multiple sources, such as glycoproteins from mucus, the epithelial cells that are shed, bile, and the digestive enzymes released into the abomasum and duodenum (Larsen et al. 2010).

Of the sources of protein leaving the rumen, microbial protein is estimated to be the largest contributor. Results from experiments estimate that 60% to 90% of the total AAs that enter the small intestine of the ruminant are from microbial proteins (Butter and Folds 1985; Nocek and Russel 1988). It is apparent that microbial protein has a large role in determining the quality of protein entering the small intestine. Because rumen fermentation leads to the production of microbial proteins, ruminants are not thought to have dietary requirements for EAAs for maintenance and low growth rates. Many of the biosynthetic pathways have been identified using in vitro experiments. It is difficult to ascertain the activity of these bacteria and their enzymes in vivo. Although ruminal bacteria can synthesize all EAAs, as noted previously, the supply of microbial EAAs may not be adequate to meet the EAA requirements of a high-producing animals (Lapierre et al. 2006). This may also be true for NEAAs (Wu 2018). Note that AA profiles in ruminal microbial proteins differ from those in the plasma of ruminants (Table 4.1), because AAs are metabolized by the small intestine at different rates during the first pass into the portal circulation (Wu 2018).

Free AAs are intermediate products in ruminal fluid as dietary protein is broken down upon entry into the rumen. These AAs can have multiple fates, including degradation to ammonia by microbes, assimilation into rumen microbes, absorption from the rumen, and being bound to microbial cells or feed particles (Chalupa 1975). There is also limited research concerning the rates of degradation of AAs within the rumen. The concentrations of free AAs in ruminal fluid are very low (e.g., ranging from 1.3 μM for taurine to 44 μM for glutamate in the ruminal fluid of adult steers; Gilbreath et al. 2020a). This is due to a number of factors, such as a limited amount of free AA in the diet, the active degradation of dietary protein-derived AAs by ruminal bacteria (via transaminases, dehydrogenases, and deaminases), the rapid microbial uptake of peptides and free AAs, as well as the constant flow of AA-containing ruminal fluid into other parts of the forestomach. The small amount of taurine present in ruminal fluid is derived from the saliva and blood, because this AA is absent from plants and is not synthesized by microbes (Wu 2013).

Several factors affect the metabolic activity of microbes, including pH, protein structure, and the predominant species of microbes in the rumen (Bach et al. 2005; Scheifinger et al. 1976). Conflicting views exist pertaining to the estimated rates of the degradation of individual AAs undergoing ruminal fermentation. Individual strains of microbes use the supply of free AAs differently and this likely results in different rates of utilization for individual AAs (Scheifinger et al. 1976). Mixtures of AAs are degraded more rapidly than individual AAs, although no explanation or mechanism has been identified to explain this result (Lewis 1955). It is possible that compared with the presence of a single AA, a mixture of AAs helps to enhance the synthesis of AA-degrading enzymes, the removal of AA metabolites via microbial protein synthesis, and overall metabolic function in ruminal microbes. Continued research on this topic reveals that all EAAs are degraded to the same extent (Macgregor et al. 1978), whereas results from other studies indicate that this assumption may not be true. EAAs are degraded more slowly than the NEAA fraction of proteins (Cozzi et al. 1995). It should be noted that rates of the degradation of individual EAAs differ among different feedstuffs, including meat meal, herring meal,

and corn gluten meal (Cozzi et al. 1995). Hydrophilic AAs, such as arginine, histidine, lysine and threonine, may be degraded more rapidly when compared to the rates of the degradation of hydrophobic AAs, such as leucine, isoleucine, methionine, phenylalanine, tryptophan, and valine (Van Soest 1994). Branched-chain AAs (BCAAs) have slower rates of degradation in comparison with other AAs (Varvikko 1986), but provide ruminants with more branched-chain fatty acids than nonruminants (Wu 2018).

In the rumen, only a small proportion of free AAs are incorporated intact into microbial proteins; therefore, the de novo synthesis of AAs by the microbial population is very important. This point is clearly shown in the case of both growing and lactating ruminants fed the diets that provided either deficient AAs or no AA (Virtanen 1966). Although the synthesis of these AAs is a continuous process, it may not be adequate to meet the EAA requirements of high-producing animals, as noted previously (Lapierre et al. 2006), as noted previously. Improving ruminant protein nutrition has always centered around the optimization of the efficient usage of dietary nitrogen to maximize growth and milk production per unit of nitrogen consumed (Wallace and Chesson 1995). This pursuit involves the adequate provision of degradable intake protein (DIP) to meet the microbial population's requirement for nitrogenous substrates and adequate provision of UIP with the correct AA balance that complements the microbial AA profile (Wallace and Chesson 1995). For this reason, supplementing rumen-protected AAs to ruminants is a viable option for meeting AA requirements and promoting optimal growth, reproduction, and lactation (Kung and Rode 1996). For ruminants to efficiently use both sources of protein, rumen-protected protein and rumen-degradable protein must be supplied at their optimal ratios, depending on reproduction, lactation, and growth (NRC 2001). There is evidence that the provision of a rumen-protected AA (RPAA) is necessary to ensure that the ruminant has sufficient nutrition to support the microbial population and allow for enzymatic digestion and absorption of the intended AA profile by the small intestine (Schwab and Broderick 2017). The use of RPAAs allows producers the opportunity to increase protein production in their livestock due to their ability to optimize the balance of AAs absorbed by the small intestine and ultimately decrease the amount of UIP needed in the diet to satisfy nutritional and physiological requirements (Wallace and Chesson 1995). There have been numerous methods to reduce the degradation of protein inside the rumen including: mild heating, chemical treatment, polyphenolic phytochemicals, and encapsulation (Wallace and Chesson 1995; Wu 2013).

Prior studies involving supplemental RPAAs mainly provided lysine and methionine. This is because the direct evidence from abomasal or duodenal infusion studies showed that lysine and methionine were often the most limiting AAs involved with growing ruminants (Merchen and Titgemeyer 1992) and lactating dairy cows (Schwab et al. 1976). A limiting AA is defined as an AA that is in the shortest supply from the diet relative to its requirement for the maintenance and growth of the animal (Wu 2018). In terms of nitrogen retention, methionine and lysine are also the first and second limiting AA within microbial proteins in growing sheep (Nimrick et al. 1970; Storm and Ørskov 1984) and growing cattle (Richardson and Hatfield 1978). There are a few reports claiming that arginine and histidine may be a factor limiting weight gain, depending upon the growth stage and the diet of the animal. Veira et al. (1988) provided theoretical calculations and recorded changes in concentrations of AAs in plasma when a combination of fishmeal and silage were fed to growing steers and suggested that both arginine and histidine were limiting in growing steers fed grass silage. However, changes in the circulating levels of AAs in animals should not be used as the sole criterion for assessing dietary requirements for AAs (Wu 2018). It should be borne in mind that the profiles of AAs in plasma differ from those in the skeletal muscle of ruminants (Table 4.1) because they differ in their metabolic fates and rates of utilization in the body (Wu 2013).

Studies involving beef cattle found an increase in their average daily gains when diets were

supplemented with RPAAs. Veira et al. (1991) observed that growing steers fed grass silage gained weight when supplemented with small quantities of RPAAs that were thought to be limiting (lysine and methionine). This weight gain was thought to result from the RPAA sufficiently meeting the AA requirements for both maintenance and growth (Veira et al. 1991). Mowat and Deelstra (1972) supplemented encapsulated methionine to lambs consuming a basal corn-alfalfa diet and found that methionine had no effect on weight gains or feed efficiency when the animals consumed the basal diet supplemented with soybean meal. However, the authors did observe an increase in gains (11%) and feed efficiency (9%) when the basal diet was supplemented with corn-urea or corn-blood meal. A metabolism trial was then conducted, and the supplemented encapsulated methionine increased protein and dry matter digestibilities, as well as the retention of dietary nitrogen in the body (Mowat and Deelstra 1972). Davenport et al. (1995) supplemented growing lambs with rumen-protected arginine or ornithine due to their actions to stimulate the secretion of somatotropin and determined its effect on growth. The supplementation of arginine and ornithine increased circulating concentrations of somatotropin and insulin-like growth factor, but failed to improve the growth performance of the lambs (Davenport et al. 1995). Although results of experiments to determine growth responses of beef cattle and other ruminants are inconsistent (Kung and Rode 1996), the various findings may be explained by the fact that AAs have metabolic roles other than for the synthesis of proteins (Wu 2013) and a possibility that some AAs are co-limiting for protein synthesis (Merchen and Titgemeyer 1992).

Researchers studying the effects of supplementation of rumen-protected methionine to dairy cattle observed mixed results, but there were beneficial effects, such as increases in milk protein synthesis (Pisulewski et al. 1996; Armentano et al. 1997; Dinn et al. 1998), milk yield and milk protein synthesis (Illg et al. 1987), as well as the yield of fat-corrected milk and milk fat (Overton et al. 1996). Other investigators supplemented diets with rumen-protected methionine and found that this nutritional treatment had no effects on milk production (Papas et al. 1984; Overton et al. 1998). Izumi et al. (2000) reported that supplementing rumen-protected methionine to the diet over a 22-week period resulted in a significant, but temporary, increase in milk yield. This temporary effect was significant only through the peak to middle periods of lactation, but over the complete lactation period there was no significant effect on milk yield, milk fat, or milk protein synthesis (Izumi et al. 2000). It is possible that there is more than one co-limiting AA for lactating ruminants.

Supplementation of diets with certain RPAAs has overcome the decrease in milk protein synthesis in dairy cattle consuming rations with higher fat contents (Canale et al. 1990). In a study of the effects of RPAAs on cow and calf production, primiparous beef cows supplemented with increasing levels of rumen-protected lysine and methionine had increased milk production (Hess et al. 1998). This increase in milk yield was also paired with decreasing body weight gain after parturition (Hess et al. 1998). Rode et al. (1993) supplemented the diet with RPAAs as a replacement for 0.5 kg of soy/blood meal and found that cows consumed less protein and increased their consumption of forage, when compared to their non-supplemented counterparts. Thus, appropriate RPAAs hold promise in improving the productivity of ruminants.

4.4 Catabolism of AAs in the Rumen of Ruminants

In ruminants, dietary protein is hydrolyzed by bacterial proteases and peptidases into small peptides and AAs in the rumen, whereas free AAs are further degraded to ammonia and their carbon skeletons by a number of bacterial enzymes, including deaminases, transaminases, hydrolases, and decarboxylases (Wu 2013). In the presence of α-ketoacids (e.g., pyruvate, oxaloacetate, and α-ketoglutarate which are products of carbohydrate metabolism) and sulfur, ammonia is utilized by ruminal bacteria for the

synthesis of new AAs and proteins (Wu 2018). The rates of AA catabolism are high in the rumen, such that all AAs studied to date (Ala, Arg, Asn, Asp, Cys, Gln, Gly, His, Ile, Leu, Lys, Met, ornithine, Phe, Pro, Ser, Thr, Tyr, and Val) do not escape the rumen. Thus, the microbial population within the rumen has long been considered to have the capability of extensively degrading dietary AAs (Chalupa 1976; Lewis and Emery 1962; Scheifinger et al. 1976).

We recently discovered that the ruminal mixed microbes of adult cattle (Gilbreath et al. 2019, 2020a) and sheep (Gilbreath et al. 2020b) do not degrade extracellular citrulline and have a limited ability to metabolize extracellular glutamate. Specifically, glutamine and arginine are extensively degraded by ruminal microbes from cattle (Fig. 4.1) and sheep (Fig. 4.2) in a time- and concentration-dependent manner. The major products of ruminal glutamine catabolism are glutamate, ammonia, and alanine, whereas the major products of ruminal arginine catabolism are ornithine, proline, and ammonia. At the end of a 2-h incubation period, no urea is present in the incubation medium containing arginine, indicating that ruminal microorganisms are highly active. In contrast, there was no detectable loss of extracellular citrulline or glutamate from the ruminal fluid during a 2-h period of incubation (Figs. 4.1 and 4.2). Consistently, the rates of formation of $^{14}CO_2$, ^{14}C-glutamine, ^{14}C-aspartate, ^{14}C-alanine, ^{14}C-ornithine, ^{14}C-proline, and ^{14}C-protein from extracellular ^{14}C-glutamate (5 mM) are negligible and there is no detectable production of ^{14}C-citrulline or ^{14}C-arginine from extracellular ^{14}C-glutamate (5 mM), compared with the formation of ^{14}C-labeled products from extracellular ^{14}C-glutamine or ^{14}C-arginine (Gilbreath et al. 2019, 2020b). Interestingly, there is little uptake of glutamate and no detectable uptake of citrulline by the ruminal microbes (Gilbreath et al. 2019, 2020b). In support of this finding, Stalon and Merceniner (1984) found that few bacteria can utilize extracellular citrulline as a nitrogen source for growth. For comparison, red blood cells of rats and humans, as well as periportal hepatocytes of mammals, do not take up extracellular glutamate (Watford 2002), and the mammalian liver does not take up extracellular citrulline (Wu and Morris 1998). Thus,

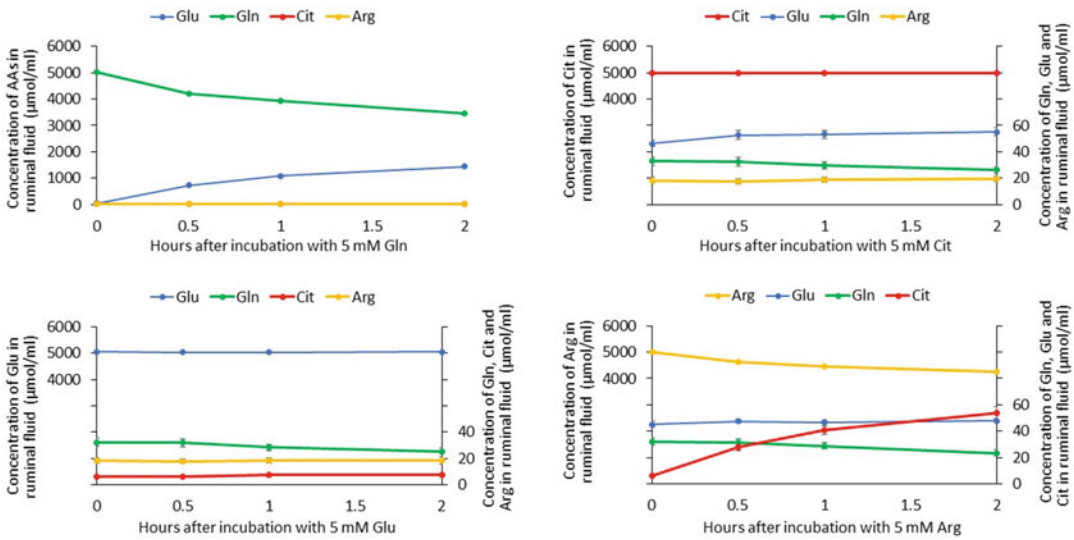

Fig. 4.1 Concentrations of amino acids in the ruminal fluid (containing microbes) of adult steers that was incubated for 0, 0.5, 1 or 2 h in the presence of 5 mM L-glutamine, 5 mM L-glutamate, 5 mM L-citrulline, or 5 mM L-arginine. Values are means ± SEM, n = 6. Adapted from Gilbreath et al. (2019). Results indicate that ruminal microbes of adult steers do not degrade extracellular L-citrulline and have a limited ability to metabolize extracellular L-glutamate. (AA = amino acid; Arg = arginine; Cit = citrulline; Gln = glutamine; Glu = glutamate)

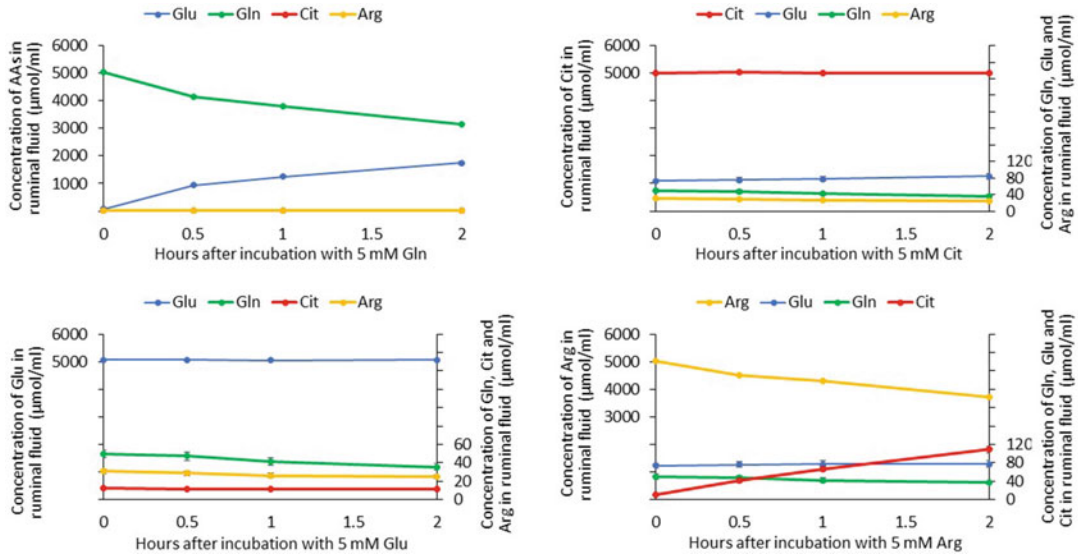

Fig. 4.2 Concentrations of amino acids in the ruminal fluid (containing microbes) of adult sheep that was incubated for 0, 0.5, 1 or 2 h in the presence of 5 mM L-glutamine, 5 mM L-glutamate, 5 mM L-citrulline, or 5 mM L-arginine. Values are means ± SEM, n = 6. Adapted from Gilbreath et al. (2019). Results indicate that ruminal microbes of adult steers do not degrade extracellular L-citrulline and have a limited ability to metabolize extracellular L-glutamate. (AA = amino acid; Arg = arginine; Cit = citrulline; Gln = glutamine; Glu = glutamate)

transporters for glutamate and citrulline are not universally expressed in all eukaryotic and prokaryotic cells. It is possible that ruminal microbes do not take up or catabolize extracellular aspartate (an acidic AA like glutamate), but experimental data are needed to test this hypothesis. Consistent with the in vitro observations, the concentrations of glutamate in the ruminal fluid of adult steers increase in response to oral administration of glutamine because of the hydrolysis of extracellular glutamine into glutamate, but there is no increase in the concentrations of arginine in the ruminal fluid of adult steers after oral administration of citrulline due to the lack of utilization of extracellular citrulline by ruminal microbes (Fig. 4.3). Likewise, the concentrations of citrulline and arginine in the plasma of adult sheep increase by 117% and 23%, respectively, at 4 h after oral administration of citrulline (8 g along with 800 g of the soybean hulls-, wheat middlings-, and corn-based diet), compared with the baseline value (Fig. 4.4). Collectively, these findings refute the traditional view that all unprotected AAs in diets are extensively catabolized by ruminal microbes and are unable to escape the rumen (Owens and Basalan 2016; Tedeschi and Fox 2016). This new concept has far-reaching implications for the nutrition of ruminants and their dietary supplementation with selected AAs. For example, glutamate, a major metabolic fuel for the small intestine and glutathione synthesis (Hou and Wu 2017, 2018), can be added to the diets of ruminants to improve digestive functions (Brake et al. 2014). In addition, dietary supplementation with citrulline plus glutamine (Keith et al. 2018) or rumen-protected arginine (130 g/day, Kirchgessner et al. 1993) enhances milk production by dairy cows, as reported for arginine supplementation to lactating sows (Mateo et al. 2008). Furthermore, citrulline, without encapsulation, can be effectively supplemented to the diets of ruminants to increase concentrations of

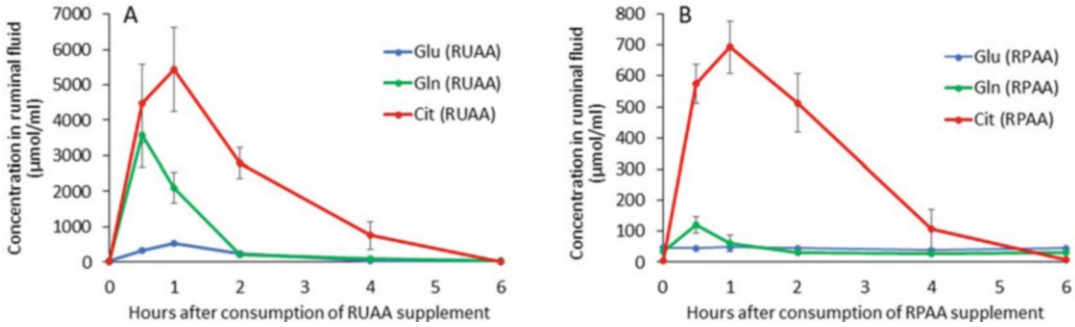

Fig. 4.3 Concentrations of amino acids in the ruminal fluid of adult steers after their consumption of a rumen-protected amino acid (RPAA; encapsulated citrulline + glutamine; Panel A) or a rumen-unprotected amino acid (RUAA; unencapsulated citrulline + glutamine; Panel B) supplement. Analyses of amino acids in the amino acid products mixed with microbe-free deionized and double-distilled water indicated that 24.2% of citrulline and 24.0% glutamine in the RPAA product were not encapsulated by the binder. Thus, concentrations of both citrulline and glutamine increased in the ruminal fluid of steers after consuming the RPAA product, but were much lower than those for steers consuming the RUAA product. Concentrations of citrulline in the ruminal fluid of RUAA and RPAA steers declined rapidly after 1 h, likely because of a rapid flow of the amino acid out of the rumen into the other compartments of the forestomach. Both a rapid flow of glutamine out of the rumen and its rapid catabolism by ruminal microbes contributed to a more rapid decline of glutamine in the ruminal fluid than citrulline. Values are means ± SEM, n = 8. (Adapted from Gilbreath et al. (2020a), Cit = citrulline; Gln = glutamine; Glu = glutamate)

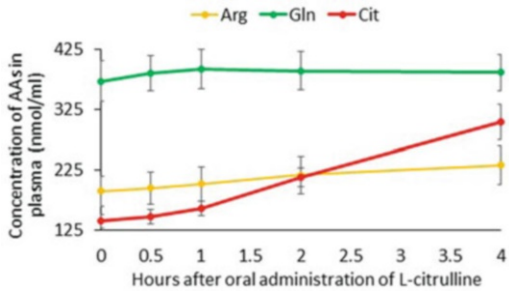

Fig. 4.4 Concentrations of amino acids in the plasma of sheep after consumption of a L-citrulline supplement. The L-citrulline supplement consisted of 8 g L-citrulline and 800 g of a soybean hulls-, wheat middlings-, and corn-based diet. Values are means ± SEM, n = 6. Adapted from Gilbreath et al. (2020b). Oral administration of L-citrulline increased the concentrations of both L-citrulline and L-arginine in the plasma of sheep at the 1-, 2- and 4-h time points when compared with the baseline (time 0 h) values, as analyzed by one-way analysis of variance for repeated measures data. (Arg = arginine; Cit = citrulline; Gln = glutamine)

arginine in plasma (Gilbreath et al. 2020b). Dietary glutamate and citrulline are functional AAs that enhance the growth and productivity (e.g., lactation and embryonic survival) of ruminants.

4.5 Benefits of AA Supplementation to Gestating Ruminants

Arginine catabolism involves multiple pathways and provides a variety of useful products to the body including ornithine, polyamines, proline, glutamate, agmatine (argamine), creatine, and NO (Wu 2013). These products along with arginine itself allow arginine supplementation to improve cardiovascular function, immunity, neurological function, wound healing, fertility in both genders, absorption of nutrients, and insulin sensitivity (Wu et al. 2013). Those benefits also allow the potential use of arginine supplementation to reduce hyperglycemia, dyslipidemia, obesity, high blood pressure, atherosclerosis, infections, embryonic and fetal death, and diarrhea (Wu 2013). NO and polyamines serve vital roles in placental angiogenesis and growth in mammals (Hosomi et al. 1987). Arginine has the potential to be a vital nutrient for both dam and fetus during pregnancy (Wu et al. 2013). Arginine is also an essential part of the urea cycle for ammonia detoxification by activating *N*-

acetylglutamate synthase to generate *N*-acetylglutamate (an allosteric activator of carbamoylphosphate synthase-I) from glutamate and acetyl-CoA (Wu and Morris 1998). Furthermore, arginine increases protein synthesis and inhibits proteolysis in ovine brown adipocytes (Ma et al. 2017), as reported for porcine mammary epithelial cells (Ma et al. 2018). Therefore, arginine is now known to be nutritionally essential for pregnancy, lactation, and rapid postnatal growth in animals (Wu et al. 2013, 2018).

Results of a study in which gilts were supplemented with 1% arginine-HCl between days 30 and 114 of gestation showed a 22% increase in live litter birth weight and a 24% increase in the number of pigs born alive (Mateo et al. 2007). Similar findings have been reported for dietary supplementation with 0.4% or 0.8% arginine to gilts between days 14 and 30 of gestation (Li et al. 2014). This enhancement in embryonic and fetal survival and growth is due, in part, to an increase in placental angiogenesis and growth during early- to mid-gestation. This improves the intrauterine environment for the maternal to fetal-placental exchange of nutrients and gases throughout pregnancy (Wu et al. 2017). Similarly, Zeng et al. (2008) conducted four separate experiments testing the effects of arginine supplementation on embryonic survival in Sprague-Dawley rats. In rats supplemented with arginine throughout their pregnancy, litter size was increased by 3.2 pups per dam (14.5 ± 0.062 vs. 11.3 ± 0.61). An increase in litter size was also observed in rats supplemented with arginine between days 1 and 7 of pregnancy (14.7 ± 0.39 vs. 11.4 ± 0.66). The arginine treatment also increased embryonic survival on day 7 of pregnancy and this was thought to be the primary factor responsible for the increase in litter size.

There has been growing interest in the role of arginine in the nutrition and metabolism of gestating sheep over the past two decades. Because intravenous administration of arginine to pregnant ewes increased the concentrations of arginine and insulin in their plasma, as well as uterine, utero-placental, and fetal uptake of arginine (Thureen et al. 2002), arginine could potentially improve pregnancy outcomes in these animals. A separate study of the effects of arginine supplementation on ovine IUGR fetuses showed that increasing circulating concentrations of arginine increased fetal protein accretion (De Boo et al. 2005). Increasing concentrations of arginine in maternal blood was thought to enhance the vasodilation of blood vessels in the uterus and placenta through NO production, which increased blood flow both to the uterus and within the fetal-placental vasculature (De Boo et al. 2005). A series of studies from our group revealed that intravenous administration of arginine-HCl to underfed, overweight or prolific ewes enhanced fetal growth, fetal brown fat, and neonatal lamb survival (Lassala et al. 2010, 2011; McKnight et al. 2020; Satterfield et al. 2012, 2013). Similar results were reported by McCoard et al. (2013, 2014, 2016) and Reynolds et al. (2019). Additionally, Sales et al. (2016) demonstrated that intravenous administration of arginine-HCl to twin-bearing ewes during late gestation enhanced placental growth and development (van der Linden et al. 2015), reduced mammary gland infections during early lactation (Sciascia et al. 2019), and promoted the postnatal growth of lambs (Sales et al. 2016).

Because arginine is extensively degraded by ruminal bacteria (Chalupa 1976), addition of unprotected arginine to the diets of ruminants cannot augment its concentration in blood. This necessitates the use of rumen-protected arginine for feeding sheep. Saevre et al. (2010) supplemented rumen-protected arginine to ewes to determine if it enhanced reproductive efficiency as reported for monogastric animals supplemented with arginine. The rumen-protected arginine product increased circulating levels of arginine and ovarian blood flow in ewes fed the arginine supplement over a 5-day period. de Chávez et al. (2015) reported that dietary supplementation with rumen-protected arginine [7.8 g Arg (as arginine-HCl)] to sheep (45 kg body weight) between the onset of estrus and day 25 after breeding enhanced embryonic and fetal survival during early pregnancy. Likewise, Zhang et al. (2016) demonstrated that dietary supplementation with rumen-protected arginine

(10 g/day) to underfed ewes (40 kg body weight; 50% of NRC (1985)-recommended nutrient requirements) between days 35 and 110 of gestation enhanced fetal weight by 18%. Similar results were obtained by Sun et al. (2018). Taken together, available results clearly indicate the promise of dietary supplementation with arginine to improve fertility and fetal growth in sheep production systems.

Recently, we determined an effect of dietary supplementation with RPAA (citrulline + glutamine) or RUAA (citrulline + glutamine) on embryonic survival in lactating beef cows that were fed a diet meeting NRC (2000) nutrient requirements (Gilbreath et al. 2018). During the entire experimental period, multiparous Brangus cows grazed green pasture and had free access to drinking water and mineral blocks. At the onset of lactation, cows received dried distillers grain (DDG) only, DDG top-dressed with the RUAA product, or DDG top-dressed with the RPAA product. After 2 months of lactation, all cows were synchronized to estrus and artificially inseminated one time. From day 1 to day 60 after artificial insemination, cows were fed daily either 0.64 kg DDG, 0.56 kg DDG + 0.28 kg RUAA (2% of estimated daily intake of 14 kg dry matter from pasture; 0.07 kg citrulline + 0.07 kg glutamine), or 0.56 kg DDG + 0.28 kg RPAA (2% of estimated daily intake of 14 kg dry matter from pasture; 0.07 kg citrulline + 0.07 kg glutamine). Once on each day of the supplementation period, cows were moved to pens to receive their respective supplement and then returned to their original pasture. Dietary supplementation of RUAA or RPAA enhanced the birth rate of live-born calves from 22% in cows fed DGG alone to 34% and 36%, respectively for the two treatment groups. The beneficial effects of the AA supplement were associated with increases in the concentrations of insulin in serum and of citrulline, arginine, ornithine and proline in plasma, but decreases in the concentrations of ammonia in plasma. Thus, dietary citrulline in either a rumen-protected or unprotected form escaped the rumen, entered the portal circulation, and served as the immediate precursor for synthesis of arginine in the extrahepatic tissues of beef cows. These findings have important implications for improving both lactation and fertility in both beef and dairy cows.

Dietary supplementation with citrulline [a neutral AA and an effective precursor of arginine (Lassala et al. 2009)] is expected to increase the reproductive efficiency of beef cows and their profitability. A successful pregnancy in beef or dairy cows is currently estimated to be worth $750 (Dr. Jason Cleere, Texas A&M AgriLife Extension, personal communication). Based on the cost of citrulline + glutamine ($10/kg) and the daily use of 0.14 kg/day for 60 days, the total expense for feeding one cow would be $84. For an operation with 1000 beef cows, the net income would be $20,250, $55,000, and $89,750, respectively, assuming a value of either $750, $1000, or $1250 per calf (Table 4.2). Dietary supplementation with L-citrulline alone, which is expected to improve the reproductive performance of cattle as effectively as with L-citrulline + L-glutamine, would double the margin of profits. Additional benefits that are not included in the margin of profit calculation include reductions in management and labor costs, improvements in herd health, an increase in cow numbers, and the prospect of improved fertility in the next breeding period. Based on the results of this research, we now know that unencapsulated citrulline is able to bypass the rumen and, therefore, will be more affordable for use by producers. Thus, the price for feed-grade citrulline without encapsulation will be substantially reduced (e.g., $5/kg), similar to that for feed-grade arginine (Wu et al. 2018). Thus, a nutrition-based management system to increase embryonic survival will have an enormous impact on the global beef industry. These findings also have important implications for enhancing both milk production and fertility in lactating dairy cows, because they also have very low pregnancy rates [e.g., 16% in the U.S. in the summer (Stewart et al. 2011)]. Large-scale experiments are warranted to optimize the supplemental doses and estimate economic returns from dietary supplementation of diets for beef and dairy cows with citrulline.

In summary, research in ruminant protein nutrition research over the past decade has

Table 4.2 Economic returns from using the Cit + Gln supplement to lactating beef cows[a]

1000 Beef cows	Live-born calves[b]	Income $	Supplement cost[c], $	Net income gain, $
$750/calf				
Control	222	166,500	0	166,500
Cit + Gln	361	270,750	84,000	186,750
Difference	139	104,250	84,000	20,250
$1000/calf				
Control	222	222,000	0	222,000
Cit + Gln	361	361,000	84,000	277,000
Difference	139	139,000	84,000	55,050
$1250/calf				
Control	222	277,500	0	277,500
Cit + Gln	361	451,250	84,000	367,250
Difference	139	173,750	84,000	89,750

Cit = L-citrulline; Gln = L-glutamine
[a]A total amount of 8.4 kg of Cit + Gln is supplemented to one cow for 60 days
[b]Taken from Gilbreath et al. (2018). These values assume an increase in the number of live-born calves by 14 per 100 beef cows due to the dietary supplementation with citrulline plus glutamine
[c]The cost of the supplemental Cit + Gln is $84 per cow for 60 days at the price of $10/kg

gradually shifted from the concept of crude protein to a focus on functional AAs, especially conditionally essential AAs. Although a majority of dietary AAs are extensively degraded by ruminal microbes and do not escape the rumen, recent studies have shown that the ruminal microbes of adult steers and sheep do not degrade extracellular citrulline and have a limited ability to metabolize extracellular glutamate due to little or no uptake of these two AAs by the microbes. Although traditional research in ruminant protein nutrition has focused on AAs (e.g., lysine and methionine for lactating cows) that are not synthesized by eukaryotic cells, there is growing interest in the nutritional and physiological roles of AAs in gestating and lactating ruminants. Intravenous administration of arginine or dietary supplementation of rumen-protected arginine to gestating ewes improves fetal growth, brown fat development in fetuses, and postnatal survival and growth of offspring. Similarly, dietary supplementation with unprotected citrulline to gestating beef cattle improved embryonic survival. The use of both citrulline and glutamate as feed additives holds great promise in improving the health (including intestinal integrity), immunity, and reproductive efficiency of ruminants.

Acknowledgments This work was supported by the Texas A&M AgriLife Research Beef Program and the Department of Animal Science Mini-Grant Program. We thank Wenliang He for assistance in the preparation of figures, as well as our colleagues, research assistants, and students for help and discussions.

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Amino Acids in the Nutrition and Production of Sheep and Goats

5

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Abstract

In sheep and goats, amino acid nutrition is essential for the maintenance of health and productivity. In this review, we analysed literature, mostly from the past two decades, focusing on assessment of amino acid requirements, especially on the balance of amino acid profiles between ruminal microbial protein and animal production protein (foetal growth, body weight gain, milk and wool). Our aim was to identify amino acids that might limit genetic potential for production. We propose that much attention should be paid to amino acid nutrition of individuals with greater abilities to produce meat, milk or wool, or to nourish large litters. Moreover, research is warranted to identify interactions among amino acids, particularly these amino acids that can send positive and negative signals at the same time.

Keywords

Foetus · Growth · Meat · Wool · Milk · Immunity · Ovine · Caprine

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Abbreviations

BCAA	branched-chain amino acids
GIT	gastrointestinal tract
MP	metabolizable proteins
PDV	portal-drained viscera

5.1 Introduction

Management of amino acids in sheep nutrition and production has two major aims – achieving genetic potential for productivity and maintaining good animal health (Liu and Masters 2000, 2003). In an industrial context, these aims must also be achieved at an acceptable cost so the enterprise is profitable, because protein feeds and amino acid additives are usually expensive, and inefficiencies in the use of such feed components must be avoided.

The sheep industry supplies meat, milk and wool, and the rates of protein retention in these products is a dominant factor in productivity. Therefore, the management of amino acid nutrition for these productive processes aims at increasing, as much as possible, protein synthesis in the mammary gland and in the wool follicle, and protein retention in body weight gain –the difference between protein synthesis and breakdown (Wu 2018). In addition, because pregnancy is fundamental for flock propagation, the survival and growth of the foeto-placental units (the foetus

G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1285, https://doi.org/10.1007/978-3-030-54462-1_5

must be neither too small nor too large), as well as associated growth and functional development of the uterus, are major targets of protein retention and therefore amino acid nutrition for ewe nutrition during gestation.

This review therefore focuses on amino acid nutrition during pregnancy and lactation, and during body weight gain and wool growth, in sheep. The relationships between these functions and the metabolism of the gastrointestinal tract (GIT) is also addressed because the GIT contains the largest immune biomass in the body. Most of the literature cited concerns sheep, but we also refer to some work on goats. To reflect the most recent advances, we primarily analysed literature published since 2000.

5.2 General Considerations in Amino Acid Nutrition

Dietary proteins are digested in the GIT and the resulting amino acids are absorbed into the body and transported to sites of protein synthesis to meet the requirements of the animal. In farm animals, the quality and quantity of dietary protein are usually referred to as 'profiles' (proportions of individual amino acids to the total amino acids or total protein) and as the amounts of essential amino acids entering the small intestine. In ruminants, in contrast to monogastric animals, dietary protein is degraded by rumen microorganisms to ammonia, amino acids and peptides that the microorganisms then use as nitrogen sources to support their own growth (Wu 2018). The host benefits from this process when the microorganisms flow into the small intestine and are digested. The amount of ruminal microbial protein that moves into the small intestine varies widely, with feed intake (as a proportion of body weight) and with the protein degradability of the dietary ingredients. Most dietary protein is degraded by the rumen microorganisms and used by those microbes as a source of nitrogen to synthesize their proteins, and this microbial protein enters the small intestine and is used by the host (Agricultural Research Council 1984), but some dietary protein can escape ruminal degradation. The amino acids contributed by this 'bypass protein' are very difficult to quantify, due to wide variation in by-pass rates, digestibility and amino acid composition of the various proteins (Ministry of Agriculture and Fisheries and Food Standing Committee on Tables of Feed Composition 1990). As a consequence, the amino acid profile of the ruminal microbial protein is used as an approximation of the profile available to the host, unless the dietary protein has been processed to enhance its rumen by-pass rate.

In farm animals, the primary purpose of the management of amino acid nutrition is to match the profile of amino acids, essential amino acids in particular, in the protein flowing into the small intestine [i.e., metabolizable proteins (MP) in ruminants)] with the amino acid profile of the products. This management is based on an assumption that the body does not need to modify the amino acid profile in MP in *do novo* protein synthesis because any such modification will lower the efficiency of utilization of dietary amino acids. To determine this supply-demand relationship, a basic strategy is to compare the amino acid profiles in MP with those in animal products. Table 5.1 lists the amino acid profiles of protein in rumen microbes, whole-body, wool, and milk for sheep, as well as milk for goats, and the uterine and umbilical uptakes of amino acids in ewes at 130 days of pregnancy. If the amount of an essential amino acid in ruminal microbial protein is much lower than the amount in the product protein, it is likely the dietary supply of this amino acid will not meet the demand of the body. As shown in Table 5.1, the pattern of most of the essential amino acids in ruminal microbial protein is similar to the patterns of body protein and milk protein, whereas the amounts of Ile and Val in microbial protein are much lower than the umbilical and uterine uptakes of these two amino acids in pregnant ewes. Among the traditionally classified dispensable amino acids, wool protein contains disproportionally high amounts of Cys (about five-fold), Arg, Pro and Ser (about two-fold), milk protein has higher proportions of Glu/Gln and Pro, and the uterus and foetus use a much

Table 5.1 Amino acid concentrations (g/kg protein) of microbial protein in the rumen, the whole-body of sheep (excluding wool) and wool protein

	Rumen microbial protein[a,b]	Whole-body protein[c]	Wool protein[d]	Goat milk[e]	Sheep milk[f]	Uterine uptake[g]	Umbilical uptake[g]
His	16–21	24	8–13	35	30	20	28
Ile	54–62	36	27–32	46	47	142	116
Leu	74–83	73	67–79	98	97	91	66
Lys	81–115	67	27–35	99	78	81	89
Met	16–25	18	4.4–6.3	22	27	12	30
Phe	49–57	39	25–36	50	42	38	55
Thr	52–66	49	54–66	40	43	46	54
Val	53–65	43	46–57	60	63	157	101
Ala	34–62	80	32–52	34	37	36	47
Arg	46–53	73	62–91	39	28	118	134
Asp[(h)]	112–129	86	55–66	72	84	0.1	−2
Cys	20–26	13	86–131	9	8	–	–
Glu[(i)]	127–141	132	111–142	200	217	97*	146*
Gly	49–65	96	46–86	16	18	−12	47
Pro	34–40	63	53–75	89	100	75	61
Ser	41–47	42	83–108	44	43	54	−27
Tyr	44–51	31	38–63	47	37	45	54

[a]Storm et al. (1983)
[b]Martin et al. (1996)
[c]MacRae et al. (1993). Calculated from the amino acid profiles of the carcass, gut, liver and skin and the corresponding protein contents in sheep by the authors. Wool protein is not included
[d]Reis (1979). Merino sheep wool
[e]Ceballos et al. (2009). Granadian goats
[f]Gerchev et al. (2005). Mean of amino acid concentrations for Tsigai and Karakachanska sheep
[g]Chung et al. (1998). Columbia-Rambouillet sheep, carrying a single fetus, pregnancy 130 days. *Including both glutamate and glutamine concentrations. Cysteine concentration was not reported in this paper. Amino acid profiles were calculated by the author based on the uterine and umbilical uptakes (g/kg fetus.d) of amino acids reported in the paper
[h]Asp = aspartate plus asparagine
[i]Glu = glutamate plus glutamine

higher proportion of Arg, compared with these amino acids in the ruminal microbial protein. The high content of these amino acids must endow special functions to proteins in the respective products – for example, Cys for disulphide bridges, Ser for hydrogen bonds, and Arg for salt bridges, in the structure of wool proteins (Popescu and Höcker 2007). The supply of these amino acids from the diet as well as from synthesis in the body, must be considered for the management of these biological processes. We will discuss some of these situations below.

A further consideration for understanding amino acid nutrition in animals is obligatory oxidation in the body after intestinal absorption – essential amino acids are either used for protein synthesis or disposed of by oxidation, including conversion to other amino acids, such as Met to Cys. The extent of oxidation therefore directly determines the utilisation efficiency of an amino acid in the body. This oxidation can be measured by using ^{13}C-labelled amino acid (Young and Borgonha 2000) and, for a given amino acid, the oxidation rate is defined as the amount oxidised as a proportion of the flux. Liu and Masters (2003) analysed published literature and calculated the oxidation rates of 0.18 for Cys, 0.03–0.04 for Leu, 0.15 for Lys, 0.16 for Met, 0.08–0.09 for Phe, and 0.04 for Thr. The sulfur-containing amino acids (Met and Cys) and Lys and have much higher oxidation rates than the others, suggesting lower efficiencies for protein synthesis and, therefore, higher dietary demands.

In essence, any essential amino acid oxidised in the body must be replenished from the diet, and the amount needed in the diet can be defined as the requirement of this amino acid for the corresponding physiological process. The requirements for the essential amino acids (Leu, Ile, Lys, Met+Cys, Phe, Tyr, Thr, and Try) for adult humans, based on measurements of their oxidation rates, is known as the Massachusetts Institute of Technology System that was proposed by Young and Borgonha (2000). For sheep, a comparable system has not been fully established – only Met and Cys requirements were reported by Liu and Masters (2000) and, for growing Kazakh lambs, the Met requirement has been estimated from Phe oxidation (Wei et al. 2017).

Another point worth noting is that amino acid nutrition is influenced by genetic potentials for productivity and dietary intake. At the same level of feed intake, animals achieving higher productivity certainly have a higher efficiency of utilization of dietary amino acids for protein deposition, compared to these with low productivity. For amino acids that are incorporated into specific products in particularly high proportions, such as wool protein and the gravid uterus, the same level of dietary intake of these amino acids may meet the demands of animals with low productivity, but could be inadequate for animals with high productivity. For example, Merino sheep of 55 kg live weight are fed 0.7 kg/day of a hay/barley/lupin diet containing 12% crude protein, providing 8% MP, an intake level that maintains body protein balance (i.e., no net protein deposition in the body) – the estimated Cys absorption would be 1.4 g/day, and after excluding obligatory oxidation, about 0.7 g/day Cys would be available for wool growth, equivalent to the amount needed for wool growth of 7 g/day (Liu and Masters 2000). This diet can thus meet the Cys demand of sheep growing up to 7 g wool per day, but not sheep growing more than 7 g wool per day.

5.3 Reproduction

In pregnant ewes, the primary purpose of amino acid nutrition is to support ovulation, fertilization, implantation, embryo development, and fetal growth through to birth (Wu 2018). This process begins with ovarian follicles and their oocytes going through a selection process regulated by an interplay of reproductive hormones, and some of the dominant follicle(s) eventually ovulate (Scaramuzzi et al. 2011). In sheep, the number of dominant follicles that ovulate, the ovulation rate, depends on the energy balance of the animal, and it seems unlikely that amino acid balance plays a role (Scaramuzzi et al. 2011). Fertilization leads to the formation of a zygote that moves into the uterus and simultaneously begins to develop into an embryo. Early embryos produce signals that lead to implantation and recognition of pregnancy about 2 weeks after fertilization. The process of embryogenesis involves consumption of nutrients, from internal reserves, oviduct fluid and uterine secretions. To create conditions for conceptus development, the glandular tissue of the uterus produces, or selectively transports from the bloodstream, a complex array of proteins and other molecules into the uterine lumen (Bazer et al. 2012). Most embryo deaths occur during this peri-implantation period so an optimal nutrient supply seems to be crucial to the success of implantation. The placenta begins to develop about 25 days after fertilization and it ensures adequate nutrition to support the growth and development of the foetus (Bazer et al. 2012).

Throughout the whole process, the nutritional status of the pregnant female is critical for the establishment and maintenance of pregnancy. The supply of amino acids plays a significant role in embryo development to the blastocyst stage. For example, studies with an *in vitro* mouse model have shown that non-essential and essential amino acids, and Gln, play opposite roles in the regulation of cleavage and blastocoel development (Lane and Gardner 1997; Van Winkle 2001). In cattle, the concentrations of both

essential and non-essential amino acids in the uterine fluid, 12–18 days post-estrus, are 2.1–3.9 fold greater than the concentrations in non-pregnant cattle (Groebner et al. 2011). In cultured ovine primary trophectoderm cells from day 15 conceptuses, concentrations of Arg, Leu, and glucose, but not Gln, seem to be critical for cell function, with Arg and Leu concentrations stimulating proliferation and migration of cells within the embryo (Kim et al. 2011). In ewes, the nutrient composition of uterine luminal fluid differs between days 3–16 of the cycle and days 10–16 of pregnancy. Similarly, in pregnant ewes, the amounts of glucose, Arg, Gln, Glu, Gly, Cys, Leu, Pro and glutathione in uterine fluid increase 3- to 23-fold between days 10 and 14 of pregnancy and remain high until day 16 (Gao et al. 2009). These observations suggest that pregnancy recognition is associated with transport of amino acids into the uterine lumen, with Arg, Leu, Val and Gln/Glu, being the most critical.

To distinguish the amino acids that are preferably used by the conceptus and associated tissues during pregnancy, Chung et al. (1998) measured the uptake of amino acids by the uterus and umbilical cord in pregnant ewes for 130 days. These observations can be compared with the amino acid profile of rumen microbial protein as a representative of dietary supply, and the amino acid profile in the whole-body protein of growing lambs (Table 5.1). It is clear that the uptakes of Arg, Ile and Val are 2–three-fold greater than their compositions in both rumen microbial protein and whole-body protein. It should be noted that any amino acid taken up can be used either for anabolism (protein deposit) or catabolism, and the amino acid profile in deposited protein would be similar to protein composition of tissue or body protein. There is no evidence that the amino acid profile of the whole-fetus differs from that of the whole-body of growing lambs. Therefore, a corollary is that these amino acids are not preferentially taken up specifically for protein deposition in the fetus, but for modulating metabolic processes in the gravid uterus, including uterine tissue as well as the fetus, the so-called "functioning amino acids" (Wu 2009). This concept is supported by the fact that the net uteroplacental uptake of Arg was about four-fold greater than the net fetal uptake in ewes at 129 days gestation (Thureen et al. 2002). The functions of Arg include: i) the synthesis of NO, an important molecule for regulating placental angiogenesis and uterine blood flow during gestation; ii) synthesis of polyamines, molecules that are essential for placental development and embryogenesis; iii) activation of the mTOR signaling pathway and regulation of hormone secretion, both thoroughly reviewed (e.g., Wu et al. 2016; Wu et al. 2014; Wu et al. 1999).

It is therefore not surprising that, in pregnant ewes, Arg supplementation has beneficial effects from the first trimester through to the birth of the foetus. In the early stages of embryogenesis, rumen-protected Arg saves weaker embryos from entering early degeneration by increasing the synthesis of NO and polyamines (Saevre et al. 2011). Parenteral administration of Arg between 100 and 121 days of gestation increases the birth weight of quadruplet lambs and improves post-natal survival (Lassala et al. 2011). The improvement in lamb survival with Arg supplementation, by feeding rumen-protected Arg or by intravenous Arg infusion, seems to be related to increased brown fat in the foetus, alleviation of slow fetal growth caused by poor maternal nutrition (restricted feeding), increased uteroplacental weight, increased birthweight, as shown in reviews and confirmed in a number of experiments, particularly in ewes that are under-fed or carrying multiple fetuses (Lassala et al. 2011; McCoard et al. 2013; Satterfield et al. 2012, 2013; Sun et al. 2018; Zhang et al. 2016; van der Linden et al. 2015). To maintain a high concentration of Arg in the maternal circulation, administration of citrulline was more effective than a direct supplement of Arg because of a longer half-life (Lassala et al. 2009). The beneficial effect continued on the post-natal growth of lambs up to about 2 months old when pregnant ewes had restricted feeding (60% of the nutrition requirement) but were supplemented with about 12 g/day rumen-protected Arg from day 54 gestation until parturition (Peine et al. 2018). Because it is now known that citrulline is not degraded by the ruminal

microbes of steers (Gilbreath et al. 2019, 2020a) and sheep (Gilbreath et al. 2020b), this amino acid (in an unprotected form) can be directly supplemented to the diets of sheep and goats to enhance their reproductive performance as suggested by these authors.

It is no surprise that there are few concerns about the supply of amino acids during early pregnancy, because so little biomass is involved in eggs, blastocysts, embryos, and early stage fetuses. The total requirement for amino acids, even those considered essential for pregnancy, would be negligible within the context of requirements for the whole body. On the other hand, evidence is accumulating for effects of periconception nutrition on the subsequent development of the embryo, the foetus, the newborn, with some effects persisting into adult life (e.g., Sen et al. 2016; Gardner et al. 2006). It is not clear whether such effects involve the supply of amino acids or energy. Moreover, some amino acids play regulatory roles that might be far more important than simply being building blocks for proteins. There are substantial changes in the reproductive endocrinology during the estrous cycle and pregnancy, and many of the hormones involved are controlled by neuronal activity in the brain, where several amino acids act as neurotransmitters (e.g., as Asp and Glu; Wu 2013) or as precursors for the synthesis of neurotransmitters (such as the large neutral amino acids, Try and Tyr; Growdom and Wurtman 1979). Indeed, in sheep, infusions of such amino acids have triggered the secretion of gonadotrophin and increased ovulation rate (Downing et al. 1995, 1996, 1997; Foster et al. 1989). One of the problems with these hypotheses is the concept that critical brain functions can be determined by normal variation in the supply of dietary amino acids, although extreme imbalances could be devastating.

In the last third of pregnancy, however, as the feto-placental units achieve significant mass and the uterus itself develops muscle and secretory tissue, protein deposition in the gravid uterus becomes significant and it becomes essential to meet this demand for amino acids. The processes that regulate metabolism prioritize the gravid uterus over other physiological processes, and preferentially allocate nutrients to it, deriving them from both nutritional sources and, where necessary, sacrificing maternal tissues. To ensure reproductive success, through the support of pregnancy and the subsequent lactation, it is essential to manage amino acid nutrition correctly. The literature mentioned above shows that supplementation of Arg can reduce the impairment of fetal growth in underfed ewes, but only partially. The effects of dietary restriction during early pregnancy on the subsequent performance of the offspring in sheep, described above, were long ago recognized for maternal undernutrition during late pregnancy (Everitt 1967). This phenomenon later became known as 'fetal programming' or 'developmental origins of health and disease (DOHAD)' in the context of human health. As in many other species, maternal undernutrition in sheep is now recognized as a factor that determines offspring performance in growth, reproduction and several aspects of homeostasis (Vinoles et al. 2014; Rhind et al. 2001; Bielli et al. 2002). Again, the role of amino acid nutrition in these phenomena is not clear, although Arg has been implicated (Sales et al. 2016). We can conclude that, because some amino acids have special physiological functions (Wu 2013), it is best to manage amino acid nutrition in a holistic fashion during pregnancy to ensure optimal life-time growth and development of offspring.

We also need to consider the role of amino acid nutrition as a determinant of the quality and quantity of colostrum and milk, an essential issue in neonatal survival and offspring growth (Banchero et al. 2015). The capacity of the mammary gland for milk synthesis depends largely on the number and efficiency of the mammary epithelial cells (Rezaei et al. 2016). The development of these cells begins in the embryo but most happens during puberty and pregnancy, when undernutrition can have profound effects subsequent milk yield and quality. We need to remember that the rates of milk synthesis and secretion are largely driven by the rate of lactose synthesis within the mammary epithelial cells, so limiting energy intake during pregnancy, even if

positive energy balance is maintained by *ad libitum* intake during lactation, will lead to reduced milk production at birth. Nevertheless, the supply of amino acids is important because they are involved in synthesis of milk proteins as well as the proliferation and function of the mammary cells. Interestingly, the amino acid profiles are very similar for milk and the rumen microbes (Table 5.1), so microbial protein can match the requirements for synthesis of milk protein without substantial modification. In other words, there does not seem to be any 'limiting' amino acids during lactation in sheep or goats. Even so, daily supplementation of multiparous Saanen dairy goats with rumen-protected Met increases milk yield (Flores et al. 2009). In another experiment with Karagouniko dairy ewes, dietary supplementation of fat plus rumen-protected Met from 2 weeks before lambing until the twelfth week of lactation increased milk yield by 37% during the first 7 weeks of lactation (Goulas et al. 2003). On the other hand, dietary supplementation with rumen-protected Met to Chios dairy ewes in last fifth pregnancy did not change milk yield. Similarly, in Danish Landrace × Saanen crossbred goats, milk yield was not affected by dietary supplementation of rumen-protected Met or Lys (Madsen et al. 2005). The same outcome has been reported for Blackface, Dorset and Comisana ewes (McCoard et al. 2016). However, the effects of Met or Lys supplementation on milk yield is likely to depend on the timing and size of the supplement. For example, the highest milk yield in these studies was less than 3 kg/day and the basal diets contained 14–18% crude protein. Mature dairy goats generally weigh 30–80 kg and daily milk yield varies among breeds, from 2.6 kg (Nigerian Dwarf) to 11.9 kg (Toggenburg) with the Alpine, Nubian, Oberhasli, and Saanen producing 7–10 kg (Park et al. 2007). Protein concentrations average at 35 g/L (Park et al. 2007). Therefore, amino acid nutrition might be an issue in dairy goats with very high milk production, but only if they are fed diets with limited crude protein.

5.4 Growing Sheep and Goats

Growth involves the accumulated outcomes of cell division and cell differentiation, and, in livestock production, is measured as growth rate and muscle gain. Critically, maternal nutrition during pregnancy influences fetal and post-natal growth, with under-nutrition seriously inhibiting the growth and development of skeletal muscle in the offspring (Wu 2018). The third trimester of pregnancy is particularly important for the proliferation of muscle cells and changes in amino acid supply might alter post-natal muscle growth (Greenwood et al. 2000). For example, nutrition-restricted ewes produce lambs with reduced body weight but, if they are provided with supplements of rumen-protected Arg during gestation, the outcome is restoration of neonatal birth weight, lamb weight at age 19 days, and brown fat reserves (Peine et al. 2018).

Overall, research on amino acid nutrition is scarce for growing lambs and kids compared to monogastric animals. It could be that growth rate is a smaller economic factor for ruminants under grazing conditions than for monogastric animals in intensive, in-door systems. Moreover, the great similarity between the amino acid profiles of whole-body protein and rumen microbial protein (Table 5.1) suggests that no particular amino acid (s) is deficient or preferably required for lamb growth. By contrast, in growing pigs, dietary supplementation of Lys is necessary for muscle protein accretion and thus muscle growth (Liao et al. 2015).

Numerous studies show that the branched-chain amino acids (BCAA; Ile, Leu, Val) have the unique ability to initiate signal transduction pathways that up-regulate translation, and therefore protein synthesis, in skeletal muscle (Kimball and Jefferson 2006; Yoshizawa 2004). However, the literature for the lamb is inconclusive. For example, van Nolte et al. (2008) fed Rambouillet wether lambs (35–46 kg body weight) a basal diet containing 14.3–15% crude protein, and then infused abomasally a mixture of

10 essential amino acids. They then removed individual essential amino acids from the infusion. Removal of Met and Thr reduced N retention (g/day) and the ratio of retained N to digested N, whereas removal of BCAA had no significant effect. On the other hand, Sang et al. (2010) found that dietary supplementation of 6-month old wether lambs (25 kg) with rumen-protected Leu (0.5, 1 or 2 g/day) for 15 days increased protein synthesis rate in *m. longissimus dorsi* and, in *m. biceps femoris*, protein synthesis was increased only at 1 g/day (Sang et al. 2010). In this experiment, the basal diet contained 11.6% crude protein. By contrast, intravenous infusion of Suffolk-cross wether lambs (32 kg; aged 8 months) with 1.3 g of a mixture of BCAA over 6 h did not change the protein synthesis rates in *vastus* muscle or *m. longissimus dorsi* (Wester et al. 2004). It appears that the effect of Leu supplementation on lamb growth varies with dietary protein level and the rate of Leu supplementation. We conclude that more work is required to resolve the issue of 'limiting' amino acids in growing lambs and kids.

5.5 Wool (Fibre) Growth

Amino acid and protein nutrition for fibre production was thoroughly reviewed a decade ago by Liu and Masters (2003) for sheep and by Galbraith (2000) for goats. Since then, there has been little new research.

The fibre is produced by follicles embedded 500–600 μm below the skin surface and, in Merino sheep, the biomass of the follicular tissue amounts to about 50 g, or 0.1% of live weight (Williams 1995). The fibre is composed almost entirely of protein and the net efficiency of dietary protein for wool growth is estimated to be 0.20–0.25 (Standing Committee on Agriculture 1990). This value is substantially lower than those for weight gain (0.59), pregnancy (0.85) and lactation (0.68) in sheep (Agricultural and Food Research Countil Technical Committee on Responses to Nutrients 1993). The low efficiency is mostly due to limits in the supply of Met+Cys from the diet, combined with the relatively low productivity (7–18 g/day) of protein retained in wool compared with values for 300 g/day body weight gain (about 45 g/day) and for 1 kg/day milk (about 50 g/day).

Wool protein contains about 10% Cys, and much higher proportions of Arg, Ser and Pro, compared with the ruminal microbial protein (Table 5.1). Most feed proteins contain at most 2% Met and 2–5% Cys (Ministry of Agriculture and Fisheries and Food Standing Committee on Tables of Feed Composition 1990). In the body, Cys can be synthesised from Met through the trans-sulphuration pathway (Finkelstein 1990) and the amount of Cys produced from Met is estimated to account for 5–22% of the Cys flux (Liu and Masters 2003). In addition, local synthesis of Cys in the skin and follicle provides substantial amounts for wool growth (Harris et al. 1997; Souri et al. 1998b). For these reasons, Cys + Met is usually considered to be the limiting amino acid for wool production.

Many studies have shown that supplementing Merino sheep with appropriate levels of Met (about 2–5 g/day) improves wool growth, but not during late pregnancy or early lactation (review: Liu and Masters 2003). In cashmere and Angora goats (Souri et al. 1998a), Met also improves fibre production. In cultured follicles, Met alone produces 80% of the response seen with Met +Cys, whereas the response to Cys alone varies – follicle growth and viability can be reduced while, with Met alone, follicle growth can reach 75% of that recorded with Met+Cys. Although the concentration of Met in wool protein is very low, it combines with Cys to play a major role, probably by initiating protein synthesis and cell division. By contrast, Cys provides a substrate for wool protein synthesis, as evidenced by the increases in expression of mRNA encoding a family of Cys-rich proteins (Fratini et al. 1994) and the synthesis of Cys-rich proteins (Harris et al. 1994).

Supplying more Met and Cys through dietary supplementation is not usually cost effective because of the high prices of the supplements and the price penalty paid because fibre diameter increases. On the other hand, the most effective way to improve feed efficiency and amino acid utilization for fibre growth in sheep and goats is probably genetic selection for high fibre growth rate. Within species, variation in fibre growth is

explained by variation in the proportion of active follicles and/or the efficiency of the follicles (fibre growth rate/follicle density). Thus, on the same plane of nutrition, sheep selected for high clean fleece weights grow more wool than sheep selected for low fleece weight, and wool growth rates are closely related to skin fractional protein synthesis rate and to skin total protein synthesis (Masters et al. 2000).

The rate of fibre production varies greatly across species. Merino sheep (53 kg body weight) produce about 4 kg greasy fleece (2.8 kg clean) per year (Mortimer et al. 2017). Angora goats (30–60 kg) can produce 1.5–4 kg mohair over 6 months and cashmere goats (30–70 kg) produce less than 1 kg of guard hair and cashmere per year (Lupton 2010). Angora rabbits (3.5–4.0 kg) can produce 1.2–1.4 kg clean wool per year at a net efficiency of 0.43 (Liu et al. 1992), and are obviously the most productive in terms of fibre produced per kg of body weight. These differences among species are associated with variation in net efficiencies of use of digested protein for fibre growth: 0.43 for Angora rabbit, 0.39 for Angora goat, and 0.20–0.25 for Merino sheep (review: Liu and Masters 2003). We do not have a value for cashmere production but it must be very low. There is a clear interaction between the genetic capacity for fibre growth and responses to dietary protein or amino acids, so we would not expect supplementation of low-productivity animals to greatly improve productivity or profitability. Experimental data supports this hypothesis: Angora goats show a substantial fibre response to supplementation with rumen-protected Met (62% vs 30%; Souri et al. 1998a, b) whereas, in cashmere goats, the fibre response is similar for a urea-based diet and a fish-meal diet (about 15% crude protein) providing similar levels of nitrogen (Galbraith, 2000), probably because the nutrient demand for fibre growth was already met by the urea. The variation among species therefore seems to be more dependent on genetic variation in follicle density and morphology than amino acid efficiency, so exploration of the molecular mechanisms that control follicle productivity is likely to be a more productive avenue towards improvements in fibre growth than dietary manipulation.

5.6 Amino Acid Nutrition for GIT Health and Nematode Infection

In sheep, the biomass of the gastrointestinal tract (GIT) is about 4–6% of body weight (Liu et al. 2005; MacRae et al. 1993) and, metabolically, GIT tissue has the highest turnover rate in the body (Lobley 1994) due to the renewal of desquamated enterocytes, and the production of secreted digestive enzymes, immune molecules and cells. Therefore, protein synthesis in the GIT accounts for 25–33% of whole-body protein synthesis (Lobley et al. 1994; Neutze et al. 1997) as well as 11%–23% of whole-body energy expenditure (McBride and Kelly 1990; Lobley 1994). There is no doubt that these high costs mean that relatively small proportions of amino acids and energy are available for anabolism of peripheral tissues, limiting body growth, the production of milk and wool, and fetal growth and development. This is an intrinsic aspect of mammalian biology. However, there is a wide range in protein turnover in the GIT in proportion to the whole body, suggesting considerable genetic variation that is likely to be closely associated with variation in whole-body efficiency of utilization of amino acids. To date, there has been little research into these issues in sheep and goats.

The use of amino acids by the GIT would be expected to have flow-on effects to the amino acid profile that is available to the peripheral tissues. In other words, does the GIT disproportionally incorporate specific amino acids into its proteins? This question may be partly answered by comparison of the amino acid profiles (g/kg total amino acids) of the GIT and the carcass, as done for sheep by, for example, MacRae et al. (1993). Using their data, we calculated the ratios of 18 amino acids in GIT to those in the carcass and found that 13 ratios fell into the range of 0.9–1.1 (i.e., close to unity). By contrast, the ratio was 1.6 for Cys, 1.2 for Met, Ser and Thr, and only 0.7 for 4-hydroxyproline. These ratios suggest that GIT proteins contain particularly

high concentrations of Cys, Met, Ser and Thr, so less of these amino acids would be available for other tissues. It should be noted that proteins secreted into the GIT lumen, such as digestive enzymes, some immunoglobulins and glycoproteins, are probably not included when GIT tissue is sampled for analysis. For example, in one study with sheep (Mukkur et al. 1985), goblet-cell mucin in the small intestine contained 94 g Cys, 243 g Thr, and 237 g Val per kg total amino acids (masses recalculated by the authors to remove ammonia). These values are over ten-fold higher than those in the sheep carcass (MacRae et al. 1993). We can see, therefore, how these proteins could affect the profile of amino acids in the GIT, but no quantitative data are available for the amounts of these proteins produced, so their impact on amino acid supply to other tissues is not known.

When not used in protein synthesis, some amino acids are oxidised in the GIT, and the oxidation rates could also alter the amounts and proportions that reach peripheral tissues. The small intestine of the sheep can catabolize Leu and Met, accounting for 26% and 10% respectively of the whole-body Leu and Met oxidation, whereas there seems to be no net catabolism of Lys and Phe (Lobley et al. 2003). Estimates of the magnitude of Leu oxidation in the GIT of sheep vary from 0–50% in the literature (Lobley et al. 2003; Yu et al. 2000), and we have not been able to find estimates for other essential amino acids in sheep and goats. This is perhaps no surprise because direct measurement of amino acid oxidation in the GIT requires surgical placement of catheters into specific positions in selected arteries and veins, as well as the small intestine, employment of isotope-labelled amino acids, and analysis of the end-product (mostly CO_2) of oxidation (Lobley et al. 2003; Yu et al. 2000). The techniques are complex, and therefore the data are rare. An alternative approach is to measure the sequestration of amino acids by the mesenteric-drained viscera (from the small intestine) and the portal-drained viscera (PDV, GIT plus spleen and pancreas; Lobley et al. 2003), as has been done in sheep (MacRae et al. 1997) and pigs (Fang et al. 2010). In sheep, the PDV recoveries of amino acids infused into the jejunum varied from 61% for His to 65% for Phe, 76% for Lys, 79% for Thr, 80% for Ile and Leu, and 83% for Val (MacRae et al. 1997). Therefore, 17–39%, depending on the amino acid, were used by the PDV. In pigs, the amount of Met used by the PDV accounted for 29–33% of dietary intake (Fang et al. 2010). It is worth noting that the sequestrated amino acid can be used either for protein synthesis or oxidation, and it is difficult to ascertain partitioning between these two processes. Yu et al. (2000) found about 14% of Leu sequestrated by the PDV in sheep was oxidized, with a slight increase to 15%–16% after infection with the helminth, *Trichostrongylus colubriformis*. Infection with *T. colubriformis* also leads to a considerable reduction in Met absorption (Liu et al. 2002).

As with the obligatory oxidation of amino acids in the liver, oxidation in the GIT is likely to serve a purpose. In the GIT, we can find all of the catabolic pathways for Met (Bauchart-Thevret et al. 2009; Liu and Masters 2003) through which S-adenosylmethionine (an important methyl donor), polyamines (spermidine and spermine), and Cys are derived. The aforementioned GIT proteins and secreted glycoproteins contain high proportions of Cys, and the GIT epithelium contains very high levels of glutathione (GSH), the synthesis of which demands Cys as a substrate (Wu et al. 2002). Cys synthesized from Met through the trans-sulphuration pathway (Finkelstein 1990) could be a significant source for GIT tissues, because the proportions of Cys are low in dietary and ruminal microbial proteins. The small intestine contains the highest levels of polyamines, spermine in particular, compared with other tissues (liver, lymph nodes, muscle, skin) in sheep (Liu et al. 2007), supporting its high turnover rate (Loest et al. 2002; Tabor and Tabor 1984). Met is catabolized through the aminopropylation pathway and provides the aminopropyl moiety for synthesis of spermidine and spermine. In rats, about 45% of spermidine and spermine are derived from *de novo* synthesis (White and Bardocz 1999). If this was also the case in sheep, synthesis of polyamines would certainly consume a considerable amount of Met. As for why Leu is oxidized in the GIT,

Lobley et al. (2003) speculated that the process might involve the interaction with signal cascades that regulate protein metabolism.

Among the dispensable amino acids, the GIT has specific and substantial demands for Gln. In the PDV of sheep, there is a net uptake of Gln when levels of protein are changed from slightly above maintenance (basal diet) to 3.8-fold maintenance by infusion of protein into the abomasum, whereas Glu was taken up at relatively low protein intakes (from maintenance to about 2.4-fold maintenance) but then released when the protein intake was more than three-fold maintenance (Freetly et al. 2010). Uptake of Gln by the PDV has also been observed in other studies with sheep (Foote and Freetly 2016; McNeil et al. 2016). The roles of Gln in GIT tissues have been thoroughly reviewed by Lobley et al. (2001) and it is clear that Gln is important for the provision of energy to rapidly growing cells. For example, it provides up to 30% of the energy needs of lymphocytes in cattle (Wu and Greene 1992). Intracellular Gln can either be deaminated to produced Glu plus ammonia, both of which are excreted out of cells, or partially oxidized to Asp, coupled with the formation of 9 ATP, far less than the 38 ATP produced from full oxidation of glucose (Rich 2003). However, Gln breakdown produces ATP at a much faster rate than oxidative phosphorylation (Aledo 2004), so it becomes an important energy source in cells with high proliferation rates, such as cancer cells and enterocytes (Aledo 2004). Gln is also a precursor that supplies half of the N required for synthesis of both purines and pyrimidines as well as aminosugars in all cell types (Calder and Newsholme 2002; Wu 2013).

In addition, the conversion of Gln to Glu (the glutaminolytic pathway) is more closely linked to cell proliferation than its intracellular concentration (Aledo 2004). High rates of conversion of Gln to Glu are seen in all lymphoid organs and cells, and Gln catabolism contributes more than a third of the energy requirement of immune cells (Duff and Daly 2002). The differentiation of B lymphocytes to plasma cells, and immunoglobulin synthesis, are Gln-dependent over the physiological range of Gln concentrations (Crawford and Cohen 1985). Since the GIT is structured to contain, or to be directly associated with, the highest biomass of immune components in the body, including the lymph nodes, Peyer's patch, immune cells, immunoglobulins and cytokines (Pastoret et al. 1998), one would expect an adequate supply of Gln to be essential for the maintenance of gut health. Indeed, in piglets weaned early (about 3 weeks of age), dietary supplementation with 1% Gln (on a fed basis) prevents jejunal atrophy, increases feed efficiency, and improves the immune responses to infection by *Escherichia coli* (Wu et al. 2011), suggesting that the Gln requirement is higher in gut-stressed animals. In calves weaned early (age 42 days), intravenous administration of Ala-Gln dipeptide at 1 g Gln per kg body weight (the total amount of Gln equivalent to 0.05% of the dietary intake of solid matter) increased blood $CD2^+$ and $CD4^+$ lymphocytes, serum IgA and IgG concentrations, and mucosal secretary IgA concentrations in jejunum and ileum, while decreasing the incidence of diarrhoea (Zhou et al. 2012). However, there is little information about the effect of dietary Gln supplementation on gut function in sheep or goats, probably because dietary Gln is destroyed by rumen microorganisms. To address this issue, we need a source of rumen by-pass Gln, although it is feasible that gut-stress is not as problematic in young sheep and goats as it is in weaning piglets.

A critical GIT health issue in grazing sheep and goats is infection by helminth nematodes. Severe infection depends on season/climate and management, but it causes chronic inflammation of GIT tissues, reduces feed intake and re-absorption of nutrients from the intestinal lumen, and can cause chronic diarrhoea, with the overall outcome being reduced productivity (Sykes and Coop 2001; Grencis et al. 2014; Williams 2011). Nematode infection also changes amino acid oxidation in the GIT. Yu et al. (2000) examined Leu metabolism in the GIT of lambs after infection with *T. colubriformis* larvae and found that, in the absence of detectable effects on whole-body leucine flux, there was a 24% increase in total GIT Leu sequestration and an increase from 22% to 41% in GIT Leu oxidation. These observations suggest that nematode

infection stimulates Leu oxidation in the GIT and reduces nutrient partitioning to the peripheral tissues. As mentioned above, infection of lambs with *T. colubriformis* reduces Met absorption into the peripheral tissues (Liu et al. 2002). Taken together, these observations suggest that nematode infection increases the consumption of some amino acids in the GIT, probably to support enhancement of metabolic processes for repair of damaged GIT tissue and to elicit immune responses.

The various pathophysiological responses to nematode infection include an increase in the secretion of mucus by the GIT (Theodoropoulos et al. 2001). The GIT is lined by a mucus layer that is continuously secreted and forms the first physical barrier that protects the GIT epithelium. The mucus has gel characteristics due to the presence of high molecular weight mucins (glycoprotein monomer or polymers), antibodies (immunoglobulin A in particular) and other molecules (Simpson et al. 2016; Theodoropoulos et al. 2001; Dharmani et al. 2009). An increase in the secretion of mucus is part of the initial nonspecific response to nematode infection, followed by activation of biosynthetic processes that involve changes in the chemical composition, and therefore structure, of the mucins; the final outcome depends on the nematode species and on the adaptation of the host to that species (Theodoropoulos et al. 2001; Menzies et al. 2010).

In the sheep small intestine, the mucin proteins contain high proportions of Cys and Pro and very high proportions of Thr, Ser and Val (Lien et al. 2001; Mukkur et al. 1985). The hydroxyl moiety in Ser, Thr and Pro services O-glycosylation, and the hydrosulphide moiety is used for forming disulphide bonds within the monomer and polymers, all of which are critical for resistance to proteolytic enzymes (Dharmani et al. 2009). In pigs, the mucin protein contributes 5–11% of the total endogenous protein in ileal digesta, depending on feed consumption, dietary protein and fibre concentration (Lien et al. 2001). The corollary is that the contribution in ruminant animals may be higher than for monogastrics due to the very high proportions of fibre in their diet. The extremely high proportions of Thr, Ser and Val in gastric and intestinal mucins means a high requirement for mucin synthesis and an increase in dietary demand, depending on the response in mucin secretion evoked by nematode infection. The problem is that there are no quantitative estimates of mucin secretion, even in healthy animals, so we have no way to assess the potential effects of these amino acids in the diet.

Investigation of the effects of supplementation with specific amino acids on epithelial barrier (mucin) and the immune responses of the GIT in ruminants is scarce. In sheep, we know that wool growth demands a high amount of SAA, particularly Cys, because they are deposited in wool, and that Cys is therefore drawn from the metabolic body pool, reducing the availability of Cys for, for example, immune competency. Indeed, in Romney sheep, the fecal worm egg counts are increased in animals selected for high fleece weight, and abomasal infusion of Cys (2 g/day) tended to increase peripheral eosinophil count, abomasal globular leukocyte count, and the immunoglobulin G response, yet no interaction between Cys supplementation and genotype was observed when selected and unselected animals were compared (Miller et al. 2000). Abomasal supplementation of 6-month old Suffolk cross lambs with both Cys (1 g/day) and Gln (5 g/day) for 12 weeks after infection with *T. colubriformis* led to an increase in nitrogen retention, along with reductions in circulating eosinophil count and peak faecal egg counts, but had no effect on final nematode counts (Hoskin et al. 2002). In lactating rats infected with *Nippostrongylus brasiliensis* (the adults reside in the small intestine), feeding the Met- and Leu-deficient diets (about 40% below the normal diet) increased the number of worm eggs in the colon, but had no effects on systemic immunoglobulin activity or the numbers of mast cells, goblet cells and eosinophils (Sakkas et al. 2013). This literature is limited, but suggests that

deficiency or supplementation of specific amino acids influences worm fecundity, as reflected in changes in fecal worm egg counts, but there is no conclusion with respect to influences on the immune responses to infection.

5.7 Concluding Remarks

Concomitant evolution of rumen microbes and their hosts over millions of years has resulted in great similarity in the amino acid profiles of microbial proteins and host whole-body protein. This similarity ensures a balance between the dominant supply of amino acids from the microbes and the host's needs for growth, survival and reproduction. However, the question is – has this balance been overridden by human interference? This question is most acute with the rapid progress in productivity driven by modern breeding practices, with changes that exceed by far the pace of evolution. The evidence is accumulating to support the view that the supply of amino acids to the host, from both rumen microbial protein and rumen by-pass protein, no longer meets the demands of high-performance animals that produce large amounts of meat, milk or wool, or need to nourish large litters. Without doubt, the pursuit of high productivity will continue, so research on amino acid nutrition needs to accelerate. Meanwhile, with regard to the processes, amino acids need to be seen as "two-way switches" that can send positive and negative signals at the same time, and thus play specific regulatory roles under specific conditions. For example, in cultured mouse mammary epithelial cells, Leu, Ile, and Val stimulated phosphorylation of ribosomal protein kinase beta-1 (S6K1), whereas Lys, His and Thr inhibited it (Prizant and Barash 2008). Similarly, in myogenic C2C12 cells, Leu and Gln have opposite regulatory effects on the phosphorylation of downstream effector S6K1 and eukaryotic translation initiation factor 4E binding protein in the mammalian target of rapamycin (mTOR) pathway (Deldicque et al. 2008). Leucine increases protein synthesis by stimulating the mTOR signaling pathway, and also enhances catabolism (Gannon and Vaughan 2016). In sheep, arginine affects both protein synthesis and proteolysis in cultured brown adipocyte precursor cells and, in those cells, the mTOR signalling pathway is promoted by an increase in the Arg concentration in maternal plasma (Ma et al. 2017). At the highest level of regulation, the brain, mTOR is deeply implicated in the control of energy homeostasis through the coordination of anabolic and catabolic processes focused on survival (Morentin et al. 2014). Therefore, much more attention must be paid to the interactions among amino acids in the regulation of biological processes.

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Amino Acids in Swine Nutrition and Production

6

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Abstract

Amino acids are the building blocks of proteins in animals, including swine. With the development of new analytical methods and biochemical research, there is a growing interest in fundamental and applied studies to reexamine the roles and usage of amino acids (AAs) in swine production. In animal nutrition, AAs have been traditionally classified as nutritionally essential (EAAs) or nutritionally nonessential (NEAAs). AAs that are not synthesized de novo must be provided in diets. However, NEAAs synthesized by cells of animals are more abundant than EAAs in the body, but are not synthesized de novo in sufficient amounts for the maximal productivity or optimal health (including resistance to infectious diseases) of swine. This underscores the conceptual limitations of NEAAs in swine protein nutrition. Notably, the National Research Council (NRC 2012) has recognized both arginine and glutamine as conditionally essential AAs for pigs to improve their growth, development, reproduction, and lactation. Results of recent work have also provided compelling evidence for the nutritional essentiality of glutamate, glycine, and proline for young pigs. The inclusion of so-called NEAAs in diets can help balance AAs in diets, reduce the dietary levels of EAAs, and protect the small intestine from oxidative stress, while enhancing the growth performance, feed efficiency, and health of pigs. Thus, both EAAs and NEAAs are needed in diets to meet the requirements of pigs. This notion represents a new paradigm shift in our understanding of swine protein nutrition and is transforming pork production worldwide.

Keywords

Amino acids · Swine · Nutrition · Metabolism · Growth · Health

Abbreviations

AA	amino acid
ASL	argininosuccinate lyase
ASS	argininosuccinate synthase
BCAA	branched-chain amino acid
BCAT	BCAA transaminase
BCKA	branched-chain α-ketoacid dehydrogenase
BW	body weight
EAA	nutritionally essential amino acid
FAA	functional amino acid

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1285, https://doi.org/10.1007/978-3-030-54462-1_6

GDH	glutamate dehydrogenase
GFAT	glutamine: fructose-6-phosphate transaminase
IDO	indoleamine 2,3-dioxygenase
IUGR	intrauterine growth restriction
KIC	α-ketoisocaproate
mTOR	mechanistic target of rapamycin
NEAA	nutritionally nonessential amino acid
NO	nitric oxide
NOS	nitric oxide synthase
NRC	National Research Council
OAT	ornithine aminotransferase
ODC	ornithine decarboxylase
OH-POX	hydroxyproline oxidase
P5C	pyrroline-5-carboxylate
POX	proline oxidase
SHMT	serine hydroxymethyltransferase
α-KG	α-ketoglutarate

6.1 Introduction

Amino acids (AAs) have traditionally been classified as nutritionally essential AAs (EAAs) or nonessential AAs (NEAAs) for animals and humans, depending on whether they can be synthesized de novo in cells of animals and can support the nitrogen balance or growth of the organism (Abderhalden 1912; Rose 1957). For those AAs that are not synthesized de novo, they must be provided in diets to sustain the life, growth and development of animals (Hou and Wu 2018a; Yao et al. 2011). With the development of modern analytical methods and biochemical research, scientists have identified that the rates of utilization of some NEAAs are greater than the rates of their synthesis under certain conditions that include early weaning, lactation, pregnancy, burns, injury, infection, heat stress, and cold stress (Hou et al. 2015, 2016a, b; Wu 2010; Wu et al. 2013c; Yi et al. 2018). Similarly, there are reports that the National Research Council (NRC 2012)-recommended requirements of swine for some EAAs (e.g., tryptophan and threonine for post-weaning pigs) are insufficient for their maximum growth or optimal health, including intestinal health (Le Floc'h et al. 2018; Liang et al. 2018, 2019; Xu et al. 2015). Therefore, it is necessary to reconsider the roles of both EAAs and NEAAs in swine nutrition and production. A growing body of evidence in the literature has led to the development of the new concept of functional amino acids (FAAs) in nutrition, which are defined as AAs that can regulate key metabolic pathways to benefit the survival, growth, development, reproduction, lactation, and health of animals and humans (Wu 2009). FAAs (e.g., arginine, cysteine, glutamine, glutamate, glycine, leucine, proline, and tryptophan) can be either EAAs or NEAAs, and play an important role in both protein synthesis and maintaining whole-body homeostasis.

Modern breeds of pigs grow faster, gain more lean body weight (BW), and gestate more fetuses; therefore, they have greater nutritional and physiological requirements for AAs, when compared with previous breeds of swine (Wu et al. 2018). However, low-protein diets widely used to reduce the production of nitrogenous wastes by swine farms may not supply sufficient AAs and may result in the suboptimal growth and productivity of pigs (Hou et al. 2016a). Furthermore, due to the extensive catabolism of AAs in the small intestine and the different metabolic fates of AAs in different extra-intestinal tissues, the pattern of AAs in the diet does not accurately reflect the composition of AAs in the body (Wu et al. 2014). Thus, the conceptual foundation for ideal protein based on the EAA composition of the body is flawed. The lack of knowledge about AA metabolism and function in pigs has precluded the use of NEAAs in pork production systems (Hou et al. 2016a). Thus, there is an urgent need to reevaluate the dietary requirements of modern breeds of pigs for AAs. To achieve this goal, the present article highlights AA metabolism and nutrition in pigs of different ages and provides a scientific basis for revising the recommendations for dietary AA requirements of the animals. Findings from studies of swine as an animal model also have important implications for improving human nutrition.

6.2 Metabolism of the Arginine Family of AAs in Pigs

The arginine family of AAs consists of glutamine (Gln), glutamate (Glu), arginine (Arg), proline (Pro), aspartate (Asp), asparagine (Asn), ornithine (Orn), and citrulline (Cit) (Wu et al. 2007). Except for Orn and Cit, all of them are substrates for protein synthesis (Wu and Morris 1998). Typically, interconversion among these AA occurs frequently via the complex interorgan metabolism of great physiological importance to maintain reproduction, growth and development of pigs (Fig. 6.1). Branched-chain AAs (BCAAs), which are highly abundant in both plant- and animal-source feedstuffs (Hou et al. 2019; Li and Wu 2020), are major donors of the amino group in Glu, Gln, Ala and Asp (Wu et al. 2016). We will also summarize the synthesis and catabolism of the arginine family of AAs to provide a better understanding of their nutritional roles in pigs.

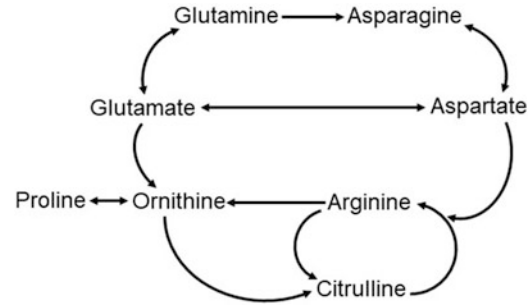

Fig. 6.1 Interconversion of the arginine family of amino acids in pigs. Aspartate is required for both the synthesis of both arginine from citrulline in all tissues (primarily the kidneys) and cell types (including enterocytes, endothelial cells, macrophages, neurons, and smooth muscle cells). Citrulline is formed from glutamine (via glutamate) and proline exclusively in the mitochondria of enterocytes, with ornithine being the common intermediate. Most of the glutamine and essentially all of the glutamate in blood are derived from branched-chain amino acids and glucose (the source of the carbon skeleton) in extra-intestinal and extra-hepatic tissues (primarily skeletal muscle). In most tissues and cell types with the exception of porcine placentae and mammary tissues, as well as mature red blood cells (without mitochondria), glutamine is hydrolyzed by phosphate-activated glutaminase into glutamate and ammonia. In red blood cells, glutamate can be generated from glutamine via glutamine:fructose-6-phosphate amidotransferase. Glutamate is converted into glutamine by glutamine synthetase or into aspartate by glutamate:oxaloacetate transaminase in all cell types, but a net synthesis of glutamine is limited in the small intestine. Arginine is metabolized to ornithine by arginases (type I and type II) and arginine:glycine amidinotransferase in a cell-specific manner, and is also oxidized by nitric oxide synthase to NO and citrulline in all cell types. The arginine-derived citrulline is recycled into arginine via argininosuccinate synthase and lyase. Note that although the mitochondria-generated ornithine is readily converted into citrulline in the small intestine, the ornithine provided from the diet and blood is a poor substrate for arginine synthesis in the body because of the complex compartmentalization of intestinal metabolism of amino acids

6.2.1 Glutamate

Glu is one of the most abundant AAs in both plant- and animal-source feedstuffs (Hou et al. 2019; Li et al. 2011a; Li and Wu 2020), as well as in tissue proteins of the body (Table 6.1; Wu 2013). Because 95–97% of dietary Glu is catabolized by the small intestine of pigs during its first pass into the portal vein (Wu 2013), essentially all of the Glu in the body is produced from other AAs via multiple metabolic pathways. Glu can be synthesized in almost all cell types of pigs. The nitrogen (N) and carbon (C) skeleton for Glu synthesis originate primarily from AAs (Gln, BCAAs, alanine and Asp) and glucose [the major source of α-ketoglutarate (α-KG)], respectively (Wu et al. 2005; Li et al. 2009). Many enzymes are involved in the reactions in a cell- and tissue-specific manner, including phosphate-activated glutaminase, glutamine:fructose-6-phosphate transaminase (GFAT), BCAA transaminase (BCAT), Glu-pyruvate transaminase, Glu-oxaloacetate transaminase and Glu dehydrogenase (GDH) (Wu 2018). Glutaminase was discovered by Krebs (1935) as a mitochondrial enzyme encoded by two different genes in the liver and kidneys that catalyze the conversion of Gln into Glu (Curthoys and Watford 1995). GFAT (a cytosolic enzyme) converts Gln and fructose-6-phosphate into glucosamine-6-phosphate and Glu, and is abundant in red blood

Table 6.1 Concentrations of free amino acids in the plasma, tissues and whole body of 42-day-old pigs and the composition of peptide-bound amino acids (PAAs) in their skeletal muscle[a]

Amino Acids (AA)	Free AA in plasma (nmol/ml)	Free AA in tissues or the whole body (nmol/g wet weight of tissue)					PAA in muscle or the body (mg/g wet weight of tissue)	
		GM muscle	Brain	Liver	JN mucosa	Whole body	GM muscle	Whole body
Ala	481 ± 14	1392 ± 47	1021 ± 39	4120 ± 135	2258 ± 69	1046 ± 40	12.2 ± 0.6	9.32 ± 0.35
Arg	124 ± 8	164 ± 6	121 ± 7	72 ± 2	966 ± 31	126 ± 6	14.1 ± 0.5	9.71 ± 0.42
Asp	30 ± 2	125 ± 4	2097 ± 64	1506 ± 58	1405 ± 44	139 ± 5	10.7 ± 0.4	5.15 ± 0.19
Asn	112 ± 7	129 ± 5	134 ± 5	823 ± 27	763 ± 32	102 ± 4	8.54 ± 0.3	6.14 ± 0.26
Cys[b]	143 ± 7	84 ± 4	203 ± 7	213 ± 8	187 ± 5	65 ± 2	3.11 ± 0.13	1.89 ± 0.08
Gln	536 ± 12	4581 ± 125	7830 ± 236	3586 ± 116	1149 ± 48	3427 ± 154	10.9 ± 0.4	6.75 ± 0.29
Glu	103 ± 6	1532 ± 68	8977 ± 258	4017 ± 124	3920 ± 127	1085 ± 50	18.6 ± 0.8	12.1 ± 0.48
Gly	1072 ± 31	2546 ± 80	1186 ± 30	6854 ± 179	2816 ± 94	1911 ± 69	7.50 ± 0.32	16.8 ± 0.62
His	76 ± 3	212 ± 9	105 ± 4	797 ± 31	709 ± 11	158 ± 6	6.27 ± 0.29	2.96 ± 0.13
Hyp	62 ± 3	45 ± 2	17 ± 1	30 ± 2	13 ± 1	17 ± 1	0.66 ± 0.03	5.42 ± 0.26
Ile	131 ± 8	78 ± 3	101 ± 4	304 ± 12	424 ± 25	61 ± 3	10.6 ± 0.5	5.07 ± 0.45
Leu	162 ± 9	121 ± 4	190 ± 7	616 ± 19	738 ± 37	93 ± 4	16.5 ± 0.6	9.81 ± 0.39
Lys	108 ± 6	139 ± 6	102 ± 4	497 ± 17	986 ± 29	106 ± 5	15.2 ± 0.7	8.64 ± 0.46
Met	85 ± 4	64 ± 2	79 ± 3	98 ± 3	297 ± 10	50 ± 2	5.81 ± 0.26	2.68 ± 0.13
Phe	113 ± 7	106 ± 3	128 ± 4	217 ± 8	440 ± 12	82 ± 3	10.1 ± 0.4	4.92 ± 0.18
Pro	242 ± 10	1836 ± 59	907 ± 35	5033 ± 181	1283 ± 39	1372 ± 56	8.02 ± 0.37	12.2 ± 0.59
Ser	174 ± 9	254 ± 8	889 ± 30	1159 ± 37	2455 ± 96	191 ± 7	8.70 ± 0.46	6.32 ± 0.26
Thr	191 ± 11	368 ± 14	1470 ± 55	914 ± 42	831 ± 28	283 ± 12	9.74 ± 0.53	5.02 ± 0.18
Trp	70 ± 3	53 ± 2	39 ± 2	112 ± 3	203 ± 4	40 ± 2	2.73 ± 0.14	1.59 ± 0.07
Tyr	105 ± 9	118 ± 5	124 ± 7	253 ± 10	368 ± 12	88 ± 4	7.66 ± 0.34	3.90 ± 0.16
Val	170 ± 10	126 ± 7	126 ± 8	657 ± 24	1075 ± 46	97 ± 5	12.0 ± 0.5	6.04 ± 0.25
GABA	0.74 ± 0.04	1.8 ± 0.1	1854 ± 96	69 ± 3	12 ± 1	124 ± 6	–	–
β-Ala	12 ± 1	749 ± 26	66 ± 3	428 ± 11	16 ± 1	566 ± 23	–	–
Cit	76 ± 2	98 ± 3	59 ± 2	60 ± 2	204 ± 8	75 ± 3	–	–
Orn	72 ± 4	80 ± 3	32 ± 1	446 ± 15	817 ± 32	129 ± 8	–	–
Tau	52 ± 2	9624 ± 312	2816 ± 107	10526 ± 328	10849 ± 185	7338 ± 305	–	–

[a]Values are means ± SEM, n = 8. Pigs (offspring of Yorkshire × Landrace dams and Duroc × Hampshire sires) were weaned at 21 days of age to a corn- and soybean meal-based diet containing 21% crude-protein. At 42 days of age, blood samples were obtained from the jugular vein at 4 h after feeding, as described by Wu et al. (1996c). Thereafter, pigs were euthanized to obtain tissues for amino acid analyses (Wu et al. 1999; Hou et al. 2019). The content of each AA was calculated on the basis of its intact molecular weight. The content of dry matter was 25% (g/g) in the GM muscle, brain, and liver, and was 22% in the jejunal mucosa. The content of true proteins plus peptides in the GM muscle and the whole body was 17.2% and 14.2% of fresh wet tissue weight, respectively

[b]Total cysteine (cysteine + ½ cystine)

Cit citrulline, *DM* dry matter, *GM* gastrocnemius, *GABA* γ-aminobutyrate, *Hyp* 4-hydroxyproline, *JN* jejunum, *Orn* ornithine, *PAA* peptide-bound AA (AA in protein plus peptides), *Tau* taurine

cells (e.g., 2.17 ± 0.13 and 2.02 ± 0.15 nmol/mg protein per min in erythrocytes of 30- and 150-day-old pigs, respectively; mean ± SEM, n = 8) and endothelial cells at high enzymatic activity, contributing to Glu production in cells that lack mitochondria (Wu et al. 2001). The activity of BCAT varies greatly among different cell types, with skeletal muscle possessing the greatest total activity per gram of tissue. In contrast to many enzymes of AA metabolism, BCAT activity in the porcine liver is low and there is little transamination of BCAAs in this organ under physiological conditions. Glu-pyruvate transaminase and Glu-oxaloacetate transaminase catalyze the reversible reactions between Glu and alanine or Asp, respectively, in various animal tissues. GDH is a mitochondrial enzyme that interconverts α-ketoglutarate (α-KG) and ammonia into Glu, with the direction of the reaction dependent on the concentrations of substrates in cells. In addition, Glu can be formed by degradation of intracellular proteins.

The contributions of metabolic pathways to Glu synthesis vary among tissues. The liver takes up little Glu from the portal vein; therefore, endogenous synthesis is the major source of Glu in this organ for release into the blood circulation in the post-absorptive state (Wu 2018). It is unlikely that under physiological conditions, the porcine liver has either a net synthesis of Glu from Gln due to the presence of the intra-organ Glu-Gln cycle or significant generation of Glu from BCAAs because of low BCAT activity (Li et al. 2009). Instead, alanine, Pro, phenylalanine and asparagine are the major substrates for Glu synthesis in the liver (Wu 2018). Other tissues, such as skeletal muscle, small intestine, and kidneys, catabolize both BCAAs and Gln to regulate their homeostasis and inter-organ AA metabolism in the body (Hou and Wu 2018b). BCAAs also undergo extensive transamination in both the mammary gland and porcine placenta to provide Glu, Gn, Ala and Asp for synthesis of milk protein and fetal-placental tissues, respectively (Li et al. 2009; Wu et al. 2013a).

Most of the enzymes in Gln-synthetic reactions are also involved in Glu degradation. In the small intestine, Glu undergoes extensive degradation, so that there is no release of Glu from the gut of post-absorptive pigs (Wu et al. 1994). In this process, Glu is metabolized into CO_2, glutathione, alanine, and Asp through oxidation via the actions of glutathione-synthetic enzymes, Glu-pyruvate aminotransferase, and Glu-oxaloacetate aminotransferase, respectively (Wu et al. 1994). Pyrroline-5-carboxylate (P5C) synthase is essential for converting Glu into Cit and Arg in enterocytes, which plays a crucial role in maintaining Arg homeostasis in milk-fed piglets (Flynn and Wu 1996), post-weaning pigs (Wu et al. 1997), and adult pigs (Wu et al. 2018). In pig enterocytes, the activity of GDH is rather low, thus little ammonia is produced from Glu (He et al. 2019a) or monosodium Glu by pig enterocytes (Blachier et al. 1999). In contrast, GDH is highly active in the liver and kidneys to produce ammonia (Hou and Wu 2018b). Ammonia is detoxified as urea via the hepatic urea cycle in the liver and is used to combine H^+ as NH_4^+ in the kidneys for control of the acid-base balance. Transamination of Glu with pyruvate and oxaloacetate also occurs in the liver, skeletal muscle, and mammary gland of pigs to produce alanine and Asp from Glu (Ytrebo et al. 2006; Li et al. 2009; Wu 2013). The Glu-derived-α-KG is used for either glucose synthesis or ATP production.

6.2.2 Glutamine

Gln is one of the most abundant AAs in the body of pigs and the third most abundant free AA in the plasma of gestating and lactating sows (0.35 to 0.5 mM), as well as fetal, neonatal, and postweaning pigs (0.4 to 0.5 mM; Wu 2018). About 67% of dietary Gln is metabolized by the pig small intestine during the first pass into the portal vein, and most of the circulating Gln is derived from endogenous synthesis (Wu et al. 2011b). The only enzyme capable of synthesizing Gln in animal cells is Gln synthetase (GS; Curthoys and Watford 1995). This ATP-dependent enzymatic reaction requires Glu and ammonia, and is present in many tissues, with skeletal muscle being the major site for Gln synthesis (Watford 2008). The lungs, adipose tissue, and the lactating mammary

glands also synthesize and release Gln (Watford 2008; Li et al. 2009). The ammonia for Gln synthesis is derived primarily from the degradation of EAAs. The liver has the capacity for both synthesis and utilization of Gln because the enzymes are compartmentalized in different populations of cells (i.e., periportal and perivenous hepatocytes) (van Straaten et al. 2006). Little Gln is synthesized in the small intestine of pigs since there is negligible Gln synthetase activity in that tissue (Chen et al. 2009; Haynes et al. 2009).

Degradation of Gln in the body primarily involves its hydrolysis into Glu and ammonia via the action of phosphate-activated glutaminase, with Glu being further metabolized to glutathione, glucose and other AAs (alanine, Orn, Pro, and Arg) or oxidized to CO_2, as noted previously. The Gln-derived ammonia is used for urea synthesis to maintain a low concentration of ammonia in the blood. A small fraction (<3%) of Gln serves as a precursor for the synthesis of purines, pyrimidines, NAD, glucosamine, and asparagine through amidotransferase pathways (Wu et al. 2011b). In the kidneys, Gln-derived ammonia is vital for regulation of the acid-base balance (Curthoys and Watford 1995). As mentioned previously, Gln can be catabolized to Glu by phosphate-activated glutaminase in tissues containing mitochondria. However, the lactating mammary gland (O'Quinn et al. 2002) and placentae (Self et al. 2004) of pigs lack glutaminase activity, which maximizes the amount of Gln available to support the production of milk and the rapid growth and development of fetal-placental tissues.

6.2.3 Arginine

Milk provides at most 40% of the total daily Arg requirements of 7-day-old sucking pigs (Wu and Knabe 1995). Substantial amounts of Arg are synthesized endogenously to support the growth and development of pigs. Arg is synthesized primarily from Cit in the small intestine and kidneys. In pig enterocytes, Gln and Pro are the main substrates for the production of Cit. This metabolic pathway is regulated by P5C synthase and *N*-acetylglutamate synthase (Wu et al. 2004). P5C synthase is expressed, almost exclusively, in enterocytes of the small intestine (Wu and Morris 1998; Wu et al. 2000), and its enzymatic activity is inhibited by the high concentrations of Orn (e.g., by 75% in the presence of 5 mM Orn; Hu et al. 1999). The oxidation of Pro by Pro oxidase (POX) in the mitochondria of enterocytes also yields P5C that is subsequently converted into Orn and Cit by Orn aminotransferase (OAT) and Orn carbamoyltransferase (Wu 1997). As noted previously, the Gln- and Pro-derived Cit is converted into Arg via argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL). In 1- to -7-d-old pigs, ASL activity is high in their enterocytes, but low in their kidneys, but the opposite is true for post-weaning pigs (Wu and Knabe 1995; Wu et al. 1997). Therefore, Arg is produced from Cit mainly in the small intestine for newborn pigs, but primarily in the kidneys for weaned pigs.

Degradation of Arg in pigs is initiated by arginase and nitric oxide (NO) synthase (NOS) (O'Quinn et al. 2002; Wu et al. 2010). Interestingly, arginase activity is absent from the porcine placenta in which degradation of Arg is initiated by NOS to promote NO synthesis, which is of great importance for the survival and growth of the conceptus (embryo/fetus and placenta) (Wu et al. 2017). In the placenta, NOS activity is quantitatively very low; therefore, a large amount of Arg is transferred from mother to fetus (Wu et al. 1996a). This explains why Arg is highly abundant in the allantoic fluid of pigs during early- and mid-gestation to support the growth and development of the conceptuses. The mammary glands of lactating sows use both arginase and NOS to actively degrade Arg to form Pro, Orn, and urea, and to a lesser extent, polyamines and NO (O'Quinn et al. 2002). Thus, the concentrations of Arg are low, but the concentrations of proline are high in sow's milk (Wu and Knabe 1994). Arg from the diet or from de novo synthesis undergoes little

catabolism in enterocytes of pre-weaning pigs because these cells have no detectable arginase activity (Wu et al. 1996b). After pigs are weaned, the expression of arginase in the intestine increases significantly in response to the cortisol surge at parturition (Flynn and Wu 1997) and 40% of the dietary Arg is catabolized by the small intestine during the first pass into the portal vein (Wu et al. 2016). Arg that is not utilized by the small intestine enters the portal vein, but only about 8% of the Arg in blood is taken up by the liver due to a low abundance of cationic AA transporters in hepatocytes (Wu et al. 2007). Within the liver, Arg is used by arginase, arginine:glycine amidinotransferase, Arg decarboxylase, and NOS to produce Orn, creatine, agmatine, and NO, respectively (Wu et al. 2018). In addition, Arg is utilized by the liver to synthesize homoarginine and lysine, possibly by arginine:glycine amidinotransferase (Hou et al. 2016b). Furthermore, Arg is an allosteric activator of *N*-acetylglutamate synthase, which catalyzes the formation of *N*-acetylglutamate (an allosteric activator of carbamoylphosphate synthase-I) from Glu and acetyl-CoA. Thus, Arg is required to maintain the hepatic urea cycle in an active state for the detoxification of ammonia. This metabolic pathway is essential for the survival of mammals such as swine.

6.2.4 Proline

Proline is the second most abundant AA in the body of pigs (after Gly) (Wu 2013). It is formed from Arg via pathways involving P5C synthase and OAT (Wu and Morris 1998). As noted previously, P5C synthase converts Glu into glutamyl-γ-semialdehyde, which spontaneously cyclizes to yield P5C. The P5C is then reduced to Pro by NADPH-dependent P5C reductase. The endogenous synthesis of Pro is highly active in the body (Bertolo et al. 2003; Wu 2010), because pigs are generally fed plant-based diets that contain low concentrations of Pro (Hou et al. 2019; Li et al. 2011a). OAT exists in all cell types throughout the body to convert Orn into glutamyl-γ-semialdehyde, which is used for Pro synthesis. The formation of Pro from Arg is active in the mammary tissue, small intestine (post-weaning animals), liver, and kidneys (Wu et al. 2011a). Of particular note, in porcine mammary tissue, Pro is synthesized from Arg and Orn via the arginase pathway, but not Glu or Gln due to the absence of glutaminase and P5C synthase (Wu and Morris 1998). This provides an additional explanation for the relatively high enrichment of Glu, Gln and Pro, but a severe deficiency of Arg in sow's milk (Wu and Knabe 1994).

Mitochondrial POX is the only known enzyme that initiates the degradation of Pro in cells of animals (Adams and Frank 1980; Phang 1985). Almost all cell types that contain mitochondria express POX, except for mammary tissue (Wu et al. 2008). In pigs, POX activity is highest in the small intestine, followed by the liver, kidneys, and placenta (Wu et al. 2008). POX generates P5C from Pro and, therefore, glutamyl-γ-semialdehyde and Orn by OAT. In porcine enterocytes, the mitochondria-derived Orn is converted into Cit and Arg. In both the intestine and the placenta, Orn is used for the synthesis of polyamines via Orn decarboxylase (ODC), spermidine synthase, and spermine synthase (Wu et al. 2005). Thus, the Pro-derived polyamines are particularly important in the placenta and in the small intestine of neonatal pigs that lack arginase activity (Wu et al. 1996b). Because the conceptus and neonatal pig grow very rapidly, both dietary and endogenously synthesized Pro play an important role in fetal and neonatal growth and development. Furthermore, the lack of conversion of Orn into Cit and of P5C into Glu due to the absence of Orn carbamoyltransferase activity and the near absence of P5C dehydrogenase maximize the synthesis of polyamines from Pro in porcine placentae (Wu et al. 2008; Wu et al. 2011a). Because polyamines are essential for syntheses of DNA and protein, Pro metabolism plays an important role in supporting conceptus growth and development in swine (Wu et al. 2005; Wu et al. 2008).

6.3 Metabolism of Glycine (Gly) in Pigs

Gly is the most abundant AA in tissue proteins of animals, including pigs (Li and Wu 2018). Interestingly, in contrast to many nonruminants (e.g., humans and rats) that have 0.2–0.3 mM free glycine in their plasma, Gly is the most abundant free AA in the plasma of fetal and postnatal pigs (0.9–1.2 mM; Wu et al. 1994). Because pigs are generally fed plant-based diets that have low concentrations of Gly (Hou et al. 2019; Li et al. 2011a; Li and Wu 2020), these animals have a high rate of Gly synthesis via multiple metabolic pathways (Hou et al. 2016a; Wu et al. 2019). Specifically, Gly is formed from: (1) serine via serine hydroxymethyltransferase (SHMT), (2) choline via the formation of sarcosine, (3) threonine via the threonine dehydrogenase pathway, (4) glyoxylate via alanine-glyoxylate aminotransferase, and (5) 4-hydroxyproline via mitochondrial hydroxyproline oxidase (OH-POX) in pigs (Walsh and Sallach 1966; Ballevre et al. 1990; Wu et al. 2011a). Gly synthesis from dietary serine, choline and threonine contributes <12% of Gly needed by young pigs (Wang et al. 2013). There is evidence that 4-hydroxyproline (abundant in sow's milk and the plasma of piglets) contributes to most Gly synthesis in 7-day-old-pigs (Wu et al. 2019). 4-Hydroxyproline is derived from the hydrolysis of proteins (primarily collagens) containing hydroxylated Pro residues. Collagen in the body and certain foods (such as milk and meat) are the major sources of 4-hydroxyproline in pigs (Wu et al. 1999; Wang et al. 2015d). The catabolism of 4-hydroxyproline into Gly occurs in multiple tissues, including the small intestine, kidneys, liver, and skeletal muscle (Hu et al. 2017b). Note that alanine-glyoxylate aminotransferase catalyzes the final, nearly irreversible reaction of the 4-hydroxyproline catabolic pathway, i.e., the formation of Gly from glyoxylate (Wu et al. 2019). This indicates that OH-POX, together with alanine-glyoxylate aminotransferase, favors the generation of Gly from 4-hydroxyproline and glyoxylate. Thus, their urinary excretion is minimal. The OH-POX pathway explains: (a) the ultimate conversion of Arg, Pro and Orn, as well as BCAAs, Glu and Gln into Gly via 4-hydroxyproline; and (b) efficient conservation of AA nitrogen and carbon as Gly.

About 30% of the dietary Gly is metabolized by the small intestine of postweaning pigs during its first pass into the portal vein (Wu 2013). Because porcine enterocytes have a limited ability to degrade Gly (Wang et al. 2014c), microbes in the lumen of the small intestine likely play an important role in utilizing dietary Gly. It appears that the rate of Gly catabolism to CO_2 and ammonia in the whole body of pigs is low, relative to the rate of Gly synthesis (Hou et al. 2016a; Wu 2010). Three enzymes are responsible for these reactions: the glycine cleavage system, SHMT, and D-amino acid oxidase (van Straaten et al. 2006). Among them, the glycine cleavage system is the main enzyme that catalyzes the degradation of Gly to NH_3 and CO_2, with the generation of 5,10-methylene-tetrahydrofolate from tetrahydrofolate, an essential cofactor for SHMT (Lamers et al. 2007). D-amino acid oxidase has a minor role in Gly degradation in animal tissues due to its low affinity for Gly (Thureen et al. 1995). In pigs, the conversion of Gly into serine is limited, likely due to insufficient amounts of methyl-group donors and folate (Wang et al. 2013).

6.4 Metabolism of Leucine (Leu) and Tryptophan (Trp) in Pigs

Leu and Trp are EAAs for monogastric animals, because their carbon skeletons cannot be formed in the body. Leu is actively transaminated with α-KG to form Glu, as noted previously, whereas Trp is the precursor of serotonin, *N*-acetylserotonin, melatonin, anthranilic acid, niacin, and indoles with enormous physiological importance (Wu 2013). Leu is highly abundant in all plant- and animal-source feedstuffs (Hou et al. 2019; Li et al. 2011a; Li and Wu 2020). In contrast, Trp is adequate in animal-source feedstuffs, but deficient in most plant-source feedstuffs. Plant-based diets generally provide more Leu than needed for protein accretion, but insufficient or barely adequate Trp for

maximum growth and optimum health of the intestine and the whole body of pigs. Therefore, pigs must degrade a substantial amount of Trp for normal neurological, endocrine, and intestinal functions, while also conserving Trp for tissue protein synthesis.

Degradation of Leu is initiated by BCAA transaminase (BCAT), which reversibly interconverts Leu and α-KG into α-ketoisocaproate (α-KIC) and Glu (Wilkinson et al. 2013). As noted previously, BCAT activity is low in the porcine liver. Therefore, Leu transamination occurs mainly in extrahepatic tissues, with skeletal muscle being quantitatively the most important site (Su et al. 2012). α-KIC is then released into the bloodstream and taken up by various tissues where it undergoes irreversible oxidative decarboxylation to isovaleryl-CoA, leading to the formation of acetoacetate (a ketone body) and acetyl-CoA (a precursor of ketone bodies and fatty acids). This reaction is catalyzed by the branched-chain α-keto acid dehydrogenase (BCKD) complex located within mitochondria. BCKD kinase regulates the activity of BCKD via protein phosphorylation (inactive form), whereas protein phosphatase maintains BCKD in the dephosphorylation state (active form; Wu 2013). BCKD is highly active in the liver, intermediate in activity in the heart and kidneys, but its activity is relatively low in skeletal muscle (Suryawan et al. 1998). In addition, a small proportion (~5%) of α-KIC is oxidized to β-hydroxy-β-methylbutyrate by α-KIC dioxygenase located within the cytosol of hepatocytes (Nissen and Abumrad 1997). In the mammary tissue of lactating sows, Leu is actively degraded to generate Glu, Gln, alanine and aspartate that support milk production (Li et al. 2009). Because all BCAAs share the same transporters for entry into cells, the proper balances of these AAs in diets and blood are particularly important to avoid antagonisms (Wu et al. 2014).

In mammals, approximately 95% of Trp is degraded through the kynurenine pathway, mediated by two rate-limiting enzymes: Trp 2,3-dioxygenase and indoleamine 2,3-dioxygenase (IDO; Brown et al. 1991; Taylor and Feng 1991; Schwarcz et al. 2001). Trp 2,3-dioxygenase is constitutively expressed in the liver, while IDO is present mainly in immune cells and is inducible by inflammatory cytokines in piglets (Ruddick et al. 2006; Bhutia et al. 2015). Through multistage enzymatic reactions, Trp is primarily converted into kynurenine, quinolinic acid, nicotinic acid, and kynurenic acid via the kynurenine pathway (Badawy 2015). In addition, 1–2% of Trp is metabolized to serotonin (5-hydroxytryptamine) by tryptophan hydroxylase, and to melatonin (Bai et al. 2017). Serotonin primarily exists in the gastrointestinal tract, and melatonin is produced mainly by the pineal gland, but also by the retina, gastrointestinal tract, skin and leukocytes (Radogna et al. 2010; Bai et al. 2017). The remaining Trp is metabolized primarily to indoleacetic acid in the gastrointestinal tract and liver. Many metabolites of Trp are involved in the regulation of immune responses (Bai et al. 2017). When pigs are challenged with endotoxins or pathogens, the concentrations of Trp in plasma decrease significantly due to high rates of utilization by immune cells and other cell types (Le Floc'h et al. 2012). Piglets are able to detect and respond to metabolic changes induced by a Trp deficiency, and Trp metabolism affects the growth and development of pigs at different stages (Ettle and Roth 2004).

6.5 Functions of AAs in Pigs

6.5.1 Regulation of Intestinal Development and Mucosal Barrier Function

The mucosa of the porcine small intestine contains high concentrations of FAAs, such as Glu, Gln, Gly and taurine (Table 6.1). Proliferation and turnover of enterocytes are essential to maintain intestinal function (Peterson and Artis 2014). AAs promote the synthesis of proteins by serving as their building blocks and activating the mechanistic target of rapamycin (mTOR) signaling (Wu 2009). mTOR is a highly conserved serine/threonine protein kinase and a master regulator of the initiation of polypeptide formation (Laplante and Sabatini 2012). Several lines of evidence support the notion that mTOR signaling is essential for intestinal growth and function. For

example, Gln stimulates protein synthesis and inhibits proteolysis (Xi et al. 2012) in porcine enterocytes by activating the mTOR signaling pathway, leading to enhanced proliferation of those cells (Xi et al. 2012; Yi et al. 2015). Glu-induced increases in the intestinal RNA/DNA ratio are associated with maintaining the mTOR signaling pathway (Qin et al. 2018). Similar findings were reported from studies involving the supplementation of culture medium for IPEC-1 cells with Gln, Glu, Arg, Trp or Gly (Wang et al. 2014c; Wang et al. 2015a; Zhu et al. 2015; Li et al. 2016b; Xiao et al. 2017). In addition to mTOR signaling, anti-apoptosis is another mechanism that supports the proliferation and survival of enterocytes. For example, Gln protects porcine enterocytes from apoptosis by activating the IRE1α-XBP1 axis, regulating glutathione-related redox homeostasis, and enhancing *glutathione S*-transferase A-mediated metabolism (Jiang et al. 2017; Liu et al. 2018b). Likewise, Gly exerts an anti-apoptotic effect on cells of the small intestine of piglets by repressing the induction of the endoplasmic reticulum stress-induced C/EBP homologous protein (a transcription factor; Fan et al. 2019). Arg also increases DNA synthesis in lipopolysaccharide-challenged enterocytes, thus contributing to the regeneration and restoration of the mucosa of the small-intestine (Tan et al. 2015). These studies provide solid evidence that FAAs are beneficial for intestinal growth, development and health.

In accordance with its role in protein synthesis, AAs are crucial for the maintenance of intestinal mucosal barrier integrity, and thus play a key role in gut homeostasis (Yang et al. 2015). Tight junctions are widely distributed in the intestinal epithelium as a primary physical barrier to selectively regulate the passage of molecules and ions via the paracellular pathway (Shen 2012). They are composed of three major tight junction proteins: occludin, claudins, and junction adhesion molecule proteins (Steed et al. 2010). Interestingly, AAs, such as Gln, Glu, Asp, Arg, Pro, Gly, and Trp, enhance the abundances of tight junction proteins in the small intestine, thus improving intestinal mucosal barrier function (Jiao et al. 2015; Wang et al. 2015b; Wang et al. 2015c; Li et al. 2016a; Wang et al. 2017; Liang et al. 2019; Zheng et al. 2018). Moreover, these AAs beneficially regulate inflammatory and oxidative responses to pro-inflammatory cytokines or reactive oxygen species to mitigate increases in intestinal permeability by enhancing the expression of tight-junction proteins (Al-Sadi et al. 2010; Wang et al. 2014a). For example, dietary supplementation with 1.0% Arg to low-birth-weight piglets improves intestinal mucosal barrier function and enhances antioxidant capacity by increasing the expression of claudin-1 and glutathione peroxidase mRNAs (Zheng et al. 2018). Similarly, dietary supplementation with Asp protects mucosal barrier function in lipopolysaccharide-challenged weaned pigs by increasing the expression of claudin-1 and occludin in the jejunum, while inhibiting TLR4 and NODs/NF-κB and p38 signaling (Wang et al. 2017). In addition, intestinal mucosal barrier and absorptive functions depend on the constant provision of a large amount of ATP (Wu 1998). Gln, Glu and Asp are primary substrates metabolized in intestinal epithelial cells of pigs and chickens to yield ATP (He et al. 2018, 2019a). Also, Gln and Glu contribute more ATP to pig and chicken enterocytes than glucose and fatty acids (He et al. 2018, 2019a; Wu et al. 1995). Therefore, AAs are indispensable for the optimal function and health of the small intestines.

6.5.2 Regulation of Gut Microbiota Composition and Diversity

The intestinal microbiota is now recognized to have broad biological effects on the health and growth of both humans and animals (Sommer et al. 2017). AAs can influence the composition and diversity of the intestinal microbiota in pigs, thus improving intestinal function. For example, dietary supplementation with 1% Gln increased the abundance of intestinal-friendly microbiota (*Bacteroidetes* and *Actinobacteria*), while decreasing the abundance of pernicious bacteria (*Oscillospira* and *Treponema*), thus alleviating constipation in sows during late gestation (Zhang et al. 2017). Supplementing 0.2% to 0.4% Trp to a corn- and soybean meal-based diet that contained 0.2% Trp reduced the

abundances of *Clostridium* species, which are potential pathogenic bacteria, and increased abundances of *Prevotella* and *Roseburia*, which can regulate homeostasis in the large intestine of weaned piglets (Liang et al. 2018). Of note, some Trp-metabolizing bacteria are enriched in both the small and large intestines (Liang et al. 2019; Liang et al. 2018). This confirms that bacteria in the intestine are primarily responsible for the use of dietary Trp (Wu 1998). Recently, Wang et al. (2020) reported that dietary supplementation of Trp (0.1 mg/g BW per day in drinking water) to mice with dextran sodium sulfate-induced colitis modulated intestinal immune response and reduced mucosal injury partly through attenuating the activation of TLR4-STAT3 signaling and nuclear p-65. These findings also provide evidence that Trp-metabolizing bacteria may contribute to the beneficial effects of dietary Trp on the integrity of the intestinal mucosa and the responses of immune cells in the intestine (Liang et al. 2019).

6.5.3 Prevention of Viral Infection

There is emerging evidence that AAs play important roles in protecting pigs from viral infections. Chen et al. (2015) reported that Gln had a positive effect to ameliorate reproductive failure caused by porcine circovirus type 2 (PCV2). Further research indicated that Gln starvation increased PCV2 replication by promoting the activation of p38 MAPK associated with the down-regulation of intracellular glutathione levels (Chen et al. 2015). The generation of reactive oxygen species, which is induced by a Gln deficiency, also activates the JAK2/STAT3 signaling pathway and induces autophagy to promote PCV2 infection (Liu et al. 2018a). Likewise, Arg suppresses viral protein interactions and promotes the inactivation of viruses using mechanisms that depend on concentrations of the virus, pH and temperature (Naito et al. 2009). Complex mechanisms are involved in the inactivation of viruses by Arg (Naito et al. 2009; Ikeda et al. 2012) and include the NO-dependent killing of viruses (Li et al. 2007). Trp also exhibits an anti-virus effect (Rabbani and Barik 2017). The degradation of Trp via the IDO pathway reduces the availability of Trp for catabolism to 5-hydoxytryptophan (a protector of viral growth) via the Trp hydroxylase pathway, thereby inhibiting human parainfluenza virus type 3 (Rabbani and Barik 2017). Interestingly, IDO is one of the genes whose expression is induced by interferons to exert antiviral effects. Thus, it is possible that AAs may not act on viruses directly, but destroy viruses and mitigate the virus-induced tissue damage through altering the production of metabolites, regulating protein synthesis, and improving immune responses. In support of this view, dietary supplementation with Arg (0.4 g/kg BW per day) to rotavirus infected piglets fed a standard milk replacer diet augmented intestinal protein synthesis by activating mTOR and p70 (S6k), thereby facilitating restitution and villus regrowth in the intestine (Corl et al. 2008). Dietary supplementation with 1% Leu improved growth performance, and alleviated diarrhea in rotavirus-challenged weanling pigs (Mao et al. 2015). Leu may exert this beneficial effect by improving the digestive and absorptive function of the small intestine and non-specific mucosal barrier mechanisms via the activation of mTOR cell signaling (Mao et al. 2015). Furthermore, dietary supplementation with both Thr and Trp to growing pigs inoculated with a modified live porcine reproductive and respiratory syndrome virus vaccine promoted the expression of TLR3 and TLR7 mRNA in lymph nodes and enhanced immune responses, thereby mitigating lung damage and improving growth performance (Xu et al. 2015). These results suggest that dietary supplementation with functional AAs is a promising strategy to protect animals and humans from viral infections (Table 6.2).

6.6 Use of Amino Acids to Improve the Nutrition, Health, and Productivity of Pigs

New knowledge of AA metabolism and function in pigs has prompted us to re-evaluate the multiple roles of AAs in the nutrition, health, and

Table 6.2 Functions of amino acids in animals, including pigs

Function	Amino acids
Energy substrate for the small intestine	Gln, Glu, and Asp
Regulation of enterocyte growth and apoptosis	Gln, Glu, Arg, Pro, Trp, and Gly
Regulation of gut microbiota composition	Gln, Arg, Trp, Pro, and Hyp
Maintenance of intestinal mucosal barrier integrity	Gln, Glu, Asp, Arg, Pro, Gly, Trp
Antiviral effects	Gln, Arg, Leu, Trp, and Pro
Antioxidant effects	Gln, Glu, Asp, Arg, Pro, and Gly
Anti-inflammatory effects	Gln, Glu, Asp, Trp, Gly, Cys, Hyp
Regulation of metabolism	Gln, Glu, Arg, Asp, Pro, Gly, Trp

Hyp 4-hydroxyproline

Table 6.3 Major mechanisms responsible for the effects of functional amino acids to improve growth and health of pigs

Pigs	Amino acids (AAs)	Active molecules or major mechanisms
Gestating swine	Arginine-family of AAs, Leu, Cit	Polyamine, NO, and MTOR signaling
Lactating sows	Gln, Glu, Arg, Leu, BCAAs, Cit	NO, MTOR signaling, downregulation of ubiquitin and proteasome expression
Postweaning pigs	Gln, Glu, Asp, Arg, Pro, Gly, Leu, Trp, Cit	Gene expression (e.g., AA transporters, and tight-junction proteins) and anti-inflammation
Growing-finishing pigs	Arg, Gly, Leu, Glu, Cit	MTOR signaling, lipid metabolism, and anti-oxidative responses
Boars	Arg, Cit	NO, polyamines, and anti-oxidative responses

BCAAs branched-chain amino acids, *Cit* citrulline, *MTOR* mechanistic target of rapamycin, *NO* nitric oxide

productivity of pigs. Indeed, intensive studies show that traditional feed ingredients cannot supply sufficient AAs to support maximum growth and development of pigs (Hou et al. 2015, 2016a; Wu et al. 2014). Instead, specialized nutritional formulas are needed to meet the requirements of pigs at different stages of their growth and production activities (e.g., reproduction and lactation), and different AAs may act through different or common mechanisms (Table 6.3).

6.6.1 Amino Acid Nutrition in Gestating Pigs

In practical swine production, the number of live-born piglets that a sow delivers is far less than the number of oocytes ovulated (10–15 vs 20–30; Ji et al. 2017). The unfavorable intrauterine conditions during gestation lead to embryo/fetus maldevelopment and losses, which represents a significant obstacle to maximizing the reproductive efficiency of gilts and sows (Vonnahme et al. 2001). Maternal nutrition is a major factor that affects the survival, growth, and development of conceptuses in pigs (Wu et al. 2006). However, maternal feed intake is restricted to avoid excessive fat accumulation in gilts or sows and prevent them from becoming overweight or obese during gestation. Moreover, AAs, such as Gln, Glu, and Arg, undergo extensive catabolism in the small intestine, and only portions (3–85%) of the dietary AAs enter the portal circulation of pregnant gilts (Stoll and Burrin 2006; Wu et al. 2014). Therefore, providing the pregnant dam with adequate amounts of AAs is vital for improving pregnancy outcomes [including the alleviation of intrauterine growth restriction (IUGR)] in pigs.

The amounts of the Arg family of AAs are particularly high in the allantoic fluid of pigs during early gestation, suggesting that they play critical roles in the growth and development of conceptuses (Wu et al. 1996a). A growing body of evidence supports this notion. First, supplementing 1% Gln to the diet of gilts between days 90 and 114 of gestation increased the litter

birth weight of live-born piglets at birth, while reducing variation in birth weights and the mortality of live-born piglets by 33% and 46%, respectively (Wu et al. 2011b). Second, sows that received 1.3% L-Arg-HCl supplementation between days 1 and 14 of gestation had more total piglets born (14.29 vs 12.22) and piglets born alive (13.24 vs 11.43) per litter than for control sows (Li et al. 2015). Third, gilts that received dietary supplementation with 1.0% L-Arg-HCl (0.83% Arg) between days 30 and 114 of gestation increased the numbers of live-born piglets by 2 per litter and litter birth weight by 24% (Mateo et al. 2007). Similar findings were obtained for gilts receiving dietary supplementation with 0.4% or 0.8% Arg between days 14 and 25 of gestation (Li et al. 2014). Fourth, supplementation with Pro (14 g/day) to the diets of sows between days 30 and 114 of gestation increased litter size and birth weights (Gonzalez-Anover and Gonzalez-Bulnes 2017). Furthermore, supplementing 1% Gln to multiparous sows from day 85 of gestation until farrowing increased the average birth weight of piglets, as well as their intestinal development and abundances of tight-junction proteins, while decreasing the within-litter variation in body weights of newborn piglets (Zhu et al. 2018). Combinations of these Arg family AAs also improved the reproductive performance of pigs. For example, adding a mixture of Arg and Gln (0.6% Gln plus 0.4% Arg) to a corn- and soybean meal-based diet increased the number of live-born piglets by 1.4 per litter and litter birth weight (+10% for all piglets born and + 15% for live-born piglets; Wu et al. 2010). In addition to Arg and Gln, other AAs may also be beneficial for fetal growth and development. For example, supplementing 0.4% to 0.8% Leu to a corn- and soybean meal-based diet for gestating swine during late gestation (day 70 to farrowing) enhanced the birth weights of piglets (Wang et al. 2018).

Scientists have made great efforts to uncover the underlying mechanisms responsible for the effects of AAs on pregnancy in swine. NO and polyamines, metabolites of Arg, play essential roles in improving pregnancy outcomes (Wu et al. 2017). Polyamines are key regulators of both DNA and protein syntheses in animal cells. Pro and Orn are the main sources of polyamines in the porcine placenta (Wu et al. 2013b). In gestating swine, Pro is degraded to Orn in maternal and fetal tissues, which is utilized for the synthesis of polyamines via ODC, spermidine synthase, and spermine synthase. Of note, expression of ODC is stimulated by Gln, and Gln is also a precursor of Orn (Wu et al. 2010). Therefore, Gln promotes polyamine synthesis in the conceptus. In addition, Arg is converted into Pro and Orn in maternal tissues, with both Pro and Orn contributing to the synthesis of polyamines in porcine placentae (Wu et al. 2006). This helps to compensate for the lack of arginase (the enzyme that hydrolyzes Arg into Orn and urea) in the porcine placenta. Consistent with this view, the concentrations of spermidine and putrescine were greater in the plasma of gestating sows on day 28 when receiving Arg supplementation between days 15 and 30 or between days 1 and 30 of gestation (Li et al. 2015). In support of this view, dietary supplementation with Pro to gestating mice also increased the concentrations of polyamines in fetal fluids and placenta (Liu et al. 2019a, b) and modulated immune responses at the placenta-uterine interface (Liu et al. 2020). Unlike polyamines, NO is generated only from Arg by NOS in the porcine placenta. NO induces angiogenesis and blood flow in the uterus and placenta, which is beneficial for the transfer of nutrients from the mother to her conceptuses to support their growth and development (Meininger and Wu 2002). Accordingly, dietary supplementation with Arg increased NO production in the placenta of gestating sows (Wu et al. 2010), as well as the angiogenesis and growth of placentae (Wu et al. 2017), resulting in increases in embryonic/fetal survival and litter size, as noted previously. In addition, Arg and other AAs (e.g., Gln, Leu, and Pro) may regulate the growth and development of skeletal muscle and other tissues of embryos and fetuses via mTOR cell signaling (Bazer et al. 2015; Ji et al. 2017), as well as the postnatal growth and development of skeletal muscle in offspring (Ji et al. 2017).

6.6.2 Amino Acid Nutrition in Lactating Pigs

In pigs, Arg undergoes intensive degradation by arginase in the lactating mammary glands (Wu et al. 2018). This necessitates an adequate provision of Arg to mammary epithelial cells for milk production. In support of this view, supplementing 1% Arg-HCl to the diets enhanced milk production by 21% in the first week of lactation and by 11% during a 21-day suckling period (Mateo et al. 2008). Laspiur and Trottier (2001) demonstrated that although dietary supplementation with Arg to lactating sows did not improve milk production, it reduced their weight loss and enhanced feed efficiency (Laspiur and Trottier 2001). The beneficial effect of Arg on milk production is partially mediated by the production of NO, which acts as a vasodilator and angiogenic factor to increase blood flow to the mammary gland (O'Quinn et al. 2002). Arg also increases protein synthesis in porcine mammary epithelial cells by activating mTOR cell signaling, while inhibiting protein degradation in these cells by down-regulating the expression of both ubiquitin and proteasomes (Ma et al. 2018). Thus, Arg enhances the synthesis and secretion of milk proteins by the lactating mammary glands (Ma et al. 2018). Furthermore, dietary supplementation with 1% Arg to lactating sows increased the concentrations of creatine in their milk by 42% (our unpublished work). This observation is highly significant for sucking piglets (particularly those with a low birth weight), because creatine is essential for the growth and development of multiple organs, particularly the skeletal muscle and brain (Wu 2020).

Lactating sows have high requirements for BCAAs (Kim et al. 2009). Interestingly, the uptake of BCAAs by porcine mammary glands substantially exceeds their output in milk (Trottier et al. 1997). Glu, Gln, Asp and Ala are the main metabolic products of BCAAs in the lactating porcine mammary glands (Li et al. 2009). In accordance with this finding, the lactating porcine mammary glands produces 125% more Gln in milk than its uptake from arterial blood, and Gln is the most abundant free AA in sow's milk (Haynes et al. 2009). Thus, dietary supplementation with 1% Gln to lactating sows increased the concentrations of Gln in milk, maternal plasma, maternal skeletal muscle, milk production, and piglet growth (Wu et al. 2011b). Likewise, supplementing Glu plus Gln to the diet of lactating sows increased the concentrations of lipids in colostrum and mature milk (Santos de Aquino et al. 2014). However, work on BCAA nutrition in lactating sows is limited and beset with conflicting results likely due to the imbalances of BCAAs in diets (Rezaei et al. 2016). Nonetheless, there are reports that supplementing BCAAs to lactating sows increases litter weaning weights (Richert et al. 1997a, b; Moser et al. 2000; Paulicks et al. 2003). Unfortunately, the milk yields of the sows were not measured in those studies. Of note, Che et al. (2019) reported that dietary supplementation with valine to gilts during late pregnancy increased the production of proteins in colostrum. Because Leu and valine can activate mTOR cell signaling in porcine mammary epithelial cells (Li et al. 2011b; Zhang et al. 2019), it is reasonable to propose that a mixture of BCAAs in proper ratios are required for maximum milk production by lactating sows.

6.6.3 Amino Acid Nutrition in Piglets

As noted previously, Gln and Glu are enriched in milk. However, milk-born Gln is insufficient for maximal growth of sow-reared piglets (Wu et al. 2011b; Hou and Wu 2018). Haynes et al. (2009) reported that Gln supplementation promoted the growth of sow-reared piglets by 12%, indicating that augmenting Gln provision beyond that from milk was beneficial for improving the growth performance of the suckling neonates (Haynes et al. 2009). Similar findings were obtained for Glu, as oral administration of monosodium glutamate to sow-reared piglets (0.5 and 1 g/kg BW per day) for 21 days increased the expression of Glu receptors and Glu transporters in their stomach and small

intestine (Zhang et al. 2013). Although Leu is highly abundant in sow's milk (Wu and Knabe 1994), this AA has a regulatory role in stimulating the syntheses of muscle proteins and syntheses of Glu and Gln in piglets (Hou et al. 2016a). Unlike Leu, Arg and Gly are relatively deficient in sow's milk (Wu and Knabe 1994). However, the growth of piglets is sensitive to the external provision of Arg, Gly and Leu. Several lines of evidence support this view. First, dietary supplementation with 0.2% and 0.4% Arg dose-dependently enhanced the concentrations of Arg in plasma (30% vs 61%), and increased the BW gain of milk-fed pigs (28% vs 66%) (Kim and Wu 2004). Second, dietary supplementation with 0.5, 1 and 2% Gly increased daily weight gains and improved intestinal health, indicating that Gly is a nutritionally essential AA for maximal growth of sucking piglets (Wang et al. 2014b). Third, oral administration of 0.7 and 1.4 g Leu/kg BW to 7- to 21-day-old sow-reared piglets increased their daily BW gains by 10.6% and 11.9%, respectively, compared with the control group, and enhanced the expression of Leu transporters in the jejunum of the sow-reared pigs (Sun et al. 2015). Further studies indicated that the beneficial effects of Leu on the growth of neonates were associated with increases in both intestinal development and lean tissue growth (Columbus et al. 2015; Sun et al. 2015). Collectively, adequate supplementation with these functional AAs is necessary for maximum and optimal growth of sucking piglets.

Piglets normally suffer from weaning stress, leading to reduced feed intake and intestinal dysfunction. Emerging evidence indicates an important role for Gln in maintaining intestinal physiology and function, but the amount of Gln from the diet and the synthesis of Gln from glucose plus BCAAs and other AAs is inadequate for maximum growth of weaning piglets (Wu et al. 1996c; Wang et al. 2008). Thus, dietary supplementation with Gln is essential for maximizing the growth performance of weanling piglets. This notion is supported by several lines of experimental evidence. First, dietary supplementation with 1.0% Gln enhanced BW gains between 21–28 day of age, as well as the integrity and villus height of the intestinal epithelium (Wu et al. 1996c; Wang et al. 2008; Wang et al. 2015b). Similarly, dietary supplementation with 1–4% monosodium glutamate (Rezaei et al. 2013) or 2% Glu (Lin et al. 2014) to weanling piglets enhanced the concentrations of glutathione, antioxidative capacity, and integrity of the small intestine, as well as its digestive and absorptive functions. In addition, other AAs can also improve the growth performance of weanling piglets by enhancing intestinal function. For example, supplementing 1% Pro to a corn- and soybean meal-based diet enhanced villus height in the jejunum, the weight of the small intestine, and the growth performance of weanling pigs (Wu et al. 2011a). Similarly, dietary supplementation with 0.5% and 1.0% Asp alleviated growth suppression and intestinal damage induced by a lipopolysaccharide challenge in weaned pigs (Pi et al. 2014). These results again highlight the crucial roles of important AAs in the intestinal development, as well as the growth and well-being of weanling piglets.

6.6.4 Amino Acid Nutrition in Growing-Finishing Pigs

NEAAs and EAAs constitute 60% and 40% of total AAs in pigs, respectively (Table 6.1). The size of the free AA pool is very small in the body, representing only 0.9% of total AAs in the body (Table 6.1). Thus, the animals must be fed regularly to provide free AAs for metabolic processes, including protein and creatine syntheses. In growing-finishing pigs, the deposition of excessive amounts of subcutaneous white adipose tissue (e.g., backfat) is a major concern in market-weight pigs fed conventional finishing diets (NRC 2012). Leu is known to be an important nutrient that stimulates protein synthesis in the skeletal muscle of pigs (Columbus et al. 2015). In addition, Hu et al. (2019) reported that dietary supplementation with 1% Leu for fattening pigs increased biceps femoris muscle weights. Conversely, reduced muscle mass was associated with decreases in the intramuscular concentrations of Leu, Thr and Val (Sales et al.

2013). These results further highlight a key role of Leu in regulating muscle protein anabolism (Volpi et al. 2003).

Besides Leu, dietary supplementation with Arg and Glu also promote skeletal muscle growth in pigs (Wu 2010; Hou and Wu 2018). For example, supplementing 0.5–2% Arg to a corn- and soybean meal-based diet for 30- to 121-d-old pigs dose-dependently reduced the concentrations of ammonia, cholesterol, free fatty acids, triglycerides in plasma, as well as white fat in the body, indicating a promising role for Arg in improving lean tissue mass (Hu et al. 2015). Similarly, supplementing 1% Arg to the diet of 110-day-old barrows for 60 days reduces serum triglycerides by 20% and whole-body fat content by 11%, while increasing whole-body skeletal-muscle content by 5.5%, and intramuscular glycogen content by 42% (Tan et al. 2009). This resulted in a 0.32 increase in the intramuscular pH at 45 min post-mortem, a greater intramuscular anti-oxidative capacity, and better meat quality (Ma et al. 2010; Tan et al. 2009). Likewise, dietary supplementation with 1% Glu favorably decreased average back fat thickness and increased intramuscular fat deposition in growing-finishing pigs (Hu et al. 2017a). Furthermore, adding 3% monosodium glutamate to a corn- and soybean meal-based diet beneficially modified the lipid content and fatty acid profile in the skeletal muscle of pigs by regulating the expression of genes related to lipid metabolism, lipid composition, and muscle fiber composition (Kong et al. 2015). Additionally, dietary supplementation with Glu plus Arg decreased average back fat thickness and the percentage of subcutaneous fat, but increased the intramuscular fat content of longissimus dorsi and biceps femoris muscles, whereas supplementation with Glu + Leu increased biceps femoris muscle mass and the concentrations of Glu and carnosine in the biceps femoris muscle (Hu et al. 2017a; Hu et al. 2019). Findings from these studies are expected to guide the development of optimal diets for growing-finishing pigs.

6.6.5 Improved Arg and Gly Nutrition to Enhance the Survival and Growth of IUGR Pigs

Among livestock species, pigs exhibit the greatest embryonic loss (up to 50%) and the most severe, naturally occurring IUGR due to inadequate uterine capacity as well as inadequate maternal and fetal AA nutrition (Wu et al. 2006). IUGR piglets represent 20–25% of all pigs born (Wu et al. 2010). At birth, runt piglets may weigh only one-half or even one-third as much as their largest littermates, and the weights of the small intestine and skeletal muscle of runt pigs are disproportionately much less than those for normal-birth-weight littermates (Wang et al. 2008). Compared to littermates with a normal birth weight, IUGR pigs have greater neonatal morbidity and mortality (representing 76% of preweaning deaths in swine), as well as lower postnatal growth and meat quality (Ji et al. 2017). Thus, preweaning survival rates decrease gradually from 95% to 15% as birth weights of piglets decrease from 1.60 to 0.60 kg (Quiniou et al. 2002). At present, IUGR piglets are culled on farms and there are no effective nutritional means to prevent their death or enhance their growth, resulting in enormous financial losses. We discovered that these compromised pigs are underdeveloped with respect to the syntheses of Arg and Gly and, therefore, are severely deficient in these two FAAs (Hu et al. 2017b). Thus, deficiencies of Arg and Gly may be major limiting factors for the postnatal growth and survival of IUGR pigs. This notion is supported by the following experimental evidence. First, the survival rates of IUGR piglets receiving oral administration of 0.0, 0.1, 0.2 or 0.4 g Arg (in the form of L-arginine-HCl) per kg BW twice daily between days 0 and 14 of life were 50%, 75%, 90% and 90%, respectively (Long et al. 2017). When compared to the control group (average daily gain = 152 g/day between days 1 and 14 of life), IUGR piglets administered 0.2 and 0.4 g Arg/day per kg BW gained 19% and 31% more BW, respectively. Thus, the growth

and survival of IUGR piglets can be improved through dietary supplementation with L-arginine-HCl. Second, supplementing 1% Gly to a corn- and soybean meal-based diet for post-weaning IUGR pigs (weaned at 21 days of age) for 127 days did not affect their feed intake (per kg BW), but increased their growth rates by 28%, 15%, and 10% during days 21–35, 35–64, and 65–120 of age, respectively (He et al. 2019b). Importantly, by day 120 of age, the BW of IUGR pigs receiving Gly supplementation did not differ from that of pigs with a normal birth weight. Thus, our results indicate that dietary supplementation with 1% Gly (a low-cost supplement) beneficially improves growth rates of, and economic returns from, IUGR piglets. With this new nutritional strategy, IUGR pigs can be saved on farms and successfully fed to a market weight.

6.6.6 Amino Acid Nutrition in Boars

The use of artificial insemination by pork producers has increased greatly over the past decades (Flower 2020). For artificial insemination, increasing the quality and quantity of sperm produced by boars is of primary importance. Arg has been identified as a nutrient to improve reproductive performance of boars through enhancing the availability of Arg (the nitrogenous precursor of NO for vasodilation of testicular blood vessels) and polyamines (essential for fertilization) in semen (Wu et al. 2009). In support of this notion, an in vitro study conducted by Funahashi (2002) found that Arg improved capacitation and acrosome reactions of boar spermatozoa via an NO-dependent mechanism. A recent feeding study further showed that dietary supplementation with 0.8% or 1.0% Arg for 42 days remarkably improved semen quality and libido of boars during the hot summer months (Chen et al. 2018). This is of great importance for pig production, because sperm are likely to be more susceptible to the effect of heat stress with reductions in their motility and numbers, as well as an increase in defects. These results indicate that adequate Arg supplementation should be taken into consideration to maximize the reproductive performance of boars.

6.7 Safety of Amino Acid Supplementation in Pigs

Appropriate doses of supplemental AAs are generally safe for animals based on food intake, behavior, as well as physiological parameters in plasma and urine (Wu 2018). The No Observed Adverse Effect Levels (NOAEL) for Arg in pigs are summarized in Table 6.4. However, excessive amounts of any AA in diets can cause AA imbalances, antagonisms, and toxicity (Wu et al. 2013c). Therefore, comprehensive and systematic studies should be conducted regarding the safety of dietary supplementation with AAs to animals, including pigs (Hou and Wu 2018a, b; Wu et al. 2016, 2018).

Pigs between 30 and 121 days of age can tolerate a large amount of supplemental Arg (2% of the diet or 630 mg/kg BW per day for 91 days) without any detectable adverse effects (Hu et al. 2015). However, supplementing 4% Arg (as the Arg base) to a corn- and soybean meal-based diet for growing pigs causes AA imbalances and reduced their growth performance (Edmonds et al. 1997). We found that supplementing up to 1% Arg to diets is safe for gestating sows (between days 14 and 114 of gestation) and lactating sows (between days 1 to 21 of lactation), and had no adverse effects on fetal or neonatal piglets (Mateo et al. 2007; Mateo et al. 2008; Li et al. 2014). For comparison, healthy adult humans (94 to 117 kg BW) can tolerate at least 30 g Arg (as Arg-HCl)/kg BW per day (in two or more divided doses daily) for at least 90 days (McNeal et al. 2018), which is equivalent to 256 to 319 mg/kg BW per day). Of note, neonatal pigs have been reported to be particularly sensitive to high intakes of Arg, as oral administration of 0.29 g Arg/kg BW per day twice daily between 1 and 16 days of age (Getty et al. 2015) or 1.80 g Arg/kg BW per day to 7-day-old enterally fed

Table 6.4 The No Observed Adverse Effect Levels (NOAELs) for supplementation with amino acids to typical diets for swine[a]

Amino acid	Young swine[b]		Adult swine (non-pregnant)[c]		Gestating swine[d]	
	% of supplemental AA in diet	Content of AA in the basal diet (%)	% of supplemental AA in diet	Content of AA in the basal diet	% of supplemental AA in diet	Content of AA in the basal diet
Ala	2.5	1.3	2.7	0.94	2.2	0.78
Arg	2.0	1.3	2.2	1.1	1.0	0.70
Asn	2.0	0.94	2.2	0.77	1.0	0.58
Asp	4.0	1.3	4.2	1.1	2.0	0.76
Cys	0.38	0.37	0.38	0.30	0.25	0.23
Gln	1.0	1.8	1.0	1.6	1.0	1.2
Glu	≥4.0	1.7	≥4.0	1.5	2.0	1.1
MSG	2.0	1.7 (Glu)	2.0	1.5 (Glu)	2.0	1.1 (Glu)
Gly	2.0	0.88	2.0	0.85	2.0	0.55
His	0.50	0.57	0.63	0.44	0.50	0.33
Ile	1.1	0.89	1.3	0.74	1.0	0.51
Leu	2.2	1.8	2.4	1.6	2.0	1.2
Lys	1.4	1.4	1.4	0.90	0.80	0.58
Met	0.40	0.36	0.4	0.28	0.25	0.18
Phe	1.0	0.99	1.1	0.86	0.80	0.60
Pro	2.0	1.6	2.2	1.4	2.0	1.0
Ser	2.0	0.79	2.0	0.83	1.5	0.45
Thr	0.80	0.85	0.80	0.65	0.50	0.49
Trp	0.40	0.25	0.40	0.21	0.30	0.17
Tyr	1.2	0.76	1.3	0.70	0.80	0.45
Val	2.0	1.0	2.2	0.82	1.5	0.65
Cit	2.0	0.0	2.0	0.0	1.0	0.0

[a]Vaues are expressed on the as-fed basis, with the dietary content of dry matter being 90%. Adapted from Hou and Wu (2017, 2018a), Wu (2018), Wu et al. (2011b, 2018)
[b]Neonatal or weanling pigs. The dietary content of crude protein is 20%
[c]Grower-finisher pigs, lactating sows, and adult boars. The dietary content of crude protein is 14–18%
[d]Early period of gestation. If 0.8% arginine is supplemented to the diet, the supplementation should not start before day 14 of gestation. The dietary content of crude protein is 12%, and dietary intake is 2 kg per day. During late gestation, dietary intake can be increased to 2.2 to 2.5 kg per day, depending on the maternal nutritional status. The total supplemental amount of nitrogen should not exceed 12.5% of the nitrogen content in the basal diet
Cit L-citrulline, *MSG* monosodium glutamate

pigs (Wilkinson et al. 2004) had adverse effects of reducing the growth of piglets. However, because the consumption of sow's milk or the dietary intake of AAs were not reported by the authors (Getty et al. 2015) and the basal enteral diet for the young pigs (Wilkinson et al. 2004) lacked both Gln and Asp and was imbalanced in AAs, caution should be exercised in interpreting these results. We did not find any adverse response of 0- to 14-day-old piglets (with either a normal or a low birth weight) to daily oral administration of up to 0.8 g Arg (as Arg-HCl)/kg BW per day (Long et al. 2017). Overall, supplementing up to 2% Arg to typical diets is safe for gestating, lactating, and growing pigs.

Gln itself is not toxic to cells because large amounts of Gln (e.g., 4 mM or approximately 8- to 10-times the physiological concentrations in plasma) are usually included in culture medium for all cell types (Curi et al. 2005). Dietary supplementation with up to 1% Gln (on an as-fed basis) for at least 34 days does not reduce feed intake, and does not contribute to any sickness or death in neonatal, post-weaning, gestating, or lactating pigs (Wu et al. 2011b). However, as with any other AA, a high dose of supplemental Gln

(such as 2% Gln to a corn- and soybean meal-based diet) may reduce the feed intake of weanling piglets, and should be avoided in swine production (Wu et al. 2011b). Collectively, Gln is now recognized as an essential nutrient to exert beneficial effects on the small intestine, as well as whole-body growth and health. Thus, a sufficient supply of dietary Gln is critical for optimum survival, growth, development, lactation, and reproduction in swine (Wu 2010).

Many studies have indicated that dietary supplementation with 0.15–4% Glu or 0.5–4% monosodium glutamate is safe in swine (Hou and Wu 2018b). Based on those studies, Hou and Wu (2018b) indicated that dietary supplementation with at least 2% Glu is safe for pigs of all ages. Because 95–97% of dietary Glu is degraded by the small intestine of pigs (Stoll and Burrin 2006), exogenous supplementation and endogenous synthesis of Glu in the extra-intestinal tissue is necessary to maintain normal intestinal physiology and support maximum growth of weaning, growing and finishing pigs. Therefore, Glu is truly a functional AA and a dietarily essential AA in swine nutrition.

Dietary supplementation with at least 2% Gly or 2.1% Pro is safe for young pigs (Wu et al. 2011a; Wang et al. 2013). This is also true for lactating and gestating sows. Moreover, dietary supplementation with 0.4% Trp or 2.5% Leu to a typical con- and soybean meal-based diet does not adversely affect growing-finishing pigs (Liang et al. 2018, 2019; Yao et al. 2011; Hu et al. 2019). The safe doses of an AA vary with the physiological state of animals, but are generally higher than its content in the basal diet (Table 6.4). Because pigs are sensitive to excessive intakes of cysteine and methionine (Hou and Wu 2018a), caution should be exercised when they are supplemented to the basal diet.

6.8 Economic Benefits of AA Supplementation to Swine Diets

Dietary supplementation with AAs that are deficient in diets or that have regulatory functions in metabolism can increase the growth rates, feed efficiency, and productivity of pigs, decrease the rates of their morbidity and mortality, and shorten the time between the weaning and marketing of hogs. This can lead to improvements in the efficiency of pork production and economic returns to the farmers, as illustrated by the examples with studies involving gestating gilts/sows and IUGR pigs. Specifically, based on the number of sows (33.4×10^6) per year worldwide, an income of US \$45/live-born piglet, one additional live-born piglet/sow per farrowing (2.4 farrowings/year), and saving 90% of IUGR piglets before weaning (Long et al. 2017), an increase in net income due to dietary supplementation with Arg to gestating swine and IUGR piglets is estimated to be US \$136.16/sow per year. For the total number of sows worldwide, the net benefits are US $\$4.55 \times 10^9$/year (Table 6.5). Of note, increasing the number of piglets at weaning allows for a reduction in the number of total sows reared globally, leading to decreases in maintenance costs (both sows and feeds) and the production of manure from swine farms. Similarly, saving one postweaning IUGR piglet/sow per farrowing through post-weaning dietary supplementation with Gly results in a net benefit of \$17.34/pig (Table 6.6). For the total number of 145×10^6 IUGR pigs worldwide, the net benefits are US $\$2.61 \times 10^9$/year (Table 6.6).

In summary, there have been exciting developments in the field of swine AA metabolism and nutrition over the past three decades. Both exogenous supplementation and endogenous syntheses via inter-organ metabolism of AAs are crucial for maintaining physiological homeostasis in the whole body. There is compelling evidence that these nutrients are essential for improving the health, survival, growth, development, lactation, and reproduction of pigs. Future studies in this exciting area of investigation are needed to elucidate the cellular and molecular mechanisms responsible for the beneficial effects of AAs on the overall health, and wellbeing of swine under physiological and pathological conditions, as well as economic returns and agricultural sustainability. Both sufficient amounts and proper ratios of EAAs and NEAAs must be provided in swine diets to achieve the goal of precision nutrition. Because pigs are similar to

Table 6.5 Economic returns from dietary supplementation with L-arginine (Arg) to gestating sows and preweaning IUGR piglets, compared with no arginine supplementation

Variable	No Arg supplementation	Arg supplementation to sows during days 14 to 30 of gestation, and to IUGR piglets during a 21-day period of suckling
A. Arginine supplementation to gestating sows		
Amount of supplemental Arg/sow per year (kg)[a]	0.0	0.614
Cost of supplemental Arg/sow per year (US $)[b]	0.0	– 6.14
Additional number of piglets born alive/sow per year (n)[c]	0.0	2.4
Value of live-born piglets per sow per year (US $)[d]	0.0	108
Net benefit per sow per year (US $)	0.0	+ 101.86
B. Arginine administration to IUGR piglets before weaning		
Number of IUGR piglets born per sow per year (n)[e]	4.8	4.8
Number of surviving IUGR piglets per sow per year (n)[f]	−4.8	4.32
Amount of supplemental Arg to IUGR piglets per sow per year (kg)[g]	0.0	0.242
Cost of supplemental Arg/sow per year (US $)	0.0	– 2.42
Additional labor cost (US $)[h]	0.0	– 181.44
Value of surviving IUGR piglets per sow per year (US $)[i]	0.0	194.4
Saving the cost of raising a sow through rescuing her IUGR offspring (US $/sow per year)[j]	0.0	23.76
Net benefit per sow per year (US $)	0.0	+ 34.3
C. Net benefit per sow per year (US $)	0.0	136.16
D. Net benefit from all sows per year worldwide (US $)[k]	0.0	4.55×10^9

IUGR intrauterine growth restriction

[a]Assuming 2.4 gestations/sow per year. During a gestation, each sow consumes 2 kg diet/day. In the case of Arg supplementation, between days 14 and 30 of gestation, each sow receives 16 g Arg/day (256 g for 16 days/gestation or 614 g Arg/2.4 gestations/year)

[b]Assuming the cost of Arg is US $10/kg. The cost of Arg for each sow is 0.614 kg/year × US $10/kg = US $6.14/year

[c]Assuming that a sow receiving Arg supplementation produces one additional live-born piglet per farrowing. The total number of additional live-born piglets per sow in a year is 2.4 (i.e., 1 additional piglet/sow per farrowing × 2.4 farrowings in a year)

[d]Assuming that the value of a live-born piglet is US $45. For 2.4 more piglets per sow in a year, the total value is $108 per sow in a year (i.e., US $45 × 2.4)

[e]The mean number of IUGR piglets born per sow is 2. The total number of IUGR piglets per sow in a year is 4.8 (i.e., 2 IUGR piglets/sow per farrowing × 2.4 farrowings in a year)

[f]All IUGR piglets are culled on farms when they are not treated with Arg. With oral administration of Arg, the preweaning survival rate of IUGR piglets is 90%

[g]Each IUGR piglet receive 0.8 g g/kg body weight per day. Assuming the mean body weight of an IUGR pig is 3 kg between days 0 and 21 of life, the piglet will receive 2.4 g Arg/day (50.4 g Arg for 21 days). For 4.8 IUGR piglets per sow per year, the use of Arg is 242 g (i.e., 50.4 × 4.8)

[h]The management requires 1.5 min/IUGR pig per administration for each of two persons. The combined time is 3 min per administration × 2 administrations per day = 6 min/IUGR piglet. For 4.8 piglets/sow and 21 days in a year, the time is 6 min/IUGR piglet per day × 4.8 IUGR piglets × 21 days = 605 min = 10.08 hours. Assuming that the additional labor cost is US $18/hour, the total labor cost is US $181.44 per sow per year (i.e., 10.08 × 18)

[i]Assuming that the value of a surviving IUGR piglet is US $45. For 4.32 surviving IUGR piglets per sow in a year, the total value is US $194.4 per sow in a year

[j]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. For 2.4 farrowings per sow per year with the survival rate of IUGR pigs being 90%, the saving is $23.76/sow per year (i.e., 2 × $5.5 × 2.4 × 0.9)

[k]Assuming that the total number of sows per year worldwide is 33.4×10^6 (FAO 2018). The net benefit per sow per year is US $4.55 × 10^9 (i.e., US $136.16/sow per year × 33.4×10^6 sows worldwide)

Table 6.6 Economic returns from dietary supplementation with glycine (Gly) to postweaning IUGR pigs until reaching market body weight[a]

Variable	No Gly supplementation	Gly supplementation supple-to postweaning mentation IUGR pigs until a market body weight
A. Direct benefit from Gly supplementation to a postweaning IUGR pig for 127 days		
Amount of supplemental Gly/IUGR pig (kg)[b]	0.0	2.56
Cost of supplemental Gly/IUGR pig (US $)[c]	0.0	– 2.56
Body weight gain due to Gly supplementation (kg/pig)	0.0	6.9
Value of body weight gain due to Gly supplementation (US $/pig)[d]	0.0	6.9
Net benefit per IUGR pig due to Gly supplementation (US $/pig)	0.0	+ 4.34
B. Indirect benefit from Gly supplementation through saving time and labor i.e., 10 less days for raising an IUGR pig to reach a 90-kg market body weight (US $/IUGR pig per 10 days)[e]	0.0	7.5
C. Indirect benefit from saving the cost of raising a sow through Rescuing her IUGR offspring (US $/pig)[f]	0.0	5.5
D. Total benefit for saving one postweaning IUGR pig and raising it to market body weight (US $/pig)	0.0	17.34
E. Total net benefit from saving and raising postweaning IUGR pigs per year worldwide (US $)[g]	0.0	2.51×10^9

[a]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., $15\% \times 966 \times 10^6$)

[b]An IUGR pig is fed a diet supplemented with 1% Gly. We determined that the average IUGR pig consumed 2.56 kg feed during a 127-day period (He et al. 2019a, b)

[c]The cost of Gly is $1/kg (Dalian Chem Imp. & Exp Group Co., Ltd., http://www.cccme.org.cn/shop/cccme4298/index.aspx)

[d]Average live-weight price of a pig is US $1/kg body weight.

[e]Taken from our previous study (He et al. 2019a, b)

[f]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. We found that all postweaning IUGR pigs receiving Gly supplementation survived to 127 days of life (He et al. 2019a, b)

[g]Calculated as US $17.34/IUGR pig $\times$ 145 $\times$ 10^6 IUGR pigs/year worldwide

humans in nutritional requirements, metabolism and physiology, results from research with swine have important implications for improving the health of human infants and adults.

Acknowledgments This work was supported, in part, by the National Key R&D Program of China (2016YFD0501210; Y. Hou), the Program of National Agricultural Research Outstanding Talents of China (2015; Y. Hou), Agriculture and Food Research Initiative Competitive Grants (2014-67015-21770 and 2015-67015-23276 to G. Wu and F.W. Bazer) from the USDA National Institute of Food and Agriculture, and Texas A&M AgriLife Research (H-8200; G. Wu). We thank students and research assistants in our laboratories for helpful discussions.

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Amino Acid Nutrition and Metabolism in Chickens

7

Wenliang He, Peng Li, and Guoyao Wu

Abstract

Both poultry meat and eggs provide high-quality animal protein [containing sufficient amounts and proper ratios of amino acids (AAs)] for human consumption and, therefore, play an important role in the growth, development, and health of all individuals. Because there are growing concerns about the suboptimal efficiencies of poultry production and its impact on environmental sustainability, much attention has been paid to the formulation of low-protein diets and precision nutrition through the addition of low-cost crystalline AAs or alternative sources of animal-protein feedstuffs. This necessitates a better understanding of AA nutrition and metabolism in chickens. Although historic nutrition research has focused on nutritionally essential amino acids (EAAs) that are not synthesized or are inadequately synthesized in the body, increasing evidence shows that the traditionally classified nutritionally nonessential amino acids (NEAAs), such as glutamine and glutamate, have physiological and regulatory roles other than protein synthesis in chicken growth and egg production. In addition, like other avian species, chickens do not synthesize adequately glycine or proline (the most abundant AAs in the body but present in plant-source feedstuffs at low content) relative to their nutritional and physiological needs. Therefore, these two AAs must be sufficient in poultry diets. Animal proteins (including ruminant meat & bone meal and hydrolyzed feather meal) are abundant sources of both glycine and proline in chicken nutrition. Clearly, chickens (including broilers and laying hens) have dietary requirements for all proteinogenic AAs to achieve their maximum productivity and maintain optimum health particularly under adverse conditions such as heat stress and disease. This is a paradigm shift in poultry nutrition from the 70-year-old "ideal protein" concept that concerned only about EAAs to the focus of functional AAs that include both EAAs and NEAAs.

Keywords

Amino acids · Protein · Nutrition · Growth · Health · Egg production

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Abbreviations

AA	amino acid
BCAA	branched-chain amino acid
BW	body weight

G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1285, https://doi.org/10.1007/978-3-030-54462-1_7

EAA	nutritionally essential amino acid
ICV	intracerebroventricular
NEAA	nutritionally nonessential amino acid
NMDA	N-methyl-D-aspartate
NRC	National Research Council
POX	proline oxidase
α-KG	α-ketoglutarate

7.1 Introduction

Amino acids (AAs) are the building blocks of protein, which is the major dry matter component of growth in chickens and their eggs (Baker 2009). As foods for humans, poultry and eggs provide high-quality protein that contains sufficient amounts and proper ratios of AAs, therefore playing an important role in the growth, development, and health of humans (McNeill et al. 2017; Réhault-Godbert et al. 2019). Chicken or poultry byproducts are also low-cost and high-quality protein feedstuffs for livestock species, fish, and companion animals (Li and Wu 2020). In addition, taurine (a nonproteinogenic AA), which is present abundantly in poultry tissues, is essential for the integrity and function of the eyes, heart and skeletal muscle, as well as the nervous, digestive, immune, and reproductive systems in both mammals and birds (Wu 2020a). As for other animals, adequate intakes of dietary AAs are crucial for the optimum efficiency of poultry production (Baker 2009). Either an excess or a deficiency of AAs has negative impacts on the health and productivity of chickens.

Compared with the chicken breeds used 30 years ago, modern breeds of broilers grow faster and gain more lean tissues, and modern breeds of leghorns lay more eggs (Applegate and Angel 2014; Bailey 2020). However, there is not much progress in our understanding of AA nutrition and metabolism in chickens over the past three decades (Bailey 2020). It is known that the patterns of free AAs in plasma and skeletal muscles of chickens (Table 7.1) differ from those in mammals (Wu 2018a, b) and that ammonia is removed primarily as uric acid in birds rather than as urea in mammals (Wu 2013). Thus, there are distinct differences in AA metabolism and nutrition between avian and mammalian species. Because improving the efficiency of poultry production and sustaining the global environment are important goals of animal agriculture (Wu et al. 2020), much attention has been paid to the formulation of low-protein diets through the addition of low-cost crystalline AAs. This necessitates renewed interest in the fundamental knowledge of cell- and tissue-specific synthesis and catabolism of AAs in chickens. Although historic nutrition research has focused on nutritionally essential amino acids (EAAs) that are not synthesized or are inadequately synthesized in the body (Baker and Han 1994), increasing evidence shows that the traditionally classified nutritionally nonessential amino acids (NEAAs; coined in 1912) such as glutamine and glutamate have physiological and regulatory roles other than protein synthesis in chicken growth and egg production (Wu 2014, 2018a, b). The major objective of this article is to highlight recent advances in AA nutrition and metabolism in meat-type and egg-laying chickens.

7.2 Digestion of Dietary Protein and Absorption of Its Hydrolysis Products in Chickens

The digestive system of chickens differs from that of pigs, but these two species share common features of digestion and absorption (Wu 2018a). In birds, ingested feed passes through the esophagus into the crop (a temporary storage pouch) and then enters the proventriculus (also known as the "true stomach"). Within the proventriculus, feed is mixed with HCl and digestive enzymes as in mammals to initiate the hydrolysis of proteins and fats. This acid is produced from NaCl and carbonic acid (H_2CO_3) by parietal cells in the gastric glands of the stomach to create an acidic environment (e.g., pH = 2.5–3.5; equivalent to $10^{-2.5}$ to $10^{-3.5}$ M HCl). Gastrin (released by the parietal cells of the stomach) and acetylcholine (released by the vagus nerve and enteric system) stimulate gastric

Table 7.1 Concentrations of free amino acids in the plasma and skeletal muscles of 6-week-old fed and 48-h fasted male White Leghorn chickens[a]

Amino acid	Fed chickens			48-h fasted chickens		
	Plasma (nmol/ml)	Gastrocnemius muscle (nmol/mg tissue)	Pectoralis muscle	Plasma (nmol/ml)	Gastrocnemius muscle (nmol/mg tissue)	Pectoralis muscle
Ala	521 ± 14	3.21 ± 0.15	1.20 ± 0.08	616 ± 8*	3.93 ± 0.10*	1.70 ± 0.09*
β-Ala	44 ± 7	1.08 ± 0.30	0.83 ± 0.13	79 ± 6*	1.49 ± 0.15	0.78 ± 0.05
Arg	461 ± 32	0.40 ± 0.03	0.24 ± 0.02	297 ± 13*	0.36 ± 0.01	0.26 ± 0.02
Asp	65 ± 5	1.55 ± 0.09	0.31 ± 0.02	152 ± 13*	0.74 ± 0.05*	0.32 ± 0.01
Asn	114 ± 13	0.41 ± 0.04	0.18 ± 0.02	86 ± 11	0.32 ± 0.03	0.21 ± 0.04
Cit	1.01 ± 0.03	0.016 ± 0.002	0.015 ± 0.001	0.74 ± 0.02*	0.014 ± 0.001	0.013 ± 0.001
Cys	251 ± 17	0.23 ± 0.02	0.21 ± 0.01	173 ± 12*	0.18 ± 0.01*	0.17 ± 0.01*
Gln	1089 ± 60	9.45 ± 0.64	1.41 ± 0.04	941 ± 26	3.02 ± 0.17*	1.38 ± 0.07
Glu	265 ± 22	3.43 ± 0.28	0.90 ± 0.06	317 ± 11*	1.69 ± 0.12*	0.89 ± 0.05
Gly	496 ± 16	1.05 ± 0.14	0.71 ± 0.09	709 ± 28*	1.37 ± 0.12	0.80 ± 0.08
His	138 ± 7	0.10 ± 0.01	0.14 ± 0.02	181 ± 12*	0.32 ± 0.03*	0.24 ± 0.01*
Hyp	103 ± 6	0.048 ± 0.002	0.043 ± 0.002	71 ± 4*	0.044 ± 0.002	0.041 ± 0.002
Ile	228 ± 10	0.21 ± 0.02	0.23 ± 0.02	241 ± 9	0.18 ± 0.02	0.19 ± 0.02
Leu	305 ± 24	0.28 ± 0.03	0.32 ± 0.05	287 ± 5	0.31 ± 0.04	0.26 ± 0.03
Lys	209 ± 13	0.47 ± 0.06	0.23 ± 0.03	446 ± 26*	0.52 ± 0.08	0.26 ± 0.04
Met	75 ± 6	0.094 ± 0.006	0.10 ± 0.01	77 ± 2	0.11 ± 0.01	0.12 ± 0.02
Orn	20 ± 2	0.046 ± 0.003	0.032 ± 0.002	21 ± 2	0.042 ± 0.003	0.030 ± 0.002
Phe	212 ± 12	0.19 ± 0.03	0.18 ± 0.02	244 ± 5	0.17 ± 0.02	0.20 ± 0.02
Pro	349 ± 21	0.29 ± 0.04	0.24 ± 0.02	251 ± 14*	0.27 ± 0.02	0.22 ± 0.01
Ser	481 ± 33	1.60 ± 0.15	0.71 ± 0.04	503 ± 31	1.17 ± 0.08*	0.84 ± 0.06*
Tau	249 ± 33	11.8 ± 0.30	0.45 ± 0.06	512 ± 29*	9.20 ± 0.42*	0.42 ± 0.05
Thr	260 ± 17	0.77 ± 0.09	0.57 ± 0.05	294 ± 10	0.76 ± 0.07	0.66 ± 0.05
Trp	68 ± 2	0.04 ± 0.01	0.05 ± 0.01	82 ± 3*	0.06 ± 0.01*	0.06 ± 0.01
Tyr	171 ± 10	0.14 ± 0.01	0.16 ± 0.01	250 ± 11*	0.21 ± 0.02*	0.23 ± 0.02*
Val	288 ± 19	0.46 ± 0.04	0.33 ± 0.03	316 ± 4	0.40 ± 0.04	0.32 ± 0.03

Cit = citrulline; Hyp = 4-hydroxyproline; Orn = ornithine; Tau = taurine
*$P < 0.05$ vs the Fed group as analyzed by unpaired t-test
[a]Taken from Watford and Wu (2005). Data are means ± SEM, n = 5

acid production. In contrast, somatostatin (also known as growth hormone-inhibiting hormone; produced by D cells in the stomach, the small and large intestine, and also the pancreas) and secretin (produced by the S cells of the duodenum) inhibit gastric acid secretion. Gastric HCl aids in protein digestion by: (1) converting inactive gastric proteases (pepsinogens A, B, C, and D and pro-chymosin, collectively called zymogens, which are synthesized and released by the chief cells of the gastric glands) to active proteases (pepsins A, B, C, and D, and chymosin); and (2) denaturing dietary proteins so that they lose their natural folded structures to expose their peptide bonds to the active proteases for hydrolysis. The specific activities of pepsinogens A, B and C in the proventriculus increase progressively during the embryonic development, reach a temporary peak several days before hatching, and increase 30-fold within 24 h after hatching, in comparison with the values at birth, regardless of enteral feeding (Yasugi and Mizuno 1981). Dietary protein, AAs, histamine, acetylcholine, gastrin, gastrin-releasing peptide, vagal stimulation, and vasoactive intestinal peptide enhance the secretion of gastric proteases (Wu 2018a).

The digesta from the proventriculus enters the gizzard (ventriculus; also known as the mechanical stomach; pH = 2.5–3.5) for grinding, mixing and mashing. The digesta includes the large

polypeptides, small peptides and free AAs resulting from the enzymatic hydrolysis by pepsins in the stomach, as well as dietary proteins that are resistant to pepsins in the stomach. The transit time of food particles through the proventriculus and gizzard is about 90 min. Food particles from the gizzard, the food particles enter the small intestine for further digestion. The pancreas plays an essential role in the digestion of dietary protein because its acinar cells secrete pro-proteases into the lumen of the duodenum (pH = 6.0–6.5). These enzymes are the zymogens of endopeptidases (trypsin, chymotrypsins A, B and C, collagenase, and elastase) and exopeptidases (carboxypeptidases A and B), and are activated in the duodenal lumen by a cascade of limited proteolysis by enterokinase to remove an N-terminal oligopeptide (2 to 6 AA residues) from each zymogen. Specifically, enterokinase (released by enterocytes of the duodenum) converts trypsinogen into trypsin through the removal of an N-terminal hexapeptide. Subsequently, trypsin converts other pancreatic zymogens into active forms (e.g., chymotrypsins A, B and C, elastase, and carboxypeptidases A and B). In addition, aminopeptidases (exopeptidases; released by the mucosa of the small intestine) cleave the last peptide bond adjacent to an AA at the NH_2 terminus. Furthermore, prolyl oligopeptidase (prolyl endopeptidase; released by the small intestine) cleaves proline or hydroxyproline from the inside of an oligopeptide that contains the imino acid.

The extracellular proteolysis occurring in the duodenum of poultry is limited due to the short length of this intestinal segment and a short transit time of food particles (about 7 min). The chyme moves into the jejunum (pH = 6.5–7.0), where most proteolysis takes place due to its long length and high protease activities. The transit time of the digesta through the jejunum is about 25 min. Continuous digestion of protein and polypeptides can occur in the ileum (pH = 7.0–7.4) if their hydrolysis is not completed in the jejunum, with the transit time of the digesta through the ileum being about 60 min. The small peptides containing 4–6 AA residues are further hydrolyzed by peptidases that are bound primarily to the brush-border of enterocytes, and to a lesser extent, in the intestinal lumen to form free AAs, dipeptides, and tripeptides. Dipeptides (not containing imino acids, i.e., proline or hydroxyproline) and tripeptides are hydrolyzed by mucosa-derived dipeptidases and tripeptidases, respectively (Wu et al. 2011a). However, dipeptides containing an imino acid are cleaved by mucosa-derived prolidases. The true ileal digestibilities of AAs in the proteins of corn grain, soybean meal, sorghum grain, and meat & bone meal are 85–89%, 86–91%, 84–88%, and 89–91%, respectively, in chickens (Wu 2014).

Absorption of tripeptides and dipeptides by the enterocytes of small intestine occurs through the apical-membrane Na^+-independent, H^+-driven peptide transporter 1 (Gilbert et al. 2010). Sodium is indirectly required for this process because the needed protons are provided by the Na^+/H^+ exchange. Within the enterocytes, tri- and di-peptides are rapidly hydrolyzed by cytosolic peptidases to form free AAs. Because of the high activity of intracellular peptidases, a nutritionally significant quantity of peptides does not transcellularly enter the portal vein or the intestinal lymphatics (Wu 2018a). It is possible that a limited amount of special small peptides [e.g., those containing an imino acid (such as Gly-Pro-OH-Pro, a degradation product of collagen) or a formyl AA (e.g., N-formyl-Met-Leu-Phe, a bacterial peptide serving as a chemotactic)] are absorbed intact from the luminal content to the bloodstream through M cells, exosomes, and enterocytes via transepithelial cell transport (Hou et al. 2017).

Free AAs in the intestinal lumen are absorbed by enterocytes primarily via (a) Na^+-independent system (facilitated system; e.g., for basic AAs as well as small and large neutral AAs) and (b) Na^+-dependent system (active transport; e.g., for acidic AAs as well as small and large neutral AAs) (Matthews 2000). There are reports that elevating dietary AA intake increases the abundance of the $b^{0,+}AT$ mRNA in the jejunum of chickens (Osmanyan et al. 2018) and that dietary supplementation with L-methionine or DL-methionine promotes the expression of the

B^0AT transporter in the small intestine of broilers (Zhang et al. 2017a). The international Nomenclature Committee has named AA transporters according to their solute carrier families based on their gene sequence similarities. Na^+-dependent AA transporters and Na^+-independent AA transporters account for the uptake of 60% and 40% of free AAs from the lumen of the small intestine into enterocytes, respectively (Wu 2018a, b). Before binding to an AA, the Na^+-dependent AA transporter binds to Na^+ first, which will increase its affinity for the AA. As a result, both Na^+ and the AA are transported into the cytoplasm of the enterocyte. To maintain the balance of electrolytes within the enterocyte, the Na^+/K^+-ATPase in its basolateral membrane is responsible for pumping Na^+ out of the cell and getting K^+ into the cell at the expenditure of ATP.

In chickens, the apical membrane of enterocytes actively takes up AAs (including glutamine, glutamate and aspartate) from the lumen of the small intestine. At present, it is unknown about the percentages of dietary AAs entering the portal circulation of any poultry species. This issue can be addressed by cannulating the portal vein of chickens and obtaining blood samples from the portal vein at various time points after feeding for AA analyses, as performed in pigs (Wu et al. 1994). Alternatively, Ussing chambers can be used to assess the transfer of AAs (e.g., 0.5–5 mM glutamine, glutamate, or aspartate) from the luminal (apical, mucosal) side of the small intestine (e.g., jejunum) of chickens to the serosal (or basolateral, facing the blood) side of the gut, as performed in the pig small intestine (Wang et al. 2014). In pigs, about 70% of dietary glutamine (Wu et al. 2011a) and 97% of dietary glutamate (Hou and Wu 2018) are utilized (primarily via oxidation to CO_2) by the small intestine during the first pass into the portal vein. If this is also true for birds, most of the circulating glutamine and glutamate in their bodies must be derived from endogenous synthesis.

Based on the intakes of digestible AAs and the accretion of AAs in the body of 14- and 42-day-old broiler chickens, we estimate that the overall efficiency of digestible AAs for their growth is 65.5% and 60.3%, respectively, with the rates for individual AAs differing greatly from 40% to 79%, depending on age and diet (Tables 7.2 and 7.3). If substantial amounts of dietary AAs are catabolized by the small intestine (either enterocytes, luminal microbes, or both) as reported for pigs (Wu 2013), these efficiency values may be greater, particularly for older birds with more active microbes in the small intestine. If nearly all of the dietary glutamate is utilized by the small intestine in chickens as reported for mammals (Wu 1998), glutamate must be synthesized endogenously from other AAs. We consider this to be highly possible. Nonetheless, in 14- and 42-day-old chickens fed rations containing 21.5% and 18.4% crude protein, respectively, all dietary AAs but glycine appear to meet requirements for growth at the rates of 36.3 and 112 g of body weight per day, respectively. This raises an important question of whether insufficient glycine intake may limit maximum growth of chickens and whether dietary supplementation with glycine can reduce the intake of total AAs by the birds without affecting their growth performance.

7.3 Amino Acid Syntheses in Chickens

Chickens, like other poultry species, do not form the carbon skeletons of the following thirteen proteinogenic AAs: arginine, cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan, tyrosine, and valine (Wu 2013). This is because the birds lack one or more of the enzymes (e.g., pyrroline-5-carboxylate synthase, carbamoylphosphate synthase-I, and ornithine carbamoyltransferase) required for the biosynthesis of those carbon skeletons from non-AA materials. Except for arginine, cysteine, lysine, and threonine, the α-ketoacids of the proteinogenic AAs can undergo transamination with glutamate to generate their corresponding L-AAs. However, chickens can convert: (1) phenylalanine into tyrosine in the liver and kidneys via the tetrahydrobiopterin-dependent phenylalanine hydroxylase, and (2) methionine into cysteine in the liver via the transsulfuration pathway

Table 7.2 Utilization of proteinogenic amino acids in diet for the growth of 14-day-old broiler chickens[a]

AAs	AAs in diet		Intake of digestible AAs in diet (g/day)[b]	AAs in the body		AA accretion in the body (g/day)	Digestible AAs catabolized to CO_2 (g/day)	Percentage (%) of digestible AA	
	% of diet (as-fed basis)	g/100 g AA		g/100 g wet weight	g/100 g AAs			Oxidized to CO_2	Deposited in the body[d]
Ala	1.31	6.16	0.491	0.951	6.61	0.345	0.145	29.6	70.4
Arg	1.44	6.77	0.546	0.975	6.78	0.354	0.192	35.2	64.8
Asn	0.93	4.37	0.345	0.521	3.62	0.189	0.156	45.2	54.8
Asp	1.31	6.16	0.491	0.618	4.30	0.224	0.266	54.3	45.7
Cys[c]	0.51	2.40	0.186	0.219	1.52	0.079	0.106	57.2	42.8
Gln	1.81	8.51	0.684	0.747	5.19	0.271	0.413	60.4	39.6
Glu	1.70	7.99	0.647	1.190	8.28	0.432	0.216	33.3	66.7
Gly	0.88	4.14	0.326	1.670	11.6	0.606	– 0.280	– 86.0	186.3
His	0.55	2.58	0.202	0.306	2.13	0.112	0.091	44.9	55.1
Ile	0.88	4.14	0.333	0.513	3.57	0.186	0.146	44.0	56.0
Leu	1.79	8.41	0.673	0.988	6.87	0.359	0.315	46.7	53.3
Lys	1.40	6.58	0.514	0.881	6.13	0.320	0.194	37.8	62.2
Met	0.52	2.44	0.196	0.273	1.90	0.099	0.097	49.3	50.7
Phe	1.01	4.75	0.385	0.496	3.45	0.180	0.204	53.2	46.8
Pro	1.52	7.14	0.564	1.224	8.51	0.445	0.120	21.2	78.8
Ser	0.81	3.81	0.307	0.641	4.46	0.233	0.074	24.2	75.8
Thr	0.87	4.09	0.317	0.521	3.62	0.189	0.128	40.4	59.6
Trp	0.24	1.13	0.088	0.167	1.16	0.061	0.027	31.2	68.8
Tyr	0.78	3.67	0.295	0.379	2.64	0.138	0.157	53.3	46.7
Val	1.02	4.79	0.385	0.598	4.16	0.217	0.168	43.7	56.3
Hyp	ND	ND		0.499	3.47	0.181	– 0.181	–	–

AA amino acid, *Hyp* 4-hydroxyproline, *ND* not detectable

[a]Male broiler chickens (Cobb) were fed a corn- and soybean meal-based diet containing 21.5% crude protein. Values are means for 10 chickens. Amino acids in the diet and the animal body were analyzed by high-performance liquid chromatography after acid and alkaline hydrolyses as previously described (Li and Wu 2020) and their values were calculated on the basis of the molecular weights of intact AAs. A negative value indicates net formation

[b]Feed intake was 170 g/kg body weight per day. The mean body weight of 14-day-old broiler chickens was 297 g, and their mean weight gain was 36.3 g/day. The true ileal digestibility (%) of AAs in the diet was: Ala, 88.1; Arg, 89.3; Asn, 87.2; Asp, 88.1; Cys, 85.6; Gln, 89.0; Glu, 89.6; Gly, 87.6; His, 86.3; Ile, 88.9; Leu, 88.5; Lys, 86.4; Met, 88.6; Phe, 89.6; Pro, 87.3; Ser, 89.1; Thr, 85.7; Trp, 86.5; Tyr, 88.9; and Val, 88.8 (Wu 2014)

[c]Total cysteine (cysteine plus ½ cystine)

[d]As protein and non-protein products

(Wu 2013). Tyrosine and cysteine can replace up to 50% of phenylalanine and methionine in the diets of chickens, respectively, depending on age and dietary nutrient composition (Baker 2009). In addition, relatively small amounts of ornithine and proline are produced from arginine via arginase, ornithine aminotransferase, and pyrroline-5-carboxylate reductase (Austic 1973; Graber and Baker 1973; Wu et al. 1995). Arginase hydrolyzes arginine into ornithine and urea. The latter is excreted in urine. Thus, despite the lack of urea cycle in avian species, the body of poultry contains urea of non-dietary origin. Furthermore, a limited amount of citrulline is generated from arginine via nitric oxide synthase. This explains why the concentrations of ornithine and citrulline in the plasma of chickens is very low and negligible, respectively (Table 7.1), in comparison with pigs (Wu 2018a).

Chickens synthesize de novo an additional group of seven proteinogenic AAs (alanine, asparagine, aspartate, glutamate, glutamine, glycine, and serine), and some nonproteinogenic AAs (e.g., taurine and γ-aminobutyrate) in a

Table 7.3 Utilization of proteinogenic amino acids in diet for the growth of 42-day-old broiler chickens[a]

AAs	AAs in diet		Intake of digestible AAs in diet (g/day)[b]	AAs in the body		AA accretion in the body (g/day)	Digestible AAs catabolized to CO_2 (g/day)	Percentage (%) of digestible AA	
	% of diet (as-fed basis)	g/100 g AA		g/100 g wet weight	g/100 g AAs			Oxidized to CO_2	Deposited in the body[d]
Ala	0.93	5.08	1.36	0.944	6.55	1.057	0.302	22.2	77.8
Arg	1.21	6.61	1.79	0.971	6.74	1.088	0.703	39.3	60.7
Asn	0.76	4.15	1.10	0.519	3.60	0.581	0.518	47.1	52.9
Asp	1.09	5.95	1.59	0.617	4.28	0.691	0.901	56.6	43.4
Cys[c]	0.43	2.35	0.61	0.226	1.57	0.253	0.357	58.5	41.5
Gln	1.62	8.85	2.39	0.741	5.14	0.830	1.561	66.3	33.7
Glu	1.50	8.19	2.23	1.183	8.21	1.325	0.905	40.6	59.4
Gly	0.76	4.15	1.10	1.712	11.9	1.917	– 0.819	– 74.6	174.6
His	0.43	2.33	0.61	0.304	2.11	0.340	0.269	44.1	55.9
Ile	0.75	4.10	1.11	0.511	3.55	0.572	0.535	48.3	51.7
Leu	1.59	8.68	2.34	0.987	6.85	1.105	1.230	52.7	47.3
Lys	1.16	6.33	1.66	0.880	6.11	0.986	0.673	40.6	59.4
Met	0.47	2.57	0.69	0.272	1.89	0.305	0.385	55.8	44.2
Phe	0.86	4.70	1.28	0.493	3.42	0.552	0.726	56.8	43.2
Pro	1.41	7.70	2.04	1.236	8.58	1.384	0.657	32.2	67.8
Ser	0.83	4.53	1.23	0.640	4.44	0.717	0.510	41.5	58.5
Thr	0.75	4.10	1.07	0.518	3.59	0.580	0.486	45.6	54.4
Trp	0.21	1.13	0.30	0.168	1.17	0.188	0.109	36.6	63.4
Tyr	0.71	3.88	1.05	0.382	2.65	0.428	0.620	59.2	40.8
Val	0.85	4.64	1.25	0.601	4.17	0.673	0.579	46.2	53.8
Hyp	ND	ND	ND	0.507	3.52	0.568	– 0.568	–	–

AA, amino acid; *Hyp*, 4-hydroxyproline; *ND* not detectable

[a]Male broiler chickens (Cobb) were fed a corn- and soybean meal-based diet containing 18.4% crude protein. Values are means for 10 chickens. Amino acids in the diet and the animal body were analyzed by high-performance liquid chromatography after acid and alkaline hydrolyses as previously described (Li and Wu 2020) and their values were calculated on the basis of the molecular weights of intact AAs. A negative value indicates net formation

[b]Feed intake was 74 g/kg body weight per day. The mean body weight of 42-day-old broiler chickens was 2245 g, and their mean weight gain was 112.0 g/day. The true ileal digestibility (%) of AAs in the diet was: Ala, 88.0; Arg, 89.1; Asn, 87.1; Asp, 88.1; Cys, 85.5; Gln, 89.0; Glu, 89.5; Gly, 87.0; His, 86.1; Ile, 88.9; Leu, 88.5; Lys, 86.2; Met, 88.4; Phe, 89.5; Pro, 87.2; Ser, 89.0; Thr, 85.6; Trp, 86.4; Tyr, 88.9; and Val, 88.7 (Wu 2014)

[c]Total cysteine (cysteine plus ½ cystine)

[d]As protein and non-protein products

cell- and tissue-specific manner (Wu 2013). To date, compelling evidence shows that chickens fed conventional diets do not adequately synthesize glycine and proline relative to their metabolic needs (Baker 2009); therefore, these two AAs are classified as EAA for the birds (Wu 2009). Note that glutamate is the major excitatory neurotransmitter in the central nervous system (He and Wu 2020). The transamination of branched-chain AAs (BCAAs; leucine, isoleucine and valine) with α-ketoglutarate (α-KG; derived primarily from glucose metabolism) by BCAA transaminase generates glutamate, which is amidated with ammonia by the ATP-dependent glutamine synthetase to form glutamine. Glutamate is also transaminated with pyruvate or oxaloacetate by glutamate-pyruvate transaminase and glutamate-oxaloacetate transaminase to yield alanine and aspartate, respectively. Asparagine is synthesized from aspartate and glutamine by the ATP-dependent asparagine synthetase. Of note, α-ketoisocaproate (the α-ketoacid of leucine) may inhibit proteolysis in chicken skeletal muscle (Nakashima et al. 2007), which fulfils another

Table 7.4 Use of dietary glycine for growth and uric acid production in broiler chickens fed corn- and soybean meal-based diets[a]

Age of chickens	Feed intake (g/kg BW per day)	Digestible glycine intake (g/kg BW per day)	Glycine accretion in the body (g/kg BW per day)	Uric acid excretion in urine (g/kg BW per day)	Glycine needed for uric acid production[b] (g/kg BW per day)	Glycine needed for weight gain and uric acid production (g/kg BW per day)	Dietary glycine meeting glycine needed for weight gain and uric acid production (%)
Days 7–14	180.5 ± 2.9	1.44 ± 0.03	2.05 ± 0.05	5.63 ± 0.19	2.51 ± 0.08	4.57 ± 0.09	30.8 ± 0.19
Days 35–42	75.6 ± 0.55	0.51 ± 0.01	0.98 ± 0.01	1.88 ± 0.04	0.84 ± 0.02	1.82 ± 0.02	28.2 ± 0.11

BW body weight

[a]Values are means ± SEM, n = 4 pens. Cobb male broiler chickens were fed 21.5% and 18.4% crude-protein diets (corn- and soybean meal-based) between days 7 and 14 and between days 35 and 42 of age, respectively. There were 10 birds per pen. The mean body weight of the chickens was 36, 118, 297, 1489 and 2245 g at 0, 7, 14, 35, and 42 days of age, respectively. The content of glycine in the whole bodies of 14- and 42-day-old broilers were 1.65 and 1.68 g/100 g of body weight, respectively

[b]Calculated on the basis of the fact that one mole of glycine is required to synthesize 1 mole of uric acid (Wu 2013). Uric acid was determined as described by Flynn and Wu (1996)

physiological function of leucine. Due to its large mass, skeletal muscle [constituting 40–45% of body weight (BW)] is the major site for the syntheses of glutamate, glutamine and alanine in chickens, with both glutamine and alanine participating in inter-organ metabolism of AAs (Wu et al. 1989). Thus, glutamine is the most abundant free α-AA in the plasma and gastrocnemius muscle (a skeletal muscle) of chickens (Table 7.1). The avian liver is also an active organ for the syntheses of glutamate, aspartate, and alanine, but contributes to little or no net synthesis of glutamine due to its use for uric acid synthesis (the major route of ammonia detoxification in birds) under physiological conditions.

Plant-based diets are deficient in glycine and proline relative to protein synthesis in chickens (Hou et al. 2019; Li et al. 2011; Li and Wu 2020). We determined that the typical corn- and soybean meal-based diets for 7- to 14-day-old and 35- to 42-day-old broiler chickens provide 30.8% and 28.2% of the glycine needed for weight gain and uric acid production in the body, respectively (Table 7.4). Assuming that the amounts of glycine used for the syntheses of creatine, purines, glutathione, hippurate, and heme as well as the oxidation to CO_2 and water (i.e., 0.91 and 0.36 g glycine/kg BW per day in 7- to 14- and 35- to 42-day-old male broiler chickens, respectively) represents 20% of the needs for weight gain plus uric acid production (Wang et al. 2013; Wu 2010), the needs for all glycine-dependent metabolic pathways are 5.48 and 2.18 g glycine/kg BW per day in 7- to 14- and 35- to 42-day-old male broiler chickens, respectively. In other words, the diets provided 25.7% and 23.5% of the glycine required by 7- to 14-day-old and 35- to 42-day-old broiler chickens, respectively. Thus, the rapidly growing bird must synthesize daily at least 74–76% of the needed glycine, as reported for young pigs (Wang et al. 2014). This AA is synthesized endogenously from threonine, serine (via glucose and glutamate), and 4-hydroxyproline (a product of collagen degradation) via multiple pathways in a cell-and tissue-specific manner involving primarily the liver, kidney, and skeletal muscle (Li and Wu 2018). For example, glycine is formed from serine in the liver and kidneys via serine hydroxymethyltransferase (present in both the mitochondria and cytosol), from threonine in the liver, and from 4-hydroxyproline in almost all tissues (Wu et al. 2019). Because of a small amount of choline in the diet, this substance is a minor source of glycine in the body. Glycine is the most abundant AA in the body of chickens (Wu 2013). This is

consistent with its diverse roles in the metabolism and physiology. For example, glycine is required for the syntheses of glutathione (the most abundant low-molecular-weight antioxidant in cells), heme (a component of hemoglobin, myoglobin, and heme-containing enzymes), and bilirubin (a vehicle for iron excretion via feces and urine). This AA also regulates the expression of proteases to inhibit protein degradation in chicken skeletal muscle (Nakashima et al. 2008). Glycine is also a major inhibitory neurotransmitter in the spinal cord and lower brainstem to regulate the behavior and function of the animals (He and Wu 2020). In addition to glycine, chickens are not able to synthesize adequately proline to meet their nutritional and physiological requirements (Baker 2009).

7.4 Amino Acid Catabolism in Chickens

In mammals (e.g., rats, pigs, and humans), it is now known that not all digestible AAs enter the portal circulation and that all individual proteinogenic AAs present in the lumen of the small intestine undergo catabolism by enterocytes, intestinal microbes, or both at various rates (Wu 2013). At present, little is known about this key aspect of protein nutrition in any poultry species. In mammals, among all the AAs in the arterial blood, only glutamine is absorbed by the basolateral membrane of the enterocyte in the post-absorptive state (Wu 2013). This is because the basolateral membrane of the enterocyte expresses glutamine transporters but no or low levels of transporters for other AAs. In adult rats and young pigs, the small intestine takes up about 30% of glutamine but no glutamate or aspartate from the arterial blood in the post-absorptive state (Wu 1998). It is unknown whether this is also true for poultry. Both in vivo (e.g., jejunal cannulation) and in vitro (e.g., Ussing chambers) techniques can be used to address this important issue.

Poultry can degrade all the twenty proteinogenic AAs in a cell- and tissue-specific manner to form ammonia and their respective carbon skeletons such as pyruvate, oxaloacetate, and α-KG (Wu 2013). For example, there is little degradation of asparagine in the small intestine (Porteous 1980) but this AA is hydrolyzed by asparaginase in the liver and kidneys of chickens to aspartate and ammonia (Coon and Balling 1984). Except for BCAAs, the avian liver is the major site for initiating and completing the catabolism of these AAs to form ammonia. This organ has a limited ability to transaminate BCAAs due to low BCAA transaminase activity under physiological conditions. In contrast, skeletal muscle converts BCAAs and α-KG into their respective α-ketoacids [i.e., branched-chain α-ketoacids (BCAAs)] and glutamate in chickens (Wu and Thompson 1987). As noted previously, glutamine and alanine (neutral AAs) are formed from glutamate as vehicles for the inter-organ transport of carbon and nitrogen atoms of AAs. In extrahepatic tissues, such as skeletal muscle, small intestine, and heart, some of the BCKAs undergo oxidative decarboxylation but most of them are released to the blood stream. The liver is the major organ to take up BCKAs in the blood for either oxidation to CO_2, glucose synthesis (except for the α-ketoacid of leucine), and ketogenesis. Tissues of birds can convert: (1) citrulline into arginine via argininosuccinate synthase and lyase, and (2) ornithine into α-KG via ornithine aminotransferase and pyrroline-5-carboxylate dehydrogenase. The latter is primarily expressed in the liver. There is negligible catabolism of taurine in animals (including poultry), and it is excreted from the body via either urine as a free AA or bile salt in feces.

In contrast to mammals, the liver of birds has a very low activity of phosphate-activated glutaminase (Coon and Balling 1984; Watford and Wu 2005) such that hydrolysis of glutamine to glutamate and ammonia is limited in this organ. Like mammals (Wu et al. 1991), the skeletal muscles of chickens express glutaminase to degrade glutamine (Wu et al. 1998). Table 7.5 summarizes the activities of glutaminase in the liver, skeletal muscle and small intestine of chickens. The low activity of hepatic glutaminase ensures the synthesis of uric acid from ammonia via the formation of glutamine and subsequently purine nucleosides in the liver (Wu 2013). Notes that adenosine and guanosine are generated from not only glutamine but also glycine, aspartate, formate, ribose-5-phosphate, bicarbonate and ATP. Compared with ureagenesis in mammals,

Table 7.5 Activities of glutaminase, glutamine synthetase, and rates of protein synthesis in tissues of 6-week-old fed and 48-h fasted male White Leghorn chickens[a]

Tissue	Nutritional state	Activity of phosphate- activated glutaminase (unit/g wet weight of tissue)	Activity of glutamine synthetase (unit/g wet weight of tissue)	Fractional rate of protein synthesis (%/day)
Liver	Fed	0.67 ± 0.02 (6)	1.68 ± 0.09 (4)	128.4 ± 7.5 (5)[b]
	48-h fasted	0.61 ± 0.05 (6)	1.75 ± 0.08 (5)	52.6 ± 3.8* (5)[b]
GM	Fed	0.39 ± 0.03 (3)	0.50 ± 0.04 (4)	36.1 ± 1.50 (5)
	48-h fasted	0.28 ± 0.04 (4)	0.73 ± 0.03* (5)	13.6 ± 0.55* (5)
PM	Fed	1.67 ± 0.09 (4)	0.07 ± 0.01 (4)	10.5 ± 0.86 (5)
	48-h fasted	1.28 ± 0.16 (4)	0.08 ± 0.01 (5)	10.3 ± 0.81 (5)
Jejunum	Fed	0.14 ± 0.02 (5)	0.034 ± 0.002 (5)	96.3 ± 4.6 (5)[b]
	48-h fasted	0.12 ± 0.02 (5)	0.031 ± 0.002 (5)	41.8 ± 2.3* (5)[b]
Kidney	Fed	8.22 ± 0.53 (6)	ND	48.6 ± 2.4 (5)[b]
	48-h fasted	6.30 ± 0.50 (7)*	ND	39.2 ± 1.7* (5)[b]

GM gastrocnemius muscle, *ND* not detected, *PM* pectoralis muscle
*$P < 0.05$ vs. the fed group
[a]Adapted from Watford and Wu (2005) and Wu et al. (1998). Values are means ± SEM. The number of animals is indicated in the parentheses. One unit represents 1 μmol of product formed per minute at 38 °C
[b]Rates of fractional protein synthesis were measured as described by Watford and Wu (2005)

more energy is required for uric acid generation per removal of one ammonia molecule, resulting in the release of more heat. This explains, in part, why the basal metabolic rate and body temperatures are higher in birds than in mammals (e.g, pigs, rats and humans). Because ammonia is toxic to the central nervous system, it must be removed via uric acid production (the primary route for detoxification) and other biochemical pathways such as glutamine and glutamate syntheses in avian species.

Physiologically important products of AA catabolism in animal cells include polyamines (putrescine, spermidine and spermine). These substances are essential to the synthesis of DNA and proteins and, therefore, the rapid growth and development of all animals, including chickens (Agostinelli 2020). However, metabolic pathways for polyamine synthesis in avian tissues are largely unknown. In chickens, expression of arginase is relatively low and pyrroline-5-carboxylate synthase is absent in all tissues (Wu et al. 1995). At present, little is known about proline oxidase (POX) for polyamine synthesis in avian tissues. Recently, we found that arginase and POX activities are present only in the mitochondrial fraction of the kidneys of chickens between 0 and 21 days of age (Furukawa et al. 2018). Renal POX activity was greater on day 7 than Day 0, but no change in renal arginase activity was detected during this period. Accordingly, there were age-dependent changes in the syntheses of ^{14}C-putrescine, ^{14}C-spermidine and ^{14}C-spermine from [U-^{14}C]arginine or [U-^{14}C]proline in the chicken kidneys. Interestingly, concentrations of putrescine, spermidine and spermine in the plasma of chickens were about 10-, 100-, and ten-fold greater, respectively, than those in plasma from mammals. Consistent with enzymatic activities and polyamine syntheses, concentrations of polyamines in the kidney and plasma were greater on day 7 than day 0, but then values decreased on days 21 and 42. Thus, results of this study reveal that polyamines are synthesized from arginine via arginase and proline via POX in the chicken kidneys and that polyamines released from the kidneys into blood provide polyamines for extrarenal tissues. This new knowledge helps to better understand the nutritional biochemistry of arginine and proline in birds.

7.5 Inter-organ Metabolism of Glutamate and Glutamine in Chickens

Because of the versatile and enormous roles of glutamine and glutamate in metabolism and physiology as noted previously, the past four decades

have witnessed growing interest in the inter-organ metabolism of glutamate and glutamine in chickens. In the skeletal muscle of chickens, glutamate and glutamine can be synthesized and degraded, with the intracellular glutamine-glutamate cycle regulating the release of glutamine from this organ (Wu et al. 1991). The rate of the oxidation of glutamate in the muscle is generally lower than the rate of the synthesis of glutamine from glutamate (Wu and Thompson 1987). In chickens, the rates of the oxidation of glutamate and glutamine are greater in the breast muscle (mainly glycolytic fibers) than in the leg muscle (mainly oxidative fibers) (Wu et al. 1991, 1998). This explains why the concentration of glutamine is much lower in the breast muscle than in leg muscles (Table 7.1). In addition, extensive metabolism of both AAs occurs in the liver, small intestine, brain, and kidneys (Smith and Campbell 1983; Tinker et al. 1986; Watford et al. 1981; Watford and Wu 2005). The synthesis of glutamine from glutamate is of physiological significance for directly scavenging free ammonia in the blood and other tissues.

In the avian small intestine, glutamine and fructose-6-phosphate are known as substrates for the synthesis of glucosamine-6-phosphate and, thus, glycoproteins (including mucins and membrane receptors) (Wu 2013). In addition, glutamine is capable of activating the mechanistic target of rapamycin signaling pathway to stimulate tissue protein synthesis and animal growth. Because of limited glutaminase activity and abundant glutamine content in common feedstuffs for poultry diets, the concentration of glutamine in the plasma of chickens is about 1 mM, which doubles the concentration of glutamine in the plasma of mammals (Wu 2018a, b). In contrast, the concentration of glutamate in plasma is relatively low (< 100 μM) in poultry, although glutamate is abundant in common feedstuffs for poultry diets. This can be now explained by a high rate of glutamate oxidation and utilization by the enterocytes of chickens (He et al. 2018), as reported for rats and humans (Reeds et al. 2000) as well as pigs (Hou and Wu 2018) and fish (Li et al. 2020a). It is likely that glutamate is utilized as a substrate for intestinal glutathione synthesis by poultry (Porteous 1980).

A previous study showed that the rate of glutamine consumption by chicken enterocytes was higher than that of proline, serine, glutamate, aspartate, asparagine, and glucose at 2.5 mM for each amino acid and 5 mM for glucose (Porteous 1980). The author also showed that the rate of glutamate consumption was only 20% of that for glucose. In contrast, Wu et al. (1995) reported that the enterocytes of growing chickens had a low activity of glutaminase and a limited ability to utilize this AA. Similarly, He et al. (2018) reported that chicken enterocytes had a low rate of catabolizing glutamine, but extensively degraded both glutamate and aspartate via reactions initiated primarily by transaminases to provide the majority of ATP. This basic research is highly significant because energy metabolism is the basis of life (Wu 2018a).

The liver of chickens takes up glutamine from the arterial blood at a higher rate in the fasting state than in the fed state (Tinker et al. 1986). In contrast, the liver of chickens in the fed state actively takes up glutamate, and the hepatic uptake of glutamate is the highest among all the amino acids measured, including glutamine, arginine, alanine and aspartate (Tinker et al. 1986). Due to the low glutaminase activity in the liver of chickens (Table 7.5), glutamine is mainly used to synthesize purine and pyrimidine nucleotides. The purine can be further converted into uric acid, which is an important antioxidant in birds (Fang et al. 2002). In contrast, the liver of chickens can readily degrade glutamate by either glutamate dehydrogenase or glutamate transaminases (e.g., glutamate-pyruvate transaminase and glutamate-oxaloacetate transaminase), with the carbon skeletons of glutamate being mainly converted into CO_2 and water. This is because, in avian hepatocytes, phosphoenolpyruvate carboxykinase is localized exclusively in mitochondria and, therefore, glutamate is not converted into glucose in these cells under fed or fasting conditions (Watford et al. 1981). This aspect of hepatic amino acid metabolism in birds is distinct from that in mammals.

In contrast to pigs (Hou and Wu 2018), the kidneys of chickens in the fed state take up glutamine from the arterial blood, but not glutamate (Tinker et al. 1986). In the long-term (6-day)

fasting state, there is no uptake of both glutamine and glutamate by the chicken kidneys (Tinker et al. 1986). In avian renal tubules, phosphoenolpyruvate carboxykinase is present in both the cytosol and mitochondria, which allows for the production of glucose from glutamate under both fed and fasting conditions (Watford et al. 1981). This is significant for the regulation of glucose homeostasis in birds (Wu 2018a). When renal glutamate dehydrogenase activity is enhanced under acidotic conditions, the glutamate-derived ammonia contributes to the regulation of acid-base balance in the whole body (Curthoys and Watford 1995).

7.6 Amino Acid Nutrition in Poultry

7.6.1 Growth Performance

Chickens grow fast and respond sensitively to the dietary intakes of AAs (Baker 2009). This is consistent with a relatively high rate of protein synthesis in their skeletal muscles (Table 7.5). In addition, growth is also associated with the accretion of free AAs (particularly taurine, γ-aminobutyrate, glutamate and glutamine) in tissues, including skeletal muscle and brain (Tomonaga et al. 2004, 2005). Because of the differences in genetic selection, environment, and dietary composition, modern breeds of chickens have different requirements for AAs than the breeds used 30 years ago (Bailey 2020). Although previous studies reported that adult roosters did not need dietary glutamate or glutamine to maintain their body at a zero or positive nitrogen balance when fed a purified diet during the 3-day experimental period (Leveille and Fisher 1959), dietary glutamate and glutamine are vital for chickens to maintain a zero or positive nitrogen balance for long-term growth and survival (Maruyama et al. 1976). Note that nitrogen balance is not highly sensitive to assess dietary requirements for all AAs in animals within a short period time (Wu 2014). One longer-term study (> a 14-day period) demonstrated that the absence of glutamate from diets decreased the BW gain of 1- to 14-day-old chickens fed a purified diet, while 10% of glutamate supplementation to a glutamate-free basal diet increased the BW gain of young chickens by four-fold (Maruyama et al. 1976).

Much evidence shows that, due to the limited activities of arginase (Klain and Johnson 1962) and proline oxidase (Furukawa et al. 2018) in chickens for glutamine synthesis from arginine and proline, dietary glutamine is of great significance for the health (including intestinal health) and growth of birds, particularly under stress conditions (Awad et al. 2014). In support of this notion, dietary supplementation with glutamine or feed-grade glutamine plus glutamate stimulates muscle protein synthesis and whole-body growth in broiler chickens (Li et al. 2010). This finding is consistent with the report that glutamine stimulates protein synthesis and inhibits proteolysis in chick skeletal muscle in vitro (Wu and Thompson 1990). Co-supplementation with glutamate and glutamine mitigated on muscle catabolism in heat-stressed broiler chickens by inhibiting intramuscular proteolysis (Furukawa et al. 2020). Similarly, supplementing 0.2%, 0.4%, or 0.8% glutamine to a corn- and soybean meal-based diet for laying hens housed at 25–30 °C improved small-intestinal and oviduct morphologies; the circulating levels of luteinizing hormone, follicle stimulating hormone, triiodothyronine and tetraiodothyronine; and egg production (Dong et al. 2010). Furthermore, dietary supplementation with 0.5% or 1% glutamine to broilers raised under hot conditions (30–34 °C) enhanced feed intake, serum insulin concentration, tissue integrity, and body-weight gain (Hu et al. 2016a), while improving the water-holding capacity, moisture and color of meat (Hu et al. 2016b). These findings establish that growing chickens cannot synthesize enough glutamine to meet their growth requirements.

Besides glutamate and glutamine, there is continued interest in the nutrition of EAAs in chickens. Dietary supplementation with lysine or methionine for 21- to 42-day-old broilers increased their growth rate through changes in metabolic pathways, as well as polygenic and pleiotropic relationships (Zhai et al. 2016).

Likewise, dietary supplementation with lysine (Zarghi et al. 2020) or tryptophan (Mund et al. 2020) above the NRC (1994)-recommended levels had positive effects on growth performance, tissue development, immune responses, and antioxidant status in broiler chickens. However, excessive supplementation with tryptophan increased risks for pulmonary arterial pressure and induced plexiform lesion (Kluess et al. 2012). Therefore, caution must be exercised to formulate AA-balanced diets for chickens. Furthermore, elevating dietary AA density for broilers enhanced feed efficiency and breast muscle yield, while reducing fat pad yield (Johnson et al. 2020). Interestingly, although dietary methionine (0.50% and 0.43% for starter and finisher diets, respectively) is sufficient to support the growth of broiler chickens with a normal hatching weight, this may not be the case for chickens with a low hatching weight. Thus, supplementing 0.1% DL-methionine to the diet for the chickens with a low hatching weight augmented their average daily BW gain, food intake, and the growth of breast muscle (Wen et al. 2014). Wen et al. (2014) explained that the beneficial effects of dietary supplementation of DL-methionine may be mediated by increases in IGF-I synthesis, as well as the expression of genes for the TOR/4EBP1 and FOXO4/atrogin-1 pathway.

Based on findings from studies with rats (Fu et al. 2005; Jobgen et al. 2009) and pig (Tan et al. 2009) that arginine reduces white fat accretion, much attention has been directed to such a novel role of this AA in poultry. For example, dietary supplementation with arginine (0.25–1.00%) from 21 to 42 days of age of broilers reduced the abdominal fat deposition without any side effect on meat flavor or quality (Fouad et al. 2013). The underlying mechanisms include: (1) reductions in the expression of lipogenic genes in the liver and abdominal fat tissue (Pirsaraei et al. 2017); (2) improvements in blood metabolic profiles (including hematology; Oso et al. 2017) and the development of immune organs (e.g., thymus and spleen; Oso et al. 2017); (3) enhancement in immunity, as shown by amelioration of immunosuppression in chickens inoculated with infectious bursal disease virus (Tan et al. 2014); (4) alleviation of oxidative stress and inflammation (Yazdanabadi et al. 2020); and (5) decreases in *Salmonella* counts in the small intestine (Oso et al. 2017).

With the availability of low-cost feed-grade EAAs, two or more of their combinations have been used to improve the growth performance of chickens. For example, Emadi et al. (2011) reported that dietary supplementation of the combination of arginine plus tryptophan above the NRC (1994) requirements not only enhanced their growth performance but also had a positive immunomodulatory effect on innate (interferon-α), cellular (interferon-γ) and humoral (immunoglobulin G) immune responses in broiler chickens challenged with an infectious bursal disease vaccine. In addition, supplementation with glycine plus threonine increased the growth performance of 21- to 35-day-old broiler chickens fed diets based exclusively on plant-source feedstuffs with low protein levels (Ospina-Rojas et al. 2013). Furthermore, supplementing a mixture of AAs (0.3% Leu, 0.2% Gly, 0.2% Pro, 0.2% Ala, 0.6% Asp, and 0.6% Glu) to a reduced-protein (18% crude protein) diet for broilers (days 6–21) enhanced body-weight gain and feed efficiency without affecting feed intake, compared with the control group fed an 18% crude protein diet (Corzo et al. 2005).

7.6.2 Neurological Function and Feed Intake

AAs are known to modulate neurological function in animals (He and Wu 2020). Intracerebroventricular (ICV) injection of L-proline inhibited spontaneous activity and increased sleeping posture of chicks in a dose-dependent manner (Hamasu et al. 2009). The sedative and hypnotic effects induced by L-Pro was mediated by N-methyl-D-aspartate (NMDA) receptors (Hamasu et al. 2010). In addition, L-Ser, L-Asp, D-Asp, L-Trp, D-Pro, L-Pro, L-Glu, glutathione, or creatine has been reported to inhibit spontaneous activity and attenuate adverse stress behaviors in chicks (Asechi et al. 2006; Erwan et al. 2012; Erwan et al. 2014; Yoshida et al.

2012; Yamane et al. 2009a, b). However, the mechanisms responsible for the sedative effects of these AAs are different. Specifically, L-Ser inhibits the social separation stress-induced behaviors, which is mediated by γ-aminobutyrate A receptors. L-Asp induces sedative and hypnotic effects via NMDA receptors, whereas L-Pro, D-Pro, and glutamate exert the same effects via NMD, glycine, and NMDA plus AMPA receptors, respectively. In contrast, D-Asp reduces stress response through the simultaneous involvement of other receptors besides the NMDA receptor. These receptors are proteins, indicating an important role of AAs in overall neural network, behavior, and food intake (Tran et al. 2019). For example, there are reports that: (1) ICV injection of L-leucine increased the food intake of neonatal chicks, while the other two BCAAs or α-ketoisocaproate had no effect (Izumi et al. 2004); (2) ICV injection of L-ornithine, carnosine, L-His, β-Ala, and histamine to neonatal chicks decrease their food intake (Tran et al. 2016; Tomonaga et al. 2004; Kawakami et al. 2000); these AAs are potential acute satiety signals in the brain of neonatal chicks; (3) the effect of L-Pro on food intake by neonatal chicks varied with feeding status, with ICV injection of L-Pro stimulating food intake under free access conditions but decreasing food intake in the fasting state (Haraguchi et al. 2007). To translate these discoveries into feeding, studies involving dietary supplementation of one or more AAs should be conducted with poultry.

7.6.3 Anti-oxidative and Anti-inflammatory Reactions

Glycine, arginine, glutamine, methionine, cysteine, tryptophan, proline, taurine, and creatine have anti-oxidative and anti-inflammatory functions in animals, including chicks (Sestili et al. 2011; Wu 2013). This line of research is still active in the field of poultry nutrition. For example, Xiao et al. (2018) reported that taurine enhanced antioxidant status in the duodenum and ameliorated lipopolysaccharide-induced intestinal inflammation in chickens by improving mitochondrial membrane permeability and goblet cell function. The anti-oxidative property of taurine also protects cardiomyocytes from oxidative injury, as taurine supplementation enhanced the levels of antioxidant molecules (e.g., glutathione, superoxide dismutase and glutathione peroxidase) and inhibited apoptosis in the cardiomyocytes of broilers with right ventricular hypertrophy (Li et al. 2020b). Furthermore, adding glycine-Zn chelates to the diet of broiler chickens enhanced the antioxidative capacity of their skeletal muscle and reduced the concentration of malondialdehyde (a product of lipid peroxidation), thereby improving meat quality (Winiarska-Mieczan et al. 2020).

7.6.4 Revisit of the Ideal Protein Concept in Chicken Nutrition

Animals have requirements for dietary AAs but not protein (Wu 2018a). Growth of poultry is characterized by the deposition of not only protein but also free AAs. The latter (e.g., free glutamate, glutamine, aspartate and asparagine) can constitute a significant proportion of the total AA pool in the body and, therefore, should not be neglected when considering dietary AA requirements (Wu 2013). Unfortunately, chemical analysis of these AAs in feedstuff and body proteins was not developed until the work of Li et al. (2011). The century-old term "NEAA" has recently been recognized as a misnomer in nutritional sciences and should be replaced by a new term, AASA (an AA that is synthesizable de novo in animal cells (Hou and Wu 2017).

The "ideal protein" concept has played a seminal role in advancing the development of AA nutrition in chickens over the past 70 years. However, this nutritional concept has flaws due to the limited knowledge of tissue-specific AA metabolism and underdeveloped analytical tools in the 1950s–1970s. The "ideal protein" concept has now been recognized to have significant shortcomings because it ignores nutritionally and physiologically important NEAAs in dietary formulations (Wu 2014). Revisiting the historic milestones in the development of the "ideal protein" concept will provide nutritional scientists with "foods" for thoughts.

Beginning in the late 1950s, researchers at the University of Illinois conceptualized an ideal protein (optimal proportions and amounts of EAAs) for diets of chickens (Glista et al. 1951; Fisher and Scott 1954). This concept concerned only EAAs but no NEAAs. Early attempts to define an ideal protein were based on the composition of EAAs in casein and chicken eggs, but were largely unsuccessful partly because of the imbalances and excessive amounts of many EAAs. Several years later, Klain et al. (1960) simulated the profile of EAAs in the chick carcass to design a revised pattern of dietary EAAs in the ideal protein. An improvement in the growth performance of broilers was achieved with the revised ideal protein, but remained largely unsatisfactory.

Subsequently, a mixture of four AAs (cystine, glycine, proline, and glutamic acid), which are synthesized from methionine or other AAs by birds and had previously been thought to be NEAAs in chicken nutrition, was used in dietary formulations to yield better results on growth performance in broilers (Baker et al. 1968; Graber and Baker 1973). The extensive research during the 1960s and the 1970s culminated in several versions of the "chick AA requirement standard" for the first 3 weeks post-hatching (Dean and Scott 1965; Huston and Scott 1968; Sasse and Baker 1973). The reference values for EAAs were revised by Baker and Han (1994) to improve their balance in diets. The common features shared by these different recommended standards of dietary requirements of chickens for EAAs are that the diets included: (a) all proteinogenic EAAs that are not synthesized de novo by poultry; (b) several AAs (glutamic acid, glycine, and proline) that are synthesized de novo by birds to various extents; and (c) no data on alanine, aspartate, asparagine, glutamine, or serine.

It is noteworthy that the patterns of AA composition in the ideal protein for chicks, as proposed by the Scott and Baker groups, differed substantially for glycine and proline, and, to a lesser extent, for branched-chain AAs, histidine, and sulfur-containing AAs (Dean and Scott 1965; Huston and Scott 1968; Sasse and Baker 1973; Baker and Han 1994). These differences may reflect variations in the AA composition of chickens reported in the literature (Price et al. 1953; Robel and Menge 1973). Because the content of proline plus hydroxyproline, as well as glutamate, glutamine, aspartate and asparagine, in the body of chickens was not known at that time, the relatively small amount of proline in the recommended ideal protein was only arbitrarily set and the diets still contained no glutamine, aspartate or asparagine, which are all highly abundant in the body (Wu 2013). In contrast, a very large amount of glutamic acid (e.g., 13 times the lysine value in the modified Sasse and Baker Reference Standard) was used to presumably meet the entire need for "nonspecific AA nitrogen". However, key questions regarding whether glutamic acid fulfilled this role and whether excessive glutamic acid might interfere with the transport, metabolism and utilization of other AAs in chickens were not addressed by the University of Illinois researchers. Possibly due to these concerns and the publication of the NRC (1994) nutrient requirements for poultry, Baker (1997) excluded glutamic acid, glycine or proline from the ideal protein for the diets of 0- to 56-day-old broiler chickens in his final version of the Ideal Ratios of Amino Acids for the birds. This is unfortunate but reflects an inadequate understanding of AA biochemistry and nutrition in poultry at the earlier times. Recent advances in nutrition research indicate that chickens, just like swine, have dietary requirements for NEAAs under certain physiological and environmental conditions (Wu 2014, 2018a). These NEAAs are now considered to be conditionally essential AAs in diets and play crucial roles in supporting the health and the maximum growth and egg-laying of chickens, as noted previously. Thus, sufficient NEAAs in diets are critical for improving the efficiency of poultry and egg production worldwide.

7.6.5 Texas A&M University's Optimal Ratios of AAs for Chickens

The composition of AAs in diets differ from that in the skeletal muscle protein of broiler chickens (Tables 7.3 and 7.6) because dietary AAs are catabolized in animal tissues to different extents

Table 7.6 Composition of amino acids in the plasma and skeletal muscles of 5-week-old male broiler chickens[a]

Amino Acid	Free AA in plasma (nmol/ml)	Free AAs in skeletal muscle (nmol/mg wet weight of tissue)			Protein-bound amino acids in skeletal muscle (g/100 g of protein AAs)		
		GM	PM	EDC	GM	PM	EDC
Ala	606 ± 28	4.95 ± 0.25	1.71 ± 0.07	4.26 ± 0.21	5.90 ± 0.28	5.79 ± 0.25	5.95 ± 0.31
β-Ala	58 ± 5	2.60 ± 0.19	2.03 ± 0.16	1.87 ± 0.14	–	–	–
Arg	336 ± 17	0.51 ± 0.02	0.51 ± 0.03	0.50 ± 0.02	6.85 ± 0.32	6.94 ± 0.35	6.80 ± 0.37
Asp	77 ± 6	0.65 ± 0.04	0.63 ± 0.03	0.61 ± 0.03	5.31 ± 0.24	5.32 ± 0.29	5.34 ± 0.33
Asn	126 ± 10	1.03 ± 0.09	0.45 ± 0.02	0.36 ± 0.01	4.28 ± 0.21	4.34 ± 0.23	4.26 ± 0.24
Cit	0.98 ± 0.02	0.015 ± 0.002	0.016 ± 0.001	0.015 ± 0.001	–	–	–
Cys	246 ± 13	0.24 ± 0.01	0.23 ± 0.02	0.24 ± 0.02	1.47 ± 0.05	1.40 ± 0.04	1.43 ± 0.06
Gln	995 ± 24	9.56 ± 0.48	2.07 ± 0.10	3.21 ± 0.15	5.58 ± 0.26	5.44 ± 0.23	5.45 ± 0.28
Glu	284 ± 13	3.07 ± 0.15	1.82 ± 0.08	1.78 ± 0.10	9.27 ± 0.37	9.20 ± 0.33	9.22 ± 0.40
Gly	433 ± 18	3.94 ± 0.17	1.85 ± 0.06	2.62 ± 0.12	4.45 ± 0.16	4.23 ± 0.14	4.29 ± 0.17
His	112 ± 6	0.57 ± 0.04	0.54 ± 0.03	0.52 ± 0.03	3.19 ± 0.09	3.08 ± 0.09	3.11 ± 0.12
Hyp	106 ± 8	0.049 ± 0.003	0.045 ± 0.003	0.046 ± 0.002	0.27 ± 0.01	0.26 ± 0.001	0.26 ± 0.02
Ile	124 ± 9	0.12 ± 0.01	0.13 ± 0.01	0.16 ± 0.01	5.27 ± 0.16	5.39 ± 0.19	5.30 ± 0.23
Leu	236 ± 11	0.35 ± 0.02	0.25 ± 0.02	0.27 ± 0.02	8.40 ± 0.30	8.57 ± 0.28	8.58 ± 0.31
Lys	162 ± 10	0.41 ± 0.03	0.40 ± 0.02	0.42 ± 0.03	7.75 ± 0.34	7.81 ± 0.31	7.83 ± 0.38
Met	70 ± 4	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	2.98 ± 0.13	3.02 ± 0.12	2.96 ± 0.16
Orn	19 ± 1	0.045 ± 0.002	0.042 ± 0.002	0.041 ± 0.002	–	–	–
Phe	174 ± 9	0.33 ± 0.02	0.31 ± 0.02	0.28 ± 0.01	4.40 ± 0.10	4.56 ± 0.09	4.49 ± 0.13
Pro	343 ± 21	0.31 ± 0.02	0.30 ± 0.02	0.31 ± 0.02	4.24 ± 0.12	4.12 ± 0.11	4.20 ± 0.15
Ser	474 ± 28	3.53 ± 0.17	1.14 ± 0.07	2.02 ± 0.09	4.44 ± 0.19	4.57 ± 0.21	4.52 ± 0.24
Tau	241 ± 17	14.6 ± 0.53	23.5 ± 0.91	19.8 ± 0.84	–	–	–
Thr	245 ± 14	0.91 ± 0.06	0.89 ± 0.04	0.87 ± 0.03	4.77 ± 0.18	4.69 ± 0.17	4.85 ± 0.22
Trp	65 ± 3	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	1.30 ± 0.06	1.31 ± 0.05	1.29 ± 0.07
Tyr	163 ± 7	0.21 ± 0.01	0.20 ± 0.01	0.22 ± 0.02	3.84 ± 0.14	3.95 ± 0.12	3.86 ± 0.18
Val	188 ± 9	0.29 ± 0.02	0.28 ± 0.02	0.27 ± 0.01	6.03 ± 0.17	6.02 ± 0.20	6.01 ± 0.26

Cit = citrulline; EDC = extensor digitorum communis; GM, gastrocnemius muscle; Hyp = 4-hydroxyproline; Orn = ornithine; PM, pectoralis muscle; Tau = taurine

[a]Values are means ± SEM, n = 10. Cobb male broiler chickens were fed a corn- and soybean meal-based diet containing 18.4% crude-protein. At 35 days of age, skeletal muscles were obtained from the birds after cervical dislocation. Free and protein-bound amino acids were analyzed as described by Li and Wu (2020). The content of each AA was calculated on the basis of its intact molecular weight

Table 7.7 Texas A&M University's optimal ratios of true digestible amino acids in diets for growing broilers[a]

AA	Age of broiler chickens			Laying hens[b]	
	0 to 21 days[c]	21 to 42 days[d]	42 to 56 days[e]	Content of digestible AAs in diet (%, as-fed basis)	Percentage of digestible lysine in diet (%)
	(% of digestible lysine in diet)				
Alanine	102	102	102	0.90	110
Arginine	105	108	108	1.03	126
Asparagine	56	56	56	0.72	88
Aspartate	66	66	66	1.03	126
Cysteine	32	33	33	0.29	35
Glutamate	178	178	178	1.45	177
Glutamine	128	128	128	1.58	193
Glycine	176	176	176	1.00	120
Histidine	35	35	35	0.41	50
Isoleucine	67	69	69	0.70	85
Leucine	109	109	109	1.52	185
Lysine	100	100	100	0.82	100
Methionine	40	42	42	0.38	46
Phenylalanine	60	60	60	0.53	65
Proline	184	184	184	1.31	160
Serine	69	69	69	0.80	98
Threonine	67	70	70	0.61	74
Tryptophan	16	17	17	0.19	23
Tyrosine	45	45	45	0.41	50
Valine	77	80	80	0.78	95

Adapted from Wu (2014)

[a]Except for glycine, all amino acids are L-isomers. Values are based on true ileal digestible amino acids

[b]A diet that consists of 60% corn grain (containing 9.3% crude protein) and 24% soybean meal (43.5% crude protein) and is supplemented with 0.2% glycine and 0.1% L-methionine can meet the requirements of laying hens for all amino acids

[c]Patterns of amino acid composition in the ideal protein are the same for male and female chickens. The amounts of digestible lysine in diet (as-fed basis; 90% dry matter) are 1.12% and 1.02% for male and female chickens, respectively

[d]Patterns of amino acid composition in the ideal protein are the same for male and female chickens. The amounts of digestible lysine in diets (as-fed basis; 90% dry matter) are 0.89% and 0.84% for male and female chickens, respectively

[e]Patterns of amino acid composition in the ideal protein are the same for male and female chickens. The amounts of digestible lysine in diets (as-fed basis; 90% dry matter) are 0.76% and 0.73% for male and female chickens, respectively

and some AAs are synthesized in a tissue-specific manner at various rates (Wu 2013). As noted previously, AAs (e.g., glutamate and glutamine) that are present in the free pool at high concentrations (Table 7.6), as well as glycine and proline (the most abundant AAs in the body), should be taken into consideration when defining dietary requirements of chickens for AAs. Based on the recent advances in the nutrition and metabolism of AAs, particularly the functional AAs (Wu 2010), Wu (2014) proposed the Texas A&M University's optimal ratios of true digestible AAs in diets for growing broiler chickens during different growth phases (Table 7.7). This is consistent with the recent findings that animals (including poultry) have particularly high requirements for dietary glutamate, glutamine, glycine and proline. These AAs are very abundant in rendered animal sources of feedstuffs, such as blood meal, feather meal, ruminant meat & bone meal, and poultry by-products (Li et al. 2011). In addition, hydrolyzed feather meal is an abundant source of both glycine and proline in chicken nutrition. In contrast, plant-source feedstuffs contain relatively low content of both glycine and proline (Hou et al. 2019; Li and Wu 2020).

The Texas A&M University's optimal ratios of dietary AAs for chickens (Wu 2014) are expected to beneficially reduce dietary protein content and

nitrogen excretion, while improving the efficiency of nutrient utilization, growth and production performance, as well as sustaining the global animal agriculture. It is noteworthy that this new nutritional concept is now widely used to guide the practice of poultry feeding worldwide (e.g., Badawi et al. 2019; Belloir et al. 2017; Chrystal et al. 2020; Dessimoni et al. 2019; Liu et al. 2016; Refaie et al. 2017; Zhang et al. 2017b).

The productivity of modern laying hens has increased but their BW has decreased, when compared with breeds used decades ago (Bailey 2020). This means that the requirements (maintenance plus production) of the hens for dietary AAs must be revised to modify those recommended by NRC (1994). As for growing chickens, the ideal protein concept without the consideration of AAs that are synthesized in the body has also been applied to the formulation of diets for laying hens (Lemme 2009). At present, only Arg, Ile, Lys, Met + Cys, Thr, Trp, and Val are considered in various ideal AA profiles proposed by different authors (see Lemme 2009 for review). This is unfortunate, because AAs (e.g., glutamate, glutamine, glycine and proline) that are synthesized by the egg-laying birds may not meet their requirements for their maximum productivity or optimum health (including intestinal health). For example, there is evidence that the provision of glutamine from corn- and soybean meal-based diets (containing 18% crude protein) is insufficient for the maintenance of a healthy gut or a healthy oviduct in laying hens and that dietary supplementation with 0.4% or 0.8% glutamine is needed to sustain their normal morphology (Dong et al. 2010). It is likely that: (1) as reported for broilers (He et al. 2018), the small intestine of laying hens uses dietary glutamate and aspartate as the major metabolic fuels; and (2) as indicated for broilers (Table 7.4), dietary glycine is inadequate for protein accretion and the detoxification of ammonia as uric acid in laying hens.

Although common feedstuffs contain both EAAs and NEAAs, dietary requirements of laying hens for all proteinogenic AAs (including glutamate, glutamine, glycine, serine, proline and tyrosine) must be recommended to guide both research and the feeding practices. Methionine is usually the first limiting AA in the typical diets for laying hens, and there is evidence that supplementing 0.1% methionine to a corn- and soybean meal-based diet containing 16% crude protein and 0.29% methionine enhances egg production (Calderon and Jensen 1990). Furthermore, supplementation with 0.4% or 0.8% glutamine to a corn- and soybean meal-based diet for laying hens for 42 days augmented their egg production (Dong et al. 2010). Similar findings were reported by Gholipour et al. (2017) for laying guinea fowls fed a corn- and soybean meal-based diet containing 18% crude protein. Based on these considerations and research findings, we proposed Texas A&M University's optimal ratios of AAs for laying eggs to further stimulate research in this field. Animal-source feedstuffs are good sources of all AAs for these animals (Li and Wu 2020). Laying hens have a particularly high requirement for glutamine, leucine, glutamate, proline, arginine aspartate and glycine, because these AAs are highly abundant in the maternal bodies and in eggs. Inclusion of 4-hydroxyproline (a precursor of glycine; Li and Wu 2018) and taurine (a product of cysteine catabolism) in diets may reduce the requirements of laying hens for dietary glycine and cysteine, respectively. Both 4-hydroxyproline and taurine are highly abundant in animal-source feedstuffs (Li and Wu 2020). These findings have important implications for improving the nutrition of zoo birds (Herring et al. 2020).

7.7 Conclusion

AAs are not only the building blocks of proteins but also signaling molecules, neurotransmitters, and regulators of metabolic pathways. Although AAs have been classified as EAAs or NEAAs for animals since 1912, growing evidence shows that a sufficient provision of NEAAs (e.g., glutamine, glutamate, glycine, and proline) is necessary for the optimal growth and health of chickens, including broilers and laying hens. Thus, the concept of "ideal protein", which was based solely on EAAs and ignored all AAs that are synthesized in the animals, is not ideal in animal nutrition. Ideal diets for poultry must provide all physiologically

and nutritionally essential AAs (including EAAs and NEAAs) to maximize their growth performance and productivity, while promoting optimum health. To achieve this goal, we have proposed the Texas A&M University's optimal ratios of dietary amino acids for growing broilers and laying hens. These data are expected to facilitate the formulation of low-protein diets and precision nutrition through the addition of low-cost crystalline AAs or their alternative sources of animal proteins. Feedstuffs of animal origin can provide AAs (including leucine, lysine, methionine, arginine, glutamate, glutamine, aspartate, glycine, and proline) to prepare AA-balanced diets for chickens and help sustain the global animal agriculture.

Acknowledgments This work was supported by Texas A&M AgriLife Research (H-8200). We thank Dr. Christopher A. Bailey and Dr. Huaijun Zhou for assistance in our studies and helpful discussions.

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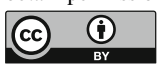

Nutrition and Functions of Amino Acids in Fish

8

Xinyu Li, Shixuan Zheng, and Guoyao Wu

Abstract

Aquaculture is increasingly important for providing humans with high-quality animal protein to improve growth, development and health. Farm-raised fish and shellfish now exceed captured fisheries for foods. More than 70% of the production cost is dependent on the supply of compound feeds. A public debate or concern over aquaculture is its environmental sustainability as many fish species have high requirements for dietary protein and fishmeal. Protein or amino acids (AAs), which are the major component of tissue growth, are generally the most expensive nutrients in animal production and, therefore, are crucial for aquatic feed development. There is compelling evidence that an adequate supply of both traditionally classified nutritionally essential amino acids (EAAs) and non-essential amino acids (NEAAs) in diets improve the growth, development and production performance of aquatic animals (e.g., larval metamorphosis). The processes for the utilization of dietary AAs or protein utilization by animals include digestion, absorption and metabolism. The digestibility and bioavailability of AAs should be carefully evaluated because feed production processes and AA degradation in the gut affect the amounts of dietary AAs that enter the blood circulation. Absorbed AAs are utilized for the syntheses of protein, peptides, AAs, and other metabolites (including nucleotides); biological oxidation and ATP production; gluconeogenesis and lipogenesis; and the regulation of acid-base balance, anti-oxidative reactions, and immune responses. Fish producers usually focus on the content or digestibility of dietary crude protein without considering the supply of AAs in the diet. In experiments involving dietary supplementation with AAs, inappropriate AAs (e.g., glycine and glutamate) are often used as the isonitrogenous control. At present, limited knowledge is available about either the cell- and tissue-specific metabolism of AAs or the effects of feed processing methods on the digestion and utilization of AAs in different fish species. These issues should be addressed to develop environment-friendly aquafeeds and reduce feed costs to sustain the global aquaculture.

Keywords

Protein · Nutrition · Metabolism · Aquatic animals

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1285, https://doi.org/10.1007/978-3-030-54462-1_8

Abbreviations

AA	amino acid
CCK	cholecystokinin
EAA	nutritionally essential amino acid
GABA	γ-aminobutyrate
GH	growth hormone
IGF	insulin-like growth factor
α-KG	α-ketoglutarate
mTOR	mechanistic target of rapamycin
NEAA	nutritionally nonessential amino acid
NO	nitric oxide
NRC	National Research Council
ROS	reactive oxygen species
SOD	superoxide dismutase

8.1 Introduction

Adequate provision of dietary protein and amino acids (AAs) is essential for the optimum health, growth, development and survival of animals (including fish) and humans (Wu 2013a, b). Traditionally, based on growth or nitrogen balance, AAs have been classified as nutritionally essential (EAAs, indispensable) or non-essential (NEAAs, dispensable) for mammals, birds and fish (Wu 2013a). However, recent studies have focused on the potential roles of functional AAs from both EAAs and NEAAs in animals (Wu 2013a; Watford 2015; Andersen et al. 2016). Some AAs and their metabolites are important regulators of key metabolic pathways that are necessary for maintenance, growth, feed intake, nutrient utilization, immunity, behavior, larval metamorphosis, reproduction, as well as resistance to environmental stressors and pathogenic organisms in various fishes (Li et al. 2009; Smedley et al. 2016). Over the past decade, there has been growing interest in the roles of functional AAs in animal nutrition and production (Wu 2013a; Watford 2015). Because protein is the major component of tissue growth and dietary protein is the most expensive nutrient in feedstuffs, animal nutritionists always pay much attention to the digestibility of dietary protein (defined as the percentages of AAs plus their small peptides released from dietary protein in the small intestine) and AA bioavailability (defined as the percentages of AAs plus their small peptides in dietary protein that are digested and absorbed in a form available for metabolic utilization by animals (Wu 2018).

Aquaculture is the fastest growing food animal sector and now contributes more high-quality protein to the human food supply (by weight) than wild caught seafood (FAO 2018). About 70% of global aquaculture (excluding aquatic plants) relies on commercial compound feeds that are produced by mixing feed ingredients (Béné et al. 2016; Li et al. 2020h). Some, but not all, fish species have a better rate of feed utilization than terrestrial animals (Fig. 8.1). However, the rate of protein retention in the body of some growing fish (e.g., largemouth bass) is even lower than that for growing pigs and chickens (Li et al. 2020c, d). This is because of the higher protein requirements in fish species (Fig. 8.1) partly to provide the bulk of energy for metabolic use (Jia et al. 2017; Li et al. 2020b). High levels of protein in diets increase the excretion of nitrogen (Cai et al. 1996; Yang et al. 2002), which is eventually discharged from aquaculture production systems. In aquaculture, fishmeal is the most important protein source and is derived from wild-harvested whole fish and shellfish (FAO 2018). There are about 20 million tons of fish destined for fishmeal production each year, and about 70% of them is directed towards aquaculture, followed by pig and chicken production (Cashion et al. 2017). Fishmeal has great values of protein digestibility and AA bioavailability. However, 90% of the fish used for fishmeal could be directed to feed humans instead (Cashion et al. 2017). Although fish production has benefits on economic development and food provision, public debate on aquaculture is dominated by concerns over resources and environmental sustainability (Béné et al. 2016). In terms of both economic returns and aquaculture sustainability, it is imperative to maximize the efficiency of protein utilization and reduce the use of fishmeal in the diets for all farmed fish species.

Understanding the digestion of dietary protein, as well as the absorption, metabolism and

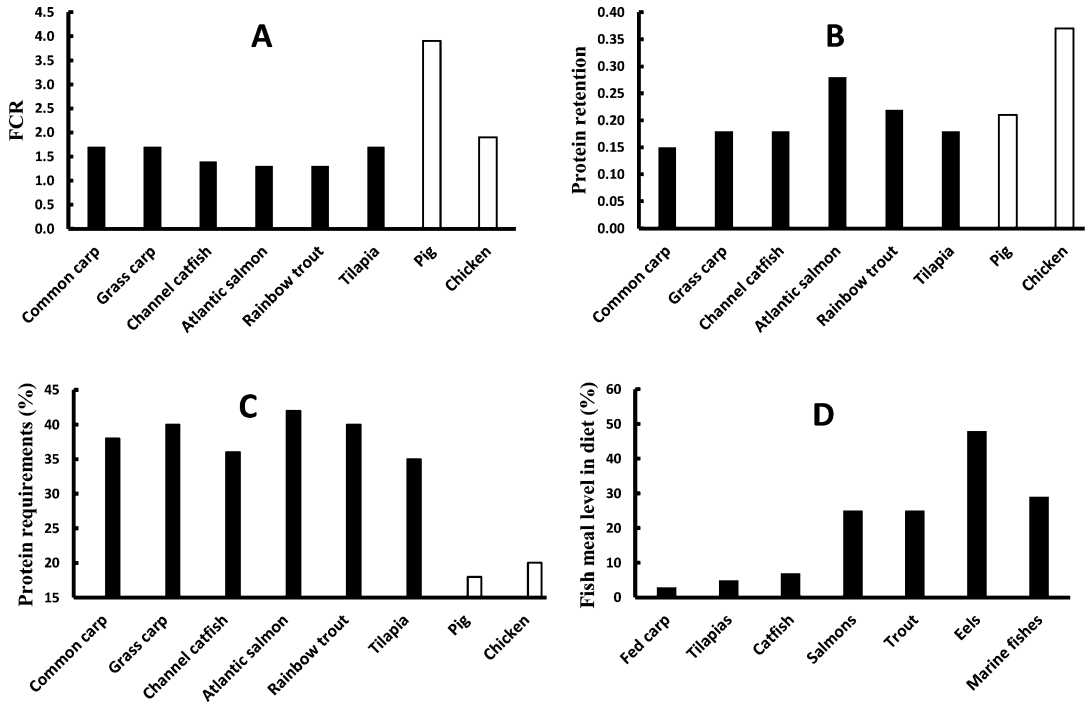

Fig. 8.1 (**a**) Feed conversion ratio (FCR, feed intake/weight gain) in different animals; (Adapted from Fry et al. 2018). (**b**) Protein retention in the edible portions of different animal species; adapted from Fry et al. (2018). (**c**) Requirements of different animal species for dietary protein (% of dry matter in the diet); adapted from Wilson and Halver (1986), Kim et al. (1991), Shelton (1971), and Applegate and Angel (2008). (**d**) Fishmeal inclusion in compound aquafeeds for different fish species and species groups; adapted from FAO (2012). All values are the means

functions of AAs (Table 8.3) is fundamental for manufacturing environment-friendly aquafeeds and reducing feed costs in animal production. The major objective of this article is to highlight current knowledge about feed processing, as well as AA nutrition and metabolism in fish species at different life stages. This will help to advance the field of protein nutrition and guide the development of future aquafeeds.

8.2 Feed Processing to Enhance Protein Digestibility in the Gastrointestinal Tract

Animal feeds are subjected to heat treatment during the processing of feedstuffs and the production of pelletized complete feeds to enhance protein digestibility in the gastrointestinal tract in fish. Extrusion cooking is the technology commonly used for the intensive production of aquafeeds as it confers good water stability and desirable flavor, improves feed utilization, and removes many anti-nutritional factors (Watanabe 2002). Appropriate heating can increase the digestibility of native proteins by unfolding the polypeptide chains and making the protein more susceptible to digestive enzymes (Opstvedt et al. 2003). However, overheated meals or feeds are undesirable because they may damage protein and AAs, thereby decreasing protein digestibility and AA bioavailability (Deng et al. 2005). Reduced protein digestibility was reported when commercial fishmeal and solvent-extracted soybean meal were subjected to additional moist heat for 30 min at 120 or 130 °C (Ljøkjel et al. 2000). Moreover, the Maillard reaction occurs between the carbonyl groups of reducing sugars and the amino groups of AAs (particularly lysine and arginine), peptides or proteins. Specifically,

lysine is bound with sugars during the Maillard reaction, resulting in the deoxyketosyl compound of lysine, which cannot be digested by fish, including rainbow trout (*Salmo gairdneri*; Plakas et al. 1988) and white sturgeon (*Acipenser transmontanus*; Deng et al. 2005). Recently, crystalline AAs have been used to balance AA composition in fish feeds. It is important to develop methods for protecting dietary AAs against for damage and loss. Fish live and consume food present in the water environment. As a result, leaching of feeds affects the nutritional values of feedstuffs and the validity of experimental results (Kaushik and Seiliez 2010; López-Alvarado et al. 1994). This issue should be taken into consideration in fish nutrition.

8.3 Digestion of Dietary Protein and the Absorption of Resulting Products

Dietary protein provides AAs for the growth and development of animals. Proteins are large polymers of AAs and have three dimensional structures. In neonatal mammals, milk-borne immunoglobulins are absorbed intact into the enterocytes of their small intestines through receptor-mediated mechanisms before "gut closure" occurs (Wu 2013b); other proteins are digested in the gastrointestinal tract. In general, dietary proteins have no nutritional value unless they are hydrolyzed by digestive enzymes (proteases and peptidases) to form free AAs and small peptides. Digestion of dietary protein is defined as its hydrolysis in the gastrointestinal tract into smaller molecules that are suitable for assimilation by the animals (Wu 2013b). The alimentary tract, including the mouth, stomach, intestines, and anus, are the organs for protein digestion, the absorption of the resultant digestion products, and the excretion of indigested feed and endogenous substances. The mucosa in the stomach of fish has a structure adapted for both food storage and digestion. Gastric parietal cells and chief cells produce hydrochloric acid (HCl) and pepsinogen, respectively (Tan and Teh 1974; Osman and Caceci 1991). The gastric HCl denatures protein, while activating pepsinogen into pepsin (an active protease) that acts on the denatured dietary proteins (Wu 2018). Gastric glands have been observed in all three regions of the stomach of *Nile Tilapia*, but have only been found in the cardiac and fundic regions of some of the other fish species (Osman and Caceci 1991). For those fish that do not have a stomach, the anterior intestine performs the function of temporary storage of ingested food (Sinha 1983). Stomach-less fish (which lack pepsin) are usually herbivores or omnivores that have a nearly mature and slightly alkaline gut (Smith 1980).

During the digestive processes, the hydrolysis of dietary proteins generates small peptides (di- and tripeptides) and free AAs at the intestinal lumen in the presence of specific proteases. The di- and tripeptides are either further hydrolyzed to free AAs by dipeptidase and tripeptidase or directly taken up in the intact form into intestinal epithelial cells. The amounts and activities of proteases and peptidases in the intestine are affected by many factors, including food intake as well as the secretion of secretin (by the S-cells of the duodenum) and cholecystokinin (CCK; by the I-cells of the duodenum; Wu 2018). Secretin regulates secretions from the stomach, pancreas and liver, whereas CCK stimulates the secretion of pancreatic digestive enzymes and the release of bile from the gallbladder (for those fish that have this organ). For example, dietary phospholipids have beneficial effects on stimulating the secretion of peptidases by increasing the level of CCK in rainbow trout (*Oncorhynchus mykiss*; Azarm et al. 2013). The true digestibilities of protein in animal- and plant-source feedstuffs are about 88–93% and 75–85% in fish, respectively, whereas the true digestibilities of free AAs in diets are 100%. In aquaculture, a rational approach to formulate diets is to supplement them with crystalline AAs (Nunes et al. 2014). However, Gu et al. (2013) reported that fish absorb crystalline AAs more rapidly and earlier in the gastrointestinal tract than protein-bound AAs. These differences in the patterns of digestion and absorption between free AAs and protein-bound AA may lead to poor growth, low feed utilization, and a suboptimal physiological

status of aquatic animals. To improve the utilization of crystalline AAs, a possible approach is to coat AAs with substances, like dextrin, β-cyclodextrin (Yuan et al. 2011), cellulose-acetate-phthalate (Fournier et al. 2003), tripalmitin-polyvinyl alcohol, acrylic resin (Chi et al. 2011), and tripalmitin (López-Alvarado et al. 1994).

Knowledge of diet and feeding habits is essential for the understanding of various aspects of fish nutrition and biology, as well as for developing cost-effective aquafeeds and feeding methods. Fish have been categorized as herbivores, omnivores, or carnivores/piscivores. Proteolytic enzymes are present in all kinds of fish species, including non-carnivorous fish (Hidalgo et al. 1999). Omnivores tolerate much higher intakes of digestible carbohydrate than carnivores, and need less dietary protein than carnivores (Kuz'mina 1990; Hidalgo et al. 1999). Moreover, some anti-nutritional factors in plant-source feedstuffs can inhibit endogenous digestive enzymes to decrease the digestibility of protein (Francis et al. 2001). For example, as the main source of protein in aquaculture production, crude or inadequately heated soybean meals contain an active Kunitz trypsin inhibitor (a 21.5-kDa protein; Roychaudhuri et al. 2004), as well as an active Bowman-Birk inhibitor (an 8 kDa protein) of both trypsin and chymotrypsin (DiPietro and Liener 1989). These soybean inhibitors reduce the digestibilities of dietary protein in juvenile starry flounder (*Platichthys stellatus*, Song et al. 2014), Japanese seabass (*Lateolabrax japonicus*, Zhang et al. 2018), and tilapia (*Oreochromis niloticus* × *O. aureus*; Lin and Luo 2011). The intestinal absorption of AAs (particularly methionine, leucine and threonine) was also decreased in rainbow trout (*Salmo gairdneri*) likely due to reductions in the expression of intestinal AA transporters when only 17.5% of fishmeal was replaced with 25% soybean meal in their diets (Dabrowski et al. 1989).

The germination and defatting of soybean meal could directly remove some of its protease inhibitors (Wassef et al. 1988). Moreover, heat treatment can inactivate and destroy some of the anti-nutritional factors found in soybean meal (El-Sayed et al. 2000; NRC 2011). For example, a combination of proper heat treatment could improve the nutritive value of defatted soy flour in young Pacific salmon (Arndt et al. 1999). Another common way to improve the digestion of dietary protein is the fermentation. Hong et al. (2004) reported that fermentation increased protein content by removing some carbohydrates, eliminated trypsin inhibitors, and reduced peptide size in soybeans and soybean meals. Replacing dietary soybean meal with its fermented soybean product has beneficial effects on the growth of largemouth bass (Jiang et al. 2018). In another study, lactic acid fermentation of soybean meal could ameliorate the effect of its trypsin inhibitors (Refstie et al. 2005). Besides, supplementation with exogenous enzymes can also improve protein digestion, leading to increases in growth performance and feed utilization. Total protease activity in the intestine of juvenile hybrid tilapia increased by dietary supplementation with a mixture of commercial enzymes (including protease), thereby augmenting protein digestibility from 78.2% to 86.7% (Lin et al. 2007). The supplementation of protease could also increase the apparent nutrient digestibility of soybean meal in rainbow trout (*Oncorhynchus mykiss*; Dalsgaard et al. 2012). Similar results have been reported for Nile Tilapia (Soltan 2009). Proteins in raw materials can be hydrolyzed by proteases to obtain bioactive peptides before mixing with other ingredients (Martínez-Alvarez et al. 2015). The protein hydrolysates of animal by-products and plant feedstuffs are promising additives to aquafeeds as flavorings, functional ingredients, and cost-effective sources of AAs (Zheng et al. 2012; Bui et al. 2014; Song et al. 2014; Cai et al. 2015; Khosravi et al. 2015).

In terrestrial mammals, dietary AAs and small peptides (i.e., di- and tri-peptides) are actively absorbed into the enterocytes via various AA transporters and peptide transporter-1, respectively, and some of them are taken up by microbes in the small intestine via similar transport mechanisms (Wu 2018). The percentages of AAs released from dietary protein that enter the blood circulation vary greatly among AAs because of their different rates of first-pass

catabolism in the small intestine (Wu 2013b). For example, 3–5% of glutamate and aspartate, 50% threonine, 60% proline and arginine, 64–66% branched-chain AAs and serine, 69% glycine and methionine, and 74–75% of asparagine and tryptophan enter the blood circulation. This is likely also true for aquatic animals (Jia et al. 2017; Li et al. 2020a). In fish species, some AAs (e.g., aspartate, glutamate and glutamine) are important energy sources for the intestine, liver, kidneys and skeletal muscle (Li et al. 2020a). Thus, most of dietary glutamate and glutamine would be oxidized in the gut for ATP production (Jia et al. 2017; Li et al. 2020a), so that only a small amount of them would pass the intestine into the blood circulation of fish (Jürss and Bastrop 1995; Jia et al. 2017). As in mammals (Wu 2013a), most of the glutamate and glutamine in plasma may be synthesized from branched-chain AAs and α-ketoglutarate (α-KG) by skeletal muscle and other tissues in aquatic animals (Li et al. 2009). Although arginine undergoes little oxidation to CO_2 in the intestine, this AA is extensively degraded by intestinal arginase to produce ornithine and urea (our unpublished work). In support of this view, almost all species of fish express arginase (Anderson 2001), but its isoforms are unknown. Oliva-Teles et al. (2017) also reported that urea-N excretion was directly related to dietary arginine intake. Recently, we identified a particularly high activity of arginase in tissues of largemouth bass, such that the concentration of arginine in their serum was very low but that of ornithine was relatively high (Table 8.1) and serum ornithine increased substantially after the feeding of diets supplemented with arginine (Fig. 8.2). Interestingly, largemouth bass are generally fed a fishmeal-based diet containing little citrulline, but have a relatively high concentration of citrulline in the serum (Table 8.1). Because this fish does not synthesize citrulline from glutamate, glutamine and proline (Li et al. 2020a) and produces citrulline from arginine via nitric oxide synthase at a very low rate, it is likely that arginine is actively metabolized to generate citrulline in its body through yet unknown pathways. Although the role of dietary glutamate and arginine in improving immunity have been well reported for many fish species (Table 8.4), little is known about their metabolism in intestinal leukocytes. It is imperative to understand the metabolism of these two AAs in a cell- and tissue-specific manner. A high activity of arginase in tissues may be a major factor limiting maximal growth of largemouth bass.

Available evidence shows that some fish species are capable of ureagenesis in the liver (Anderson 2001). It is unknown whether this also occurs in the small intestine of fish as reported for pigs (Wu 1995). However, there are species differences in the tissue-specific expression of enzymes involved in the conversion of ammonia or glutamine into urea and nucleotides. For example, fish and invertebrates possess carbamoylphosphate synthase-III (a mitochondrial enzyme), which utilizes the amide group of glutamine as the nitrogen-donating substrate and requires N-acetylglutamate for activity. For comparison, carbamoylphosphate synthase-I (a mitochondrial enzyme in mammalian liver and enterocytes) uses NH_3 as the nitrogenous substrate and requires N-acetyl-glutamate for activation, whereas carbamoylphosphate synthase-II (a cytosolic enzyme in mammalian liver, enterocytes and many other cell types) uses glutamine as the nitrogenous substrate but does not require N-acetyl-glutamate for activity. All these three isozymes produce carbamoylphosphate.

8.4 Protein Synthesis

After absorption, AAs will enter either catabolic (oxidation to CO_2) or anabolic (protein and peptide syntheses) pathways. The process of protein synthesis in both fish and other animals include five steps: (1) gene transcription; (2) initiation of translation; (3) peptide elongation; (4) termination, and (5) posttranslational modification (Wu 2013b). The supply of AAs, ATP, and GTP, as well as the number of ribosomes and the formation of polyribosomes will affect protein synthesis (Green and Noller 1997). The availability of AAs in plasma and cells has a direct relationship with the rate of protein synthesis. Ten AAs (arginine, histidine, isoleucine, leucine,

Table 8.1 Concentrations of free amino acids (AAs) in serum as well as free and peptide-bound AAs in the whole body of juvenile largemouth bass[a]

AAs	Free AAs in serum (nmol/ml)	Free AAs in in the whole body (μg/g of wet weight)	Total AAs (free plus peptide- bound) in the whole body[b]		AAs in protein (mg/g of PAAs)	Ratio of free AAs to total AAs in the whole body (%, g/g)
			mg/g of wet weight	mg/g of total PAAs		
Proteinogenic AAs						
Ala	521 ± 28	132 ± 12	9.60 ± 0.07	68.4 ± 0.53	68.6 ± 0.56	1.37 ± 0.12
Arg	38 ± 1.0	14.8 ± 1.6	9.66 ± 0.05	68.8 ± 0.36	69.9 ± 0.95	0.15 ± 0.02
Asn	78 ± 2.4	21.0 ± 1.6	4.95 ± 0.09	35.3 ± 0.63	35.7 ± 0.64	0.42 ± 0.03
Asp	20 ± 1.2	33.7 ± 1.4	6.25 ± 0.12	44.5 ± 0.83	45.0 ± 0.84	0.54 ± 0.02
Cys	152 ± 8.5	36.1 ± 1.8	1.98 ± 0.06	14.1 ± 0.45	14.0 ± 0.46	1.83 ± 0.10
Gln	194 ± 9.7	315 ± 8.6	8.06 ± 0.15	57.4 ± 1.09	56.1 ± 1.13	3.92 ± 0.14
Glu	34 ± 2.2	163 ± 3.5	12.7 ± 0.19	90.2 ± 1.37	90.6 ± 1.39	1.29 ± 0.03
Gly	369 ± 23	412 ± 15	13.0 ± 0.15	92.7 ± 1.07	91.3 ± 1.12	3.17 ± 0.13
His	125 ± 3.8	352 ± 11	3.47 ± 0.06	24.7 ± 0.45	22.6 ± 0.47	10.1 ± 0.37
Ile	148 ± 9.1	18.6 ± 0.7	5.52 ± 0.08	39.3 ± 0.57	39.9 ± 0.58	0.34 ± 0.01
Leu	242 ± 10	38.3 ± 1.3	9.62 ± 0.14	68.5 ± 0.96	69.4 ± 0.98	0.40 ± 0.01
Lys	185 ± 7.0	89.9 ± 4.3	8.65 ± 0.12	61.6 ± 0.83	62.0 ± 0.83	1.04 ± 0.05
Met	53 ± 2.3	12.5 ± 0.9	4.02 ± 0.08	28.6 ± 0.58	29.0 ± 0.59	0.31 ± 0.02
Phe	92 ± 2.6	30.1 ± 2.4	5.63 ± 0.13	40.1 ± 0.96	40.6 ± 0.96	0.53 ± 0.04
Pro	257 ± 13	204 ± 8.5	9.43 ± 0.16	67.2 ± 1.15	66.3 ± 1.36	2.17 ± 0.09
OH-Pro	42 ± 1.6	15.3 ± 0.5	3.07 ± 0.09	21.9 ± 0.67	21.2 ± 0.96	0.50 ± 0.02
Ser	179 ± 5.5	72.2 ± 5.7	6.87 ± 0.12	48.9 ± 0.86	49.2 ± 0.84	1.05 ± 0.07
Thr	143 ± 4.7	99.7 ± 9.0	5.76 ± 0.11	41.0 ± 0.76	41.0 ± 0.76	1.73 ± 0.15
Trp	28 ± 1.1	21.6 ± 1.5	1.62 ± 0.06	11.5 ± 0.42	11.6 ± 0.42	1.33 ± 0.06
Tyr	72 ± 2.2	49.8 ± 4.3	4.10 ± 0.09	29.2 ± 0.64	29.3 ± 0.67	1.22 ± 0.12
Val	268 ± 15	36.7 ± 2.6	6.46 ± 0.10	46.0 ± 0.69	46.9 ± 0.68	0.57 ± 0.04
Nonproteinogenic AA						
β-Ala	12 ± 0.7	5.97 ± 0.66	–	–	–	–
Cit	70 ± 2.5	23.4 ± 2.1	–	–	–	–
Orn	124 ± 6.0	131 ± 9.4	–	–	–	–
Tau	1016 ± 42	1587 ± 43	–	–	–	–

[a]Values are means ± SEM, n = 6. Juvenile largemouth bass were fed a diet consisting of the following (dry matter basis): 55.63% Menhaden fishmeal, 14.03% soybean protein concentrate, 9.2%, dextrinized starch, 0.57% soybean oil, 3.17% poultry fat, 1% vitamin, 1% mineral premix, 14.27% cellulose, and 1% sodium carboxy methyl cellulose (Li 2020). The composition of the vitamin premix (g/kg premix) was: vitamin A, 2.31; vitamin D3, 2.02; vitamin E, 20.00; vitamin K3, 1.2; vitamin C, 30.00; vitamin B5, 10.87; inositol, 15.00; niacin, 14.00; vitamin B6, 3.04; vitamin B2, 3.00; vitamin B1, 3.26; biotin, 0.15; folic acid, 0.6; vitamin B12, 0.02; Choline chloride, 135.00; Cellulose, 894.53. The composition of the mineral premix (g/kg premix) was: NaCl, 363.88; $MgSO_4.7H_2O$, 586.67; $FeSO_4.7H_2O$, 22.22; $AlCl_3.6H_2O$, 0.67; KI, 0.67; $CuSO_4.5H_2O$, 2.22; $MnSO_4$, 4.67; $CoCl.6H_2O$, 0.86; $ZnSO_4.7H_2O$, 18.09; Na_2SeO_3, 0.06. The crude-protein content of the diet was 50% (dry matter basis). Blood samples were obtained from the caudal vein of the fish (~ 50 g) at 24 h after the last feeding. Free AAs in serum as well as free and peptide-bound AAs in the whole body were analyzed as described by Li and Wu (2020). The amounts of amino acids in the whole body and the protein were calculated on the basis of their intact molecular weights. The content of dry matter in the whole fish was 31.0%

[b]*Cit* citrulline, *OH-Pro* 4-hydroxyproline, *Orn* ornithine, *PAAs* proteinogenic amino acids

lysine, methionine, phenylalanine, threonine, tryptophan, and valine) are not synthesized from any dietary AAs in fish. If one of these AAs is in excess, it will be oxidized to CO_2 (NRC 2011). If one of them is deficient (limiting), it will limit the use of all AAs for intracellular protein synthesis, therefore increasing their oxidation to CO_2. Although AAs synthesizable in tissues are

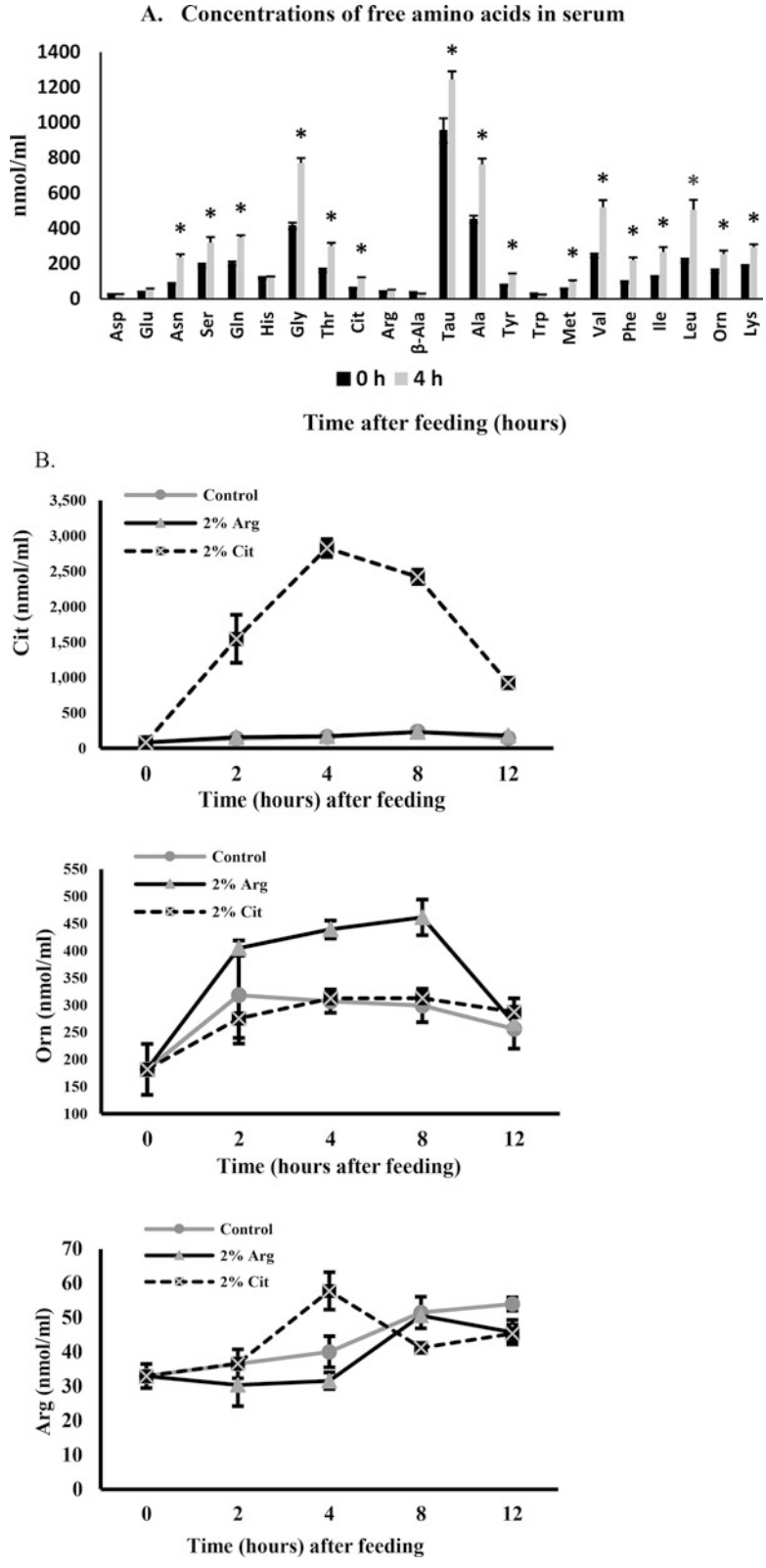

Fig. 8.2 Post-prandial concentrations of free amino acids in the serum of largemouth bass (*Micropterus salmoides*). The body weight of the fish was 51.4 ± 2.6 g (mean ± SEM, n = 6). (**a**) The fish were fed a diet consisted of the following (%, dry matter basis): fish meal menhaden, 53.73; soybean protein concentrate, 12.62; soybean oil:fish oil (2:1), 3; poultry fat, 1.23; dextrinized starch, 5; vitamin premix, 1; mineral premix, 1; cellulose, 19.17; choline chloride, 0.24; and carboxymethyl cellulose, 2. The crude-protein content was 45% (dry matter basis). Data are represented as mean ± SEM (n = 6). * $P < 0.05$ vs the value for 0 h. (**b**) Post-prandial concentrations of citrulline (Cit), arginine (Arg) and ornithine (Orn) in the serum of largemouth bass (*Micropterus salmoides*). The body weight of the fish was 52.0 ± 1.6 g (mean ± SEM, n = 3). Time indicates hours after feeding. Data are represented as mean ± SEM (n = 3). Control: fishmeal based diet with 45% crude protein and 10% lipids (dry matter basis, Li et al. 2020c); 2% Arg: dietary supplementation with 2% arginine; 2% Cit: dietary supplementation with 2% citrulline. Arg or Cit was added to the basal diet at the expense of 2% starch

traditionally regarded as NEAAs, they play a critical role in protein synthesis, as well as cell growth and development. For example, the percentage of proliferating muscle cells was markedly enhanced in response to supplementation with glutamine (Østbye et al. 2018).

Protein synthesis requires a large amount of energy. A minimum energetic cost of protein synthesis has been estimated to be 40 mmol ATP equivalents per gram of protein synthesized, and even 50 mmol ATP equivalents per gram of protein synthesized when the use of energy for post-translational modifications and intracellular trafficking is considered (Wu 2018). Thus, relationships between protein and energy intakes are critical to the efficiency with which dietary protein is partitioned into growth (Ballantyne 2001). For example, the protein-sparing effect of dietary lipids has been well reported in many fish species, including giant croaker (*Nibea japonica*) (Li et al. 2015), starry flounder (*Platichthys stellatus*; Ding et al. 2010), grouper (*Epinephelus coioides*; Luo et al. 2005). However, such an effect of dietary lipids has not been observed in largemouth bass (Li et al. 2020d).

In fish as in terrestrial mammals, protein synthesis is a major energy-demanding physiological process (Wu 2018). The balance between the rates of protein synthesis and proteolysis in tissues (primarily skeletal muscle) determines the rate of protein accretion in the body and, therefore, its growth. For example, a 300-g Atlantic cod that gains the body weight of 1.0%/day (i.e., 3 g of body weight/day) synthesizes 1.25 g of protein and degrades 0.81 g of protein per day, with a net deposition of 0.4 g of protein (plus 1.2 g of associated water) for growth (Houlihan et al. 1988). The other components (1.2 g) of the growth includes lipids, minerals, and glycogen, as well as water associated with non-lipids and non-protein nutrients. The proportion of total synthesized protein retained in the body increases with increasing growth rate due to little or no change or even a reduction in the rate of protein breakdown, such that at a maximum growth rate of 2%/day (i.e., 6 g of body weight/day), over 40% of the synthesized protein is retained in the body. Among the tissues studied, liver, gills, intestine, spleen, ventricle, stomach, gonads, and white muscle, the white muscle has the highest efficiency of protein retention and accounts for 40% of the total protein accretion in the fish body. Interestingly, in contrast to terrestrial mammals and birds, starving Altantic cods exhibit increased rates of proteolysis in the whole animal and white muscle as the rate of weight loss increases but at a constant rate of protein synthesis, irrespective of the rate of weight loss (Houlihan et al. 1988).

8.5 Amino Acid Metabolism

8.5.1 Oxidation to CO_2 and Ammonia

In view of animal production, the most important role of AAs is to serve as the building blocks of proteins. However, most of fish species are carnivorous which use primarily AAs as energy substrates to provide ATP (Ballantyne 2001; Li et al. 2020a). For example, 35–40% of leucine is oxidized for ATP production in fish (Fauconneau and Arnal 1985). Likewise, the oxidation of AAs as an entity may contribute to 50–70% of total energy needs in the marine fish embryos and yolk-sac larvae (Rønnestad and Fyhn 1993; Rønnestad et al. 1999). We have recently shown that glutamate, glutamine, leucine, aspartate, and alanine together contribute to ~80% of ATP production in the liver, proximal intestine, kidney, and skeletal muscle of zebrafish (Jia et al. 2017), hybrid striped bass (Jia et al. 2017), and largemouth bass (Li et al. 2020b). Individual AAs have their own catabolic pathways because of their different structures. However, the catabolism of many AAs shares a number of common steps to generate pyruvate, oxaloacetate, α-KG, fumarate, succinyl-CoA, and acetyl-CoA. For example, the carbon backbones of some AAs are converted into α-KG by glutamate dehydrogenase and transaminases. Glutamate dehydrogenase is also quantitatively a major enzyme for glutamate and glutamine catabolism in fish (Ballantyne 2001). Alanine transaminase and aspartate transaminase play an important role in initiating the degradation of alanine and aspartate to yield pyruvate and oxaloacetate, respectively

(Wu 2013b). For the catabolism of leucine in mammals, it undergoes active transamination with α-KG to form α-ketoisocaproic acid and glutamate primarily in skeletal muscle. Then, the α-ketoisocaproic acid is converted into acetyl-CoA by the branched-chain α-ketoacid dehydrogenase complex primarily in the liver (Wu 2013b). Little is known about the inter-organ metabolism of leucine and other branched-chain AAs in fish. Results of our recent studies indicated that leucine was extensively transaminated and decarboxylated in the liver of largemouth bass (Li and Wu 2019). It is possible that patterns of the catabolism of branched-chain AAs differ between fish and terrestrial mammals.

The major end product of AA metabolism in fish is ammonia, which is highly toxic and is directly excreted into the water environment (Ip et al. 2001). However, most teleost fish also release a significant proportion of their total excreted nitrogen as urea (5–20%). The latter is formed via the hepatic urea cycle and the catabolism of dietary arginine by arginase. Largemouth bass excrete a higher percentage of their total nitrogen as urea (about 30%) likely due to a high activity of arginase (Anderson 2001). This enzyme generates ornithine, which is used to synthesize proline and polyamines for the production of connective tissue and protein.

8.5.2 Gluconeogenesis and Lipogenesis

Although AAs are important metabolic fuels in carnivorous fish species, glucose is still required for aerobic oxidation in the nervous system and certain other cell types (e.g., red blood cells) and as a precursor for the syntheses of glycogen and mucopolysaccharides (Bever et al. 1981). Most AAs are quantitatively important glucogenic substrates in fish, which consume only a small amount of dietary carbohydrate in their liver (Cowey et al. 1977; Bever et al. 1981). The major AA for gluconeogenesis in the fish liver may be alanine, as its concentration in serum is the greatest among all AAs in both largemouth bass (Table 8.1) and hybrid striped bass (Table 8.2). Intracellular serine, glutamate, glutamine and aspartate are also important substrates for glucose synthesis in fish. In the pathway of gluconeogenesis, there are four unidirectional rate-controlling steps that are catalyzed by pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase and glucose-6-phosphatase, respectively. Gluconeogenesis from alanine or glutamate is increased by fasting in the kelp bass (*Paralabrax sp.*; Bever et al. 1981). In another study, the whole-body synthesis of glucose from [U-^{14}C]glutamate was markedly increased by the low-carbohydrate diet or starvation in rainbow trout (*Salmo gairdneri*; Cardenas 1985). During prolonged starvation plus exercise, the rates of gluconeogenesis from AAs increased two-fold and, simultaneously there was a corresponding increase in the activity of phosphoenolpyruvate carboxykinase in the liver of rainbow trout (French et al. 1981). All of these results support the view that the primary function of gluconeogenesis from AAs is to meet the needs of the body for glucose when dietary carbohydrate intake is inadequate.

Exogenous glucose and AAs could produce acetyl-CoA, which could increase fatty acid synthesis in fish. Interestingly, de novo lipogenesis from glucose in the liver is limited in some fish, especially when they have low carbohydrate intake (Jürss and Bastrop 1995). AAs are the preferred precursors for lipid synthesis, compared to glucose in some fish species (Nagai and Ikeda 1972; Nagai 1973). For example, in juvenile carp (*Cyprinus carpio*), glutamate was preferentially incorporated into hepatopancreatic lipids than glycogen (Nagai and Ikeda 1972). Thus, the partitioning of AAs into glucose or lipid synthesis likely varies among different fish species.

8.5.3 Derivatives of AAs

AAs serve as substrates for the synthesis of many substances with enormous physiological importance (Wu 2013b). These metabolites re essential for the health, growth, and development of animals (Table 8.3). For example, γ-aminobutyric acid (GABA) is synthesized from glutamate, which is

Table 8.2 Concentrations of free amino acids (AAs) in serum as well as free and peptide-bound AAs in the whole body of juvenile hybrid striped bass[a]

AAs	Free AAs in serum (nmol/ml)	Free AAs in in the whole body (μg/g of wet weight)	Total AAs (free plus peptide- bound) in the whole body[b]		AAs in protein (mg/g of PAAs)	Ratio of free AAs to total AAs in the whole body (%, g/g)
			mg/g of wet weight	mg/g of total PAAs		
Proteinogenic AAs						
Ala	554 ± 31	81.3 ± 4.3	9.75 ± 0.11	68.7 ± 0.76	68.8 ± 0.77	0.83 ± 0.05
Arg	140 ± 8.6	21.3 ± 1.5	9.80 ± 0.15	69.1 ± 1.07	69.6 ± 1.09	0.22 ± 0.02
Asn	66 ± 2.7	3.18 ± 0.32	5.14 ± 0.08	36.3 ± 0.60	36.6 ± 0.60	0.06 ± 0.006
Asp	28 ± 1.4	19.8 ± 3.3	6.47 ± 0.11	45.6 ± 0.77	45.9 ± 0.76	0.30 ± 0.05
Cys	157 ± 9.3	37.4 ± 1.4	2.01 ± 0.09	14.2 ± 0.63	14.0 ± 0.64	1.87 ± 0.08
Gln	206 ± 10	164 ± 6.4	8.11 ± 0.12	57.2 ± 0.85	56.6 ± 0.87	2.02 ± 0.09
Glu	67 ± 3.5	74.2 ± 4.0	12.8 ± 0.29	90.3 ± 2.07	90.5 ± 2.11	0.58 ± 0.04
Gly	292 ± 24	320 ± 20	13.1 ± 0.33	92.7 ± 2.34	91.0 ± 2.27	2.43 ± 0.12
His	237 ± 15	142 ± 7.0	3.49 ± 0.07	24.6 ± 0.52	23.8 ± 0.54	4.08 ± 0.24
Ile	148 ± 8.7	10.4 ± 1.7	5.56 ± 0.05	39.2 ± 0.37	39.4 ± 0.37	0.19 ± 0.03
Leu	235 ± 12	13.2 ± 0.38	9.76 ± 0.22	68.8 ± 1.52	69.3 ± 1.53	0.14 ± 0.004
Lys	176 ± 9.1	34.3 ± 1.1	8.71 ± 0.15	61.4 ± 1.09	61.7 ± 1.09	0.39 ± 0.01
Met	55 ± 2.8	11.8 ± 0.42	4.01 ± 0.13	28.3 ± 0.88	28.5 ± 0.89	0.30 ± 0.01
Phe	80 ± 2.2	11.2 ± 1.2	5.71 ± 0.12	40.2 ± 0.82	40.5 ± 0.82	0.20 ± 0.02
Pro	231 ± 11	188 ± 5.7	9.49 ± 0.22	66.9 ± 1.56	66.2 ± 1.57	1.98 ± 0.07
OH-Pro	43 ± 2.0	14.3 ± 0.75	3.03 ± 0.16	21.4 ± 1.16	21.5 ± 1.17	0.48 ± 0.04
Ser	172 ± 6.8	37.3 ± 1.7	6.93 ± 0.13	48.8 ± 0.91	49.0 ± 0.91	0.54 ± 0.02
Thr	133 ± 6.0	26.4 ± 1.3	5.73 ± 0.09	40.4 ± 0.65	40.6 ± 0.66	0.46 ± 0.02
Trp	29 ± 1.3	9.29 ± 0.71	1.60 ± 0.06	11.3 ± 0.45	11.4 ± 0.47	0.59 ± 0.06
Tyr	72 ± 2.9	10.4 ± 0.46	4.12 ± 0.13	29.0 ± 0.94	29.2 ± 0.95	0.25 ± 0.01
Val	264 ± 17	22.1 ± 0.75	6.49 ± 0.17	45.8 ± 1.19	46.0 ± 1.20	0.34 ± 0.02
Nonproteinogenic AA						
β-Ala	10 ± 0.8	2.18 ± 0.32	–	–	–	–
Cit	69 ± 3.2	4.75 ± 0.50	–	–	–	–
Orn	120 ± 7.5	8.86 ± 1.3	–	–	–	–
Tau	979 ± 54	1098 ± 87	–	–	–	–

[a]Values are means ± SEM, n = 6. Juvenile hybrid striped bass were fed a diet consisting of the following (dry matter basis): 60.0% Menhaden fishmeal, 0.7% fish oil, 0.3% soybean oil, 4.7% poultry fat, 20% dextrinized starch, 1% vitamin, 1% mineral premix, 8.3% cellulose, 2.3% carboxymethyl cellulose, 0.8% $Ca(H_2PO_4)_2{\cdot}H_2O$, 0.5% K_2HPO_4, and 0.5% $CaHPO_4$. The vitamin premix provided the following (mg/kg of the complete diet): vitamin A acetate, 23.06; cholecalciferol, 20.24; DL-α-tocopheryl acetate, 200; menadione, 12; ascorbic acid, 300; DL-calcium pantothenate, 109; myo-inositol, 150; niacin, 140; pyridoxine-HCl, 30.38; riboflavin, 30; thiamine mononitrate, 32.6; biotin, 1.5; folic acid, 6; vitamin B_{12}, 0.2; and carnitine, 0.08. The mineral premix Provided the following (mg/kg of the complete diet): chromium(III) chloride, 7.3; $CuSO_4.5H_2O$, 35; $FeSO_4.7H_2O$, 498; $MnSO_4.H_2O$, 82; Na_2SeO_3, 3; $ZnSO_4.7H_2O$, 258; sodium molybdate, 0.26; sodium fluoride, 1.3; $CoCl.6H_2O$, 5.2; KI, 7.8; and NiCl, 2.2. The crude-protein content of the diet was 38% (dry matter basis). Blood samples were obtained from the caudal vein of the fish (~ 50 g) at 24 h after the last feeding. Free AAs in serum as well as free and peptide-bound AAs in the whole body were analyzed as described by Li and Wu (2020). The amounts of amino acids in the whole body and the protein were calculated on the basis of their intact molecular weights. The content of dry matter in the whole fish was 32.4%

[b]*Cit* citrulline, *OH-Pro* 4-hydroxyproline, *Orn* ornithine, *PAAs* proteinogenic amino acids

the major inhibitory neurotransmitter in the central nervous system (Wagner et al. 1997) and plays an important role in the control of pituitary hormone secretion, anoxic metabolic depression, sex steroidal regulation and excitatory responses (Nilsson 1992; Lariviere et al. 2005). Carnosine (β-alanyl-

Table 8.3 Amino acid derivatives and their functions

Derivatives	Amino acids sources	Functions
Nitric oxide	Arg	A killer of pathogens; a signaling molecule; a neurotransmitter
Carnosine	β-Ala, L-His	Scavenge reactive oxygen species
Glutathione	Cys, Glu, Gly	Antioxidants, initiation of cell differentiation
Polyamines	Arg, Met, Pro, Orn	Protein and nucleic acid syntheses, protection from oxidative damage, activity of ion channels, cell proliferation, differentiation, and apoptosis
Creatine	Arg and Met	Energy storage and metabolism
Carnitine	Lys, Met and Ser	Long-chain fatty acid transport
Purines and pyrimidines	Gln, Gly and Asp	Energy for cells, and are essential for production of DNA and RNA
Heme	Gly	Transport and storage of oxygen molecule
Histamine	His	Immune responses, neurotransmitter
Melatonin and serotonin	Trp	Modulate cortisol release, behavior and feeding
Epinephrine	Phe and Tyr	Increase heart rate, muscle strength, blood pressure, and sugar metabolism
Triiodothyronine and thyroxine	Phe and Tyr	Regulation of basal energy metabolism, metamorphosis and growth
γ-aminobutyrate	Glu	Major inhibitory neurotransmitter

Adapted from Wu (2013b)

L-histidine), with a characteristic imidazole-ring, is a dipeptide molecule, made up of β-alanine and histidine. Carnosine is an antioxidant and important buffer in the skeletal muscle of aquatic animals, especially migratory pelagic marine fishes (Snyder et al. 2012). Glutathione (L-glutamyl-L-cysteinyl-glycine) is a tripeptide formed from glycine, cysteine, and glutamate. Glutathione is capable of protect cellular components from damage by reactive oxygen species, such as free radicals, peroxides, lipid peroxides, and heavy metals in fish species (Peña-Llopis et al. 2003).

8.6 Functions of Dietary AAs in Fish

8.6.1 Survival, Growth and Muscle Development

Protein is an essential component for every cell in the body and undergoes continuous turnover (synthesis and degradation). AAs not only serve as the building blocks of protein but also play an essential role in whole-body homeostasis (Wu 2018). At present, the NRC (2011) recommends dietary EAA requirements for fish, but does not provide any values for NEAAs, including glutamate, glutamine, glycine and proline. However, using a chemically purified diet that provides all EAAs in NRC (2011)-recommended amounts, all NEAAs but no glutamate and glutamine, as well as sufficient amounts of fatty acids, carbohydrate, minerals and vitamins, juvenile hybrid-striped bass grew poorly in comparison with fish fed the purified diet containing no glutamate or glutamine (Jia et al. 2019). Beginning on Day 18 of the experiment, deaths of the fish occurred in all tanks of fish fed the purified diet without glutamate or glutamine. By Day 35 of the experiment, mortality rates in the different treatment groups of the juvenile hybrid-striped bass were as follows: the 60% fishmeal diet, 97%; the complete purified diet (containing all AAs), 89%; the purified diet without glutamate, 39%; the purified diet without glutamine, 39% (Jia et al. 2019). These results indicate that the endogenous synthesis of glutamate or glutamine is insufficient for the growth or survival of the hybrid-striped bass and that these two AAs are nutritionally essential for the fish.

In growing fish, protein synthesis exceeds protein degradation, resulting in protein deposition (NRC 2011). Intracellular protein synthesis requires AAs and energy supply. Traditionally, the requirements for dietary AAs were determined based on the growth performance or protein deposition in fish fed different levels of a given AA. It should be borne in mind that protein deposition is the main determinant of body weight gain in growing fish (Dumas et al. 2007). As in other animals, fish need 20 different proteinogenic AAs to synthesize protein. About 25 to 55% of dietary AAs are used for protein accretion in growing fish (NRC 2011). Generally, the rates of lean tissue gain and protein retention in fish increase progressively when the content of protein or AAs in the diet increases from a suboptimal to an optimal level, beyond which the rates of lean tissue gain and protein retention either remains at the plateau or declines.

Fish continue to grow throughout their lives, but the relative growth rate (%/day) decreases with age. Both hyperplasia (increases in fiber number) and hypertrophy (increases in fiber size) contribute to adult myotomal muscle growth for fish (Johnston 2001). Skeletal muscle formation or myogenesis involves the specific control of several myogenic regulatory factors (MRFs) which control a series of events, including the specification, activation, and differentiation of myogenic cells. The maintenance of formed muscle fibers is dependent on a balance between protein synthesis and protein degradation (Fuentes et al. 2013). The mechanistic target of rapamycin (mTOR) plays a key role in cell physiology, acting primarily at the initiation of polypeptide synthesis (Wang and Pround 2006; Duan et al. 2015). The pathway can be directly activated by intracellular AAs through the mediators of Rag, GTPase, Rheb, hVps34, and MAP 4K3 (Duan et al. 2015). Dietary AAs, such as leucine, glycine, glutamine, and arginine are capable of regulating mTOR signaling pathway in fish species (Chen et al. 2015; Liang et al. 2018a, b; Li et al. 2019). For example, increasing dietary levels of leucine enhanced mTOR expression, growth performance and whole-body protein gain in juvenile blunt snout bream (Liang et al. 2018a). Myostatin is a negative regulator of myogenesis, and its mRNA expression in fish could be suppressed by proper supplementation with histidine to the diet (Michelato et al. 2017). Glutamate and glutamine are important in the growth of proliferating muscle cells, as well as the acceptable firmness and quality of fish fillets (Østbye et al. 2018; Ingebrigtsen et al. 2014). The effects of these two AAs may be mediated, in part, through activating the mTOR pathway.

The composition of AAs in tissue proteins is generally similar among all fish species, as shown for largemouth bass (Table 8.1) and hybrid striped bass (Table 8.2). As fish grow, the free AA pool in their bodies also expands. Most of free AAs represent < 5% of their total AAs in largemouth bass (Table 8.1) and hybrid striped bass (Table 8.2), with the exception of histidine in largemouth bass. In the largemouth bass, free histidine represents 10% of total histidine in the whole body. This AA, along with taurine (also a highly abundant free AA), may play a role in osmotic regulation and the maintenance of acid-base balance. Thus, the profiles of both free plus peptide-bound AAs in the whole body, rather than the "ideal protein" that concerns only EAA composition in tissue proteins, should be considered when formulating AA-balanced diets for fish.

8.6.2 Release of Hormones

AAs regulates muscle growth and development through direct actions on myogenic regulatory factors and mTOR signaling, or indirectly via the growth hormone (GH)/insulin-like growth factor (IGF) axis (Vélez et al. 2017). As in other animal species, the GH/IGF axis plays an important role in muscle protein synthesis, as well as muscle cell growth through both hyperplasia and hypertrophy. GH can exert a direct effect on the muscle or indirectly through IGF-I secreted by the liver. IGF-1 modulates cell metabolism (e.g. nutrient uptake) and the mTOR signaling pathway, which controls both protein turnover and muscle cell proliferation. Previous studies have demonstrated that there is cross-talk between ghrelin and

neurotransmitters (such as AAs and serotonin) to regulate GH secretion (Pinilla et al. 2003). In humans, AA intake increased ghrelin secretion to further stimulate the GH/IGF axis (Knerr et al. 2003). Dietary AAs and protein are also important nutrients that positively influence the GH-IGFs axis in fish (Picha et al. 2008). For example, Bower and Johnston (2010) have shown that AAs can enhance the expression of many genes in the IGF signaling pathway in the Atlantic salmon. In another study, a deficiency of AAs, especially lysine, affects the expression of genes in the IGF system and of myogenic factors in gilthead sea bream (Azizi et al. 2016). In rainbow trout, dietary methionine could increase the expression of genes involved in the GH/IGF axis response and protein turnover (Rolland et al. 2015). More details about the effects of AAs on GH/IGF axis and muscle development in fish are presented in Fig. 8.3.

As noted previously, the CCK plays an important role in controlling digestion in vertebrates. In humans, the most potent stimulants of CCK secretion are the partial digestion products of fat and protein, including di- and tri-peptides (Liddle 2000). In sea bass larvae, different levels of protein or its hydrolysates in diets modulate trypsin expression and affect CCK content (Cahu et al. 2004). There are reports that the ingestion of liposomes that contain free AAs, protein or their combinations effectively stimulates CCK production in first-feeding herring larvae (Koven et al. 2002). In mammals, the secretion of other hormones [e.g., insulin, gonadotropin-releasing hormone (GnRH) and cortisol] may also be affected by intakes of dietary protein and AAs (Bourguignon et al. 1989; Kraemer et al. 2006; Veldhorst et al. 2009). However, such studies are limited in fish species (Fig. 8.4).

8.6.3 Attractants

The use of AAs as dietary attractants has received considerable attention because the replacement of fishmeal with plant-source protein feedstuffs often reduces the feed intake of aquatic animals. Vertebrates express two families of G-protein-coupled receptors, taste receptors type 1 (T1R) and type 2 (T2R), in their taste buds (Oike et al. 2007). In mammals, the heteromeric taste receptor type 1 members 1 and 3 (T1R1/3) respond to umami tastants, such as glutamate and nucleic acids, whereas the taste receptor type 1 members 2 and 3 (T1R2/3) respond to sweet tastants, such as sugars (Oike et al. 2007; Yarmolinsky et al.

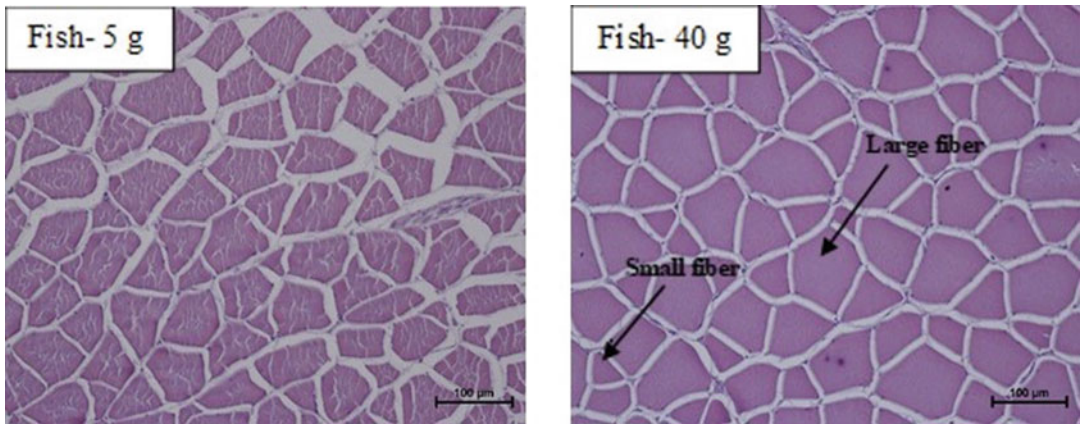

Fig. 8.3 Different sizes of white skeletal muscle fibers in largemouth bass at 5 g or 40 g of body weight. The fish were fed a diet containing 45% crude protein and 10% lipids (dry matter basis, Li et al. 2020c). The white skeletal muscle (near the dorsal fin region) was obtained and stained with haemotoxylin and eosin. The histology slides were examined with the use of a microscope at 100X magnification. Both large and small fibers are present in the skeletal muscle of 40-g fish, indicating that both hyperplasia and hypertrophy contribute to myotomal muscle growth for this fish

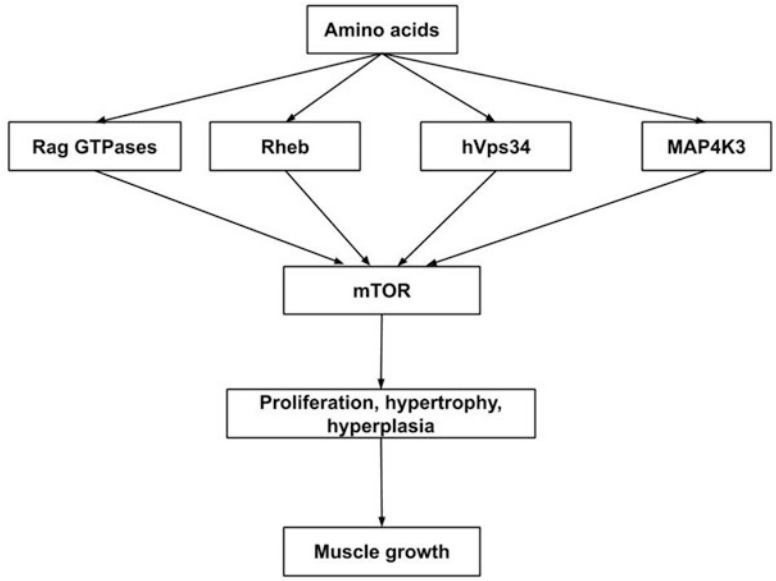

Fig. 8.4 Molecular and cellular mechanisms whereby amino acids stimulate muscle development and growth through the mechanistic target of rapamycin (mTOR) signaling pathway. mTOR is a highly conserved protein kinase. Activated mTOR (in the phosphorylated form) phosphorylates downstream proteins [eIF4E-binding protein-1 (4E-BP1) and ribosomal protein S6 kinase-1 (p70S6K1)] to initiate protein synthesis. Activated mTOR also contributes to the inhibition of intracellular proteolysis. Accretion of intracellular protein results in the growth of cells and tissues

2009). The T2Rs respond to bitter tastants, including poisonous chemicals (Chandrashekar et al. 2000; Mueller et al. 2005). However, both T1R1/3 and T1R2/3 act as receptors for AAs but not sugars in fish (Oike et al. 2007). The facial nerve of zebrafish respond strongly to the administration of alanine and proline; moderately to cysteine, glycine, serine, tyrosine, quinine-HCl, and denatonium; and weakly to other AAs. Glycine and some L-AAs (e.g., alanine, glutamate, and arginine) possess dietary attractant properties (Wu 2020b), which can trigger reflexive snapping and biting behaviors (Kasumyan and Morsi 1996; Polat and Beklevik 1999; Derby and Sorensen 2008). Similarly, Shamushaki et al. (2007) reported that alanine and glycine are potent attractants for Persian sturgeon juveniles. Some non-proteinogenic AAs (e.g., D-glutamine, D-asparagine, D-glutamate, and β-alanine) are also strong attractants for glass eels (Sola and Tongiorgi 1998). DL-alanine is also an attractant that has a very strong effect on improving the survival or growth of post-larval African catfish (*Clarias gariepinus*; Yilmaz 2005) and juvenile Sea Bass (*Dicentrarchus labrax*; Tekelioglu et al. 2003).

8.6.4 Immune Responses

Immunity is the ability of an organism to resist attacks by pathogens. Generally, there are three levels of immune defense in fish. The first line consists of physical and epithelial barriers (such as the scales, skin, and mucus), gastric acid, and chemical mediators [such as lysozyme, transferrin, complement systems, reactive oxygen species (ROS), and reactive nitrogen species]. Dietary AAs regulate the production of these tissues and substances (Table 8.4). The second line of defense involves cells, including phagocytes, natural cytotoxic cells, and inflammatory response. The third line of defense is the development of a specific immune response through the production of antibodies by B-cells against specific pathogens or the development of T-cell responses

Table 8.4 Immune functions of amino acids in different fish species

Amino acids	Fish	Main functions	
Arginine	Red drum (*Sciaenops ocellatus*)	Increases in the production of reactive oxygen species by neutrophils; higher serum lysozyme activity	Cheng et al. (2011)
	Channel catfish (*Ictalurus punctatus*)	Increase in the resistance of channel catfish to infection by *E. ictalurid*	Buentello and Gatlin (2001)
	Hybrid striped bass (Morone chrysops × M. saxatilis)	Increases in serum lysozyme activity and in superoxide anion production by neutrophils	Cheng et al. (2012)
	Senegalese sole (*Solea senegalensis* Kaup, 1858)	Increases in respiratory burst and nitric oxide production by head–kidney leucocytes	Costas et al. (2011)
	Yellow catfish (*Pelteobagrus fulvidrac*)	Increases in lysozyme activities, as well as the phagocytic index and the respiratory burst of head–kidney leucocytes	Zhou et al. (2015)
	Turbot (*Scophthalmus maximus* L.)	Increases in lysozyme and glutathione peroxidase activities	Zhang et al. (2017)
	Jian carp (*Cyprinus carpio* var. Jian).	Increases in mRNA levels for inflammatory cytokines, the phosphorylation of mTOR and 4E-BP, and humoral and cellular immunities	Chen et al. (2015)
	Channel catfish (*Ictalurus punctatus*)	Increases in phagocyte superoxide production and neutrophil respiratory burst	Pohlenz et al. (2014)
	Golden pompano (*Trachinotus ovatus*)	Increases in the activities of total nitric oxide synthase and lysozyme in the serum and liver and in survival rate, in response to *Vibrio harveyi* challenge	Lin et al. (2015)
	Turbot (*Scophthalmus maximus*)	Increases in respiratory burst and NO production by blood monocytes	Costas et al. (2013)
	Nile tilapia (*Oreochromis niloticus*)	Increases in NO metabolites, as well as total NO synthase and lysozyme activities in plasma	Yue et al. (2015)
	Orange-spotted grouper (*Epinephelus coioides*)	Regulating mRNA levels for immune-associated genes, and enhancing humoral and cellular immunities	Han et al. (2018)
Glutamine	Red drum (*Sciaenops ocellatus*)	Increases in the production of reactive oxygen species by neutrophils; higher serum lysozyme activity	Cheng et al. (2011)
	Hybrid striped bass (Morone chrysops× M. saxatilis)	Increases in the production of superoxide by neutrophils; higher serum lysozyme activity	Cheng et al. (2012)
	Turbot (*Scophthalmus maximus* L.)	Increases in the respiratory burst of head-kidney macrophages, and in serum lysozyme and glutathione peroxidase activities	Zhang et al. (2017)
	Hybrid sturgeon (*Acipenser schrenckii* ♀ × *Huso dauricus*♂)	Increases in the concentrations of complement-3 (C3) and complement-4 (C4) in serum	Zhu et al. (2011)
	Jian carp (*Cyprinus carpio* var. Jian)	Increases in serum lysozyme activity and C3 concentration	Hu et al. (2015)
Methionine	Jian carp (*Cyprinus carpio* var. Jian)	Increases in survival rate; leukocyte phagocytic activity; and lysozyme activity, acid phosphatase activity, total iron-binding capacity, haemagglutination titer, complements 3, 4, and immunoglobulin M concentrations in serum	Kuang et al. (2012)
	Jian carp (*Cyprinus carpio var.* Jian)	Increases in lysozyme activity, lectin potency, immunoglobulin M concentration, compliments C3, C4, total iron-binding capacity in serum; decreases in intestinal *Escherichia coli* and Aeromonas counts	Tang et al. (2009)
	European seabass (*Dicentrarchus labrax*)	Increases in peripheral leucocyte responses, complement activity and bactericidal capacity; cellular recruitment to the inflammatory site; and peroxidase and bactericidal activities in plasma	(Machado et al. 2015)
	Yellow catfish (*Pelteobagrus fulvidraco*)	Increases in serum lysozyme activity; serum immune globulins; as well as the phagocytic activity and respiratory burst of head-kidney phagocytic cells	(Elmada et al. 2016)

(continued)

Table 8.4 (continued)

Amino acids	Fish	Main functions	
Other AAs			
Lysine	Cobia (*Rachycentron canadum*)	Increase in blood leukocyte number	Zhou et al. (2007)
Taurine	Yellow catfish (*Pelteobagrus fulvidraco*)	Increases in growth and red blood cells; serum lysozyme activity and immunoglobulin concentrations; and phagocytic index and respiratory burst of macrophages	Li et al. (2016a)
Leucine	Blunt snout bream (*Megalobrama amblycephala*)	Increases in plasma C3 immunoglobulin M (IgM) concentrations; and decreases in the expression of genes for pro-inflammatory factors in the hepatopancreas	Liang et al. (2018a)
Leucine	Golden pompano Trachinotus ovatus	Increases in growth and protein deposition; increase in serum lysozyme activity; improvements in intestinal morphology	Tan et al. (2016)
Leucine	Black carp *Mylopharyngodon piceus*	Increases in mRNA levels or enzyme activities of immune defense effectors and in non-specific immunities; decrease in oxidative stress	Wu et al. (2017)

(Trichet 2010; Webster and Thompson 2015). The nutritional status of the host can influence the severity of impacts from pathogens (e.g., viruses, bacteria, fungi, and parasites), as well as immunity acquisition (Webster and Thompson 2015). AAs hold great promise in improving health and preventing infectious diseases in animals (including fish and shrimp) and humans (Li et al. 2007).

AAs play fundamental roles in the immune systems of fish (Li et al. 2009). In fish, glutamine is crucial to the immune response as it is a major energy substrate to support optimal lymphocyte proliferation and production of cytokines by lymphocytes and macrophages (Table 8.5), as reviewed previously (Alejo and Tafala 2011; Li et al. 2009; Reyes-Cerpa et al. 2012). Macrophage-mediated phagocytosis is influenced by glutamine availability (Calder and Yaqoob 1999). Glutamine is essential for the proliferation of T and B cell lymphocytes in fish, as dietary glutamine increased the proliferation of lymphocytes from the head-kidney and spleen of the channel catfish (Pohlenz et al. 2012b). Results of *in vitro* studies have shown that arginine and glutamine are important immunomodulators of both innate and adaptive responses in fish leukocytes (Pohlenz et al. 2012a, b). In recent years, the positive function of dietary glutamine on the immune responses has been well studied in several fish species (Table 8.4). Arginine is an abundant AA in tissue proteins and plays an important role in the immunity of the host directly through the production of nitric oxide (NO) and polyamines by macrophages, or indirectly via affecting gene expression and endocrine status (Li et al. 2009; Andersen et al. 2016). For example, NO is a cytotoxic molecule of macrophages and mediates inflammation (Wu 2013a, b). Both *in vivo* and *in vitro* experiments in channel catfish indicated that arginine has positive effect on the immune system, as dietary arginine supplementation enhanced the pathogen-killing and phagocytosis abilities of macrophages (Buentello et al. 2007; Pohlenz et al. 2012a, b). Higher serum lysozyme activity was observed in fish fed the diet supplemented with 1% arginine, 2% arginine, 1% glutamine, or 1% arginine plus 1% glutamine in hybrid striped bass (Cheng et al. 2012). Methionine also has beneficial effects on the immune system by improving both cellular and humoral immune responses (Rubin et al. 2007). As noted previously, methionine is involved in polyamine and glutathione syntheses, which may also affect the proliferation of lymphocytes and inflammatory processes in cells (Grimble and Grimble 1998). The

positive function of dietary methionine on the immune response has been well studied in several fish species (Table 8.4). Some studies also reported that certain AAs, such as taurine and lysine, may modulate immune responses in aquatic animals. More studies are necessary to understand the complex relationship between AAs and the immune system (Fig. 8.5).

8.6.5 Anti-oxidative Defenses

Free radicals play a beneficial role in biological evolution, metabolism, and physiology, but pathological levels of these substances also have an adverse effect on oxidative damages to protein, lipids and DNA, leading to cell injury and death (Fang et al. 2002). The production and deleterious

Table 8.5 Primary sources and functions of major cytokines and chemokines produced in fish

Cytokine	Primary source	Primary functions
Pro-inflammatory cytokines		
TNFα	Macrophages, NK-cells, and T-cells	Modulating cell proliferation; inducing necrosis and apoptosis; promoting the synthesis of other cytokines; killing infected cells; inhibiting the intracellular replication of pathogens
IL-1β	Blood monocytes and tissue macrophages	Regulating the expression of other cytokines, lymphocyte activation, leucocyte migration, phagocytosis, and bactericidal activity
IL-6	Macrophages, lymphocytes fibroblasts, neurons, glial cells, and endothelial cells	Modulating the production of immunoglobulins, the differentiation of lymphocytes and monocytes, the secretion of chemokines, and the migration of leukocytes to infected tissues
IL-11	Intestine and gills (constitutive); spleen, head kidney, and liver (induced following infection)	Exerting anti-microbial and anti-viral defenses
IL-12	Dendritic cells, macrophages	Stimulating the secretion of IFNγ from T-cells; activating NK cells and neutrophils; promoting the maturation of naïve T-cells into cytotoxic T-cells; regulating T-cell development
IL-17	Head kidney, spleen, gills, testis, ovary and skin	Exerting pro-inflammatory actions
IL-18	M1 macrophages	Inducing the synthesis of IFNγ by Th1 and NK cells in concert with IL-12; promoting the maturation of T-cells and NK cells; activating neutrophils; enhancing Fas ligand-mediated cytotoxicity
GM-CSFs	M1 macrophages and T-cells	Stimulating stem cells to produce granulocytes and monocytes; hematopoietic growth factors; promoting neutrophils, eosinophils, and M1 macrophages to produce pro-inflammatory cytokines; activating NO production by iNOS
Regulatory cytokines		
IL-2	Th1 cells	Primarily promoting proliferation, activation and differentiation of T-cells; required for the activation of NK cells and the synthesis of immunoglobulins by B-cells
IL-4	T-cells, mast cells, and basophils	Regulating the functions of B-cells, T-cells, and macrophages, as well as hematopoietic and non-hematopoietic cells; serving as a key cytokine to drive Th2 differentiation as well as mediating humoral immunity and allergic responses
IL-7	Different stromal cell types (e.g., those in thymus, head kidney, spleen, liver, gill, intestine, and skeletal muscle)	Regulating the development, survival, proliferation, and homeostasis of lymphocytes

(continued)

Table 8.5 (continued)

Cytokine	Primary source	Primary functions
IL-15	Leukocytes	Regulating the functions of T-cells, dendritic cells, and NK cells; serving as a key regulator of the innate immune response
IL-21	Th1 and Th2 cells	Acting on $CD4^+$ and $CD8^+$ cells, B-cells, NK cells, dendritic cells, and myeloid cells; enhancing the proliferation of $CD4^+$ and $CD8^+$ cells; exerting anti-tumor effects
Anti-inflammatory cytokines		
IFNα/β (type-I IFN)	Most cells (induced by viruses)	Exerting anti-viral, anti-proliferative, and immunomodulatory activities
IFNγ (type-II	NK cells and T-cells (in response to IL-12, IL-18, mitogens or pathogens)	Activating leukocytes (including M1 macrophages); mediating cellular resistance against viral pathogens; modulating both innate and adaptive immune responses; enhancing NO synthesis by iNOS
IL-10	M2 macrophages, monocytes, T-cells, and keratinocytes	Inhibiting the production of Th1 cytokines (mediated by induction of regulatory T-cells); serving as a major anti-inflammatory cytokine
IL-20	Immune cells (e.g., macrophages) and certain other cell types (e.g., keratinocytes and brain)	Mediating crosstalk between epithelial cells and tissue-infiltrating immune cells
TGF-β	Leukocytes (including M2 macrophages, monocytes, T-cells)	Serving as a suppressive cytokine; inhibiting the proliferation of Th1 and Th2 cells; promoting the generation of T regulatory cells; maintaining immune tolerance; regulating the development, proliferation, differentiation, migration, and survival of leukocytes (e.g., thymic lymphocytes, dendritic cells, NK cells, macrophages, and granulocytes; inducing the generation of Th17 cells
Chemokines		
MCP-1	Monocytes and macrophages	The most important chemokine that regulates the migration and infiltration of monocytes and macrophages
IL-8	Macrophages and many other cell types	Recruitment of neutrophils, lymphocytes and basophils to infected tissues; stimulating respiratory burst in neutrophils
CK-1	Macrophages and many other cell types	Inducing chemotaxis in leucocytes

$CD4^+$ cells, T helper cells (T_h cells) that play an important role in the immune system, particularly in the adaptive immune system; they help the activity of other immune cells by releasing T cell cytokines; $CD8^+$ cells, cytotoxic T-cells that induce apoptosis in cells; *CK-1* cytokeratin-1, *GM-CSFs* granulocyte-macrophage colony-stimulating factors, *IFN* interferon, *IL* interleukin, *iNOS* inducible nitric oxide synthase, *M1 macrophages (iNOS)* pro-inflammatory macrophages, M2 macrophages (arginase-2), anti-inflammatory macrophages that promote wound healing and tissue repair, *MCP-1* monocyte chemotactic protein-1, *NK* natural killer, *NO* nitric oxide, *TGF-β* transforming growth factor-β, *Th* helper T-cell, *TNFα* tumor necrosis factor-α

effects of free radicals are illustrated in Fig. 8.6. ROS, including superoxide anion (O_2^-), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2), contribute to radiation and oxidant-induced cytotoxicity. Fish species are highly susceptible to ROS as their tissues contain higher levels of polyunsaturated fatty acids than those in mammals and birds (Enser et al. 1996). To prevent these harmful effects, ROS should be rapidly removed by non-enzymatic and enzymatic antioxidants (Fang et al. 2002; Martinez-Alvarez et al. 2005). Glutathione peroxidase acts to reduce lipid hydroperoxides to their corresponding alcohols and convert free H_2O_2 to H_2O. Thus, this enzyme is crucial for efficient protection against lipid peroxidation. Besides

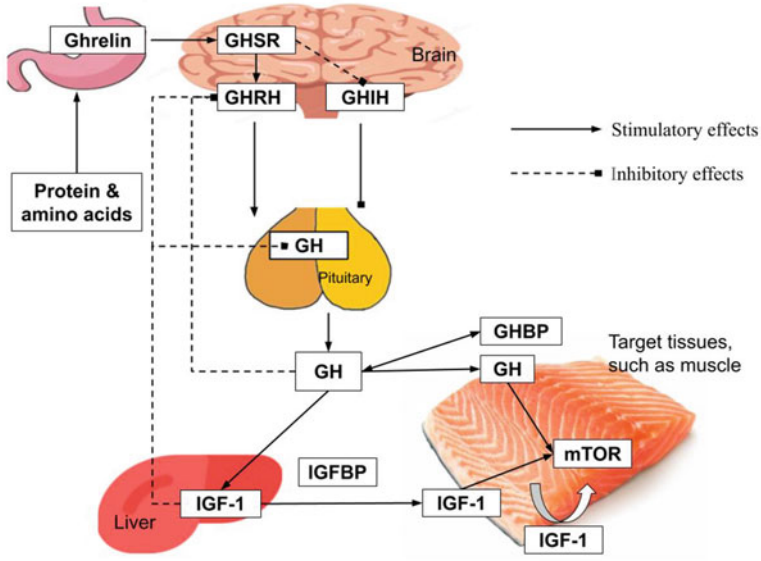

Fig. 8.5 The endocrine axis (GH/IGF axis) that regulates the growth of teleost fish. Amino acids act through the ghrelin signaling pathway and also directly on the pituitary gland to stimulate the release of growth hormone and on the liver to promote the production of insulin-like growth factor (IGF)-1. *GH* growth hormone, *GHBP* growth hormone-binding protein, *GHRH* growth hormone-releasing hormone, *GHIH* growth hormone-inhibiting hormone, *GHSR* growth hormone secretagogue receptor, *mTOR* mechanistic target of rapamycin, *IGFBP* insulin-like growth factor-binding protein

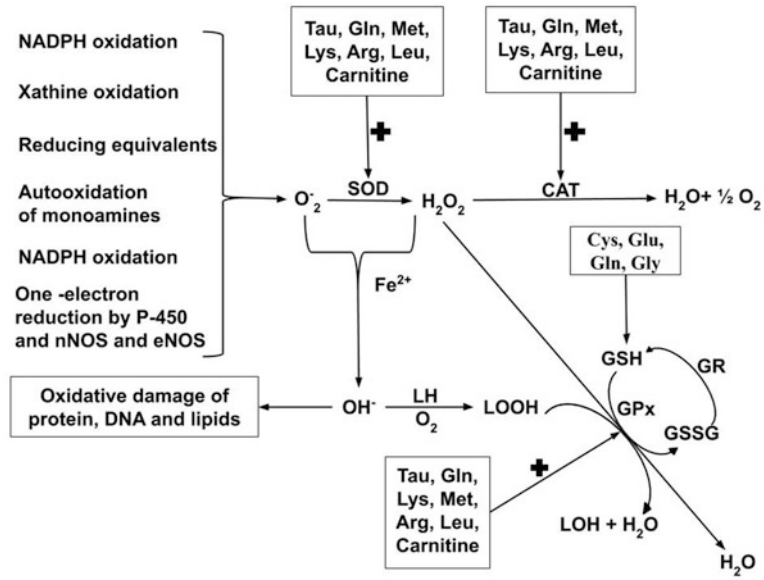

Fig. 8.6 Roles of amino acids and their metabolites as antioxidants in fish (adapted from Fang et al. 2002). *Arg* arginine, *Cys* cystine, *CAT* catalase, *Gln* glutamine, *Glu*, glutamate, *Gly* glycine, *GPx* glutathione peroxidase, *GR* glutathione reductase, *GSH* glutahione, *GSSG* oxidized glutathione, *Lys* lysine, *Leu* leucine, *LH* lipids (unsaturated fatty acids), *LOOH* lipid hydroperoxide, *Met* methionine, *SOD* superoxide dismutase, *Tau* taurine

glutathione peroxidase, catalase and superoxide dismutase (SOD) are two other major antioxidant enzymes of the antioxidant defense system. SOD catalyzes the conversion of O_2^- into H_2O_2, which is further converted to H_2O by catalase (Fang et al. 2002).

AAs are important nutrients for anti-oxidative defense as they can be the building blocks for the synthesis antioxidant enzymes. Some AAs (arginine, citrulline, glycine, proline, 4-hydroxy-proline, taurine and histidine) can directly remove oxygen free radicals (Fang et al. 2002; Wu et al. 2019a). The antioxidant ability of fish could be improved by AAs, such as arginine (Liang et al. 2018b), glutamine (Zhu et al. 2011; Han et al. 2014), taurine (Han et al. 2014; Pinto et al. 2010), methionine (Elmada et al. 2016), leucine (Deng et al. 2016), lysine (Li et al. 2014), histidine (Feng et al. 2013), citrulline (Li et al. 2013), and proline (Li et al. 2013). In most of those studies, authors made conclusions based on the activities and gene expression of antioxidant enzymes. For example, Liang et al. (2018b) reported that dietary supplementation with arginine enhanced mRNA mRNA levels for Cu/Zn-SOD, glutathione peroxidase and catalase, as well as antioxidant defense in juvenile blunt snout bream. In another study with juvenile yellow catfish, optimum dietary methionine decreased peroxidative damage in tissues, because SOD and glutathione peroxidase activities decreased with increasing dietary methionine levels (Elmada et al. 2016). Recovery from oxidative damage can be associated with a reduction in inflammatory molecules. Thus, changes in the expression or activities of antioxidative enzymes may reflect either an increase or decrease in oxidative stress. For this reason, the evaluation of effects of AAs on antioxidative responses should be carefully performed with fish under different conditions. Besides AAs, small peptides (glutathione and carnosine) and nitrogenous metabolites (creatine) are also important compounds for scavenging oxygen free radicals (Wu 2013a, b; Li and Wu 2018). For example, dietary supplementation with L-carnitine elevated the levels of enzymatic antioxidants, such as SOD, catalase, glutathione S-transferase (GST) activities, in tissues of juvenile black sea bream (Ma et al. 2008). Glutamate (derived from diet, synthesis, or glutamine hydrolysis), cysteine and glycine are required for the synthesis of glutathione, thereby improving the ability of fish enterocytes to repair, proliferate, and migrate in response to oxidants (Hu et al. 2014).

In all animals, including fish, the normal function of organs depends on their structural integrity, which can be affected by radiation-induced injury (Wen et al. 2014). In other words, an increase in antioxidant capacity brought about by dietary AAs is important for the health and growth of aquatic animals. Li et al. (2016b) suggested that lysine plays a significant role in protecting the intestine of fish in vivo and in vitro through the induction of expression of key antioxidant genes. Similarly, Rimoldi et al. (2016) reported that taurine supplementation to soybean meal-based diets could increase the length of villi folds, reduce the number of vacuoles, and increase the number of goblet cells. Decreases in the length of villi and the number of goblet cells were observed in turbot (*Scophthalmus maximus* L.) fed Met-deficient diets (Gao et al. 2019). Our results also indicated that low fishmeal diets could cause structural damage in the intestine and liver (Li et al. 2020f) and that this nutritional problem could be alleviated by dietary supplementation with methionine or, to a lesser extent, taurine (Fig. 8.7; Li et al. 2020g). Moreover, some traditionally nonessential AAs, such as glutamate and glutamine, could promote the antioxidant capacity in fish, which could further enhance intestinal development and growth (Li et al. 2013; Jiang et al. 2015; Zhao et al. 2015). It should be noted that an improvement in antioxidant capacity is not the only variable for assessing the positive functions of AAs in different organs, because AAs are also major substrates for ATP production and essential for protein synthesis (Jia et al. 2017; Li et al. 2020b).

8.6.6 Lipid Digestion and Metabolism

As in terrestrial animals, AAs influence the nutrition and metabolism of dietary lipids in fish, including digestion, absorption, transport, lipogenesis, and biological oxidation (primarily the mitochondrial β-oxidation) (Wu 2018). The release of CCK is augmented by the entry of long-chain fatty acids into the stomach or duodenum of fish. CCK has two major functions in lipid digestion: (1) stimulating the gallbladder to contract and release the stored bile acids into the

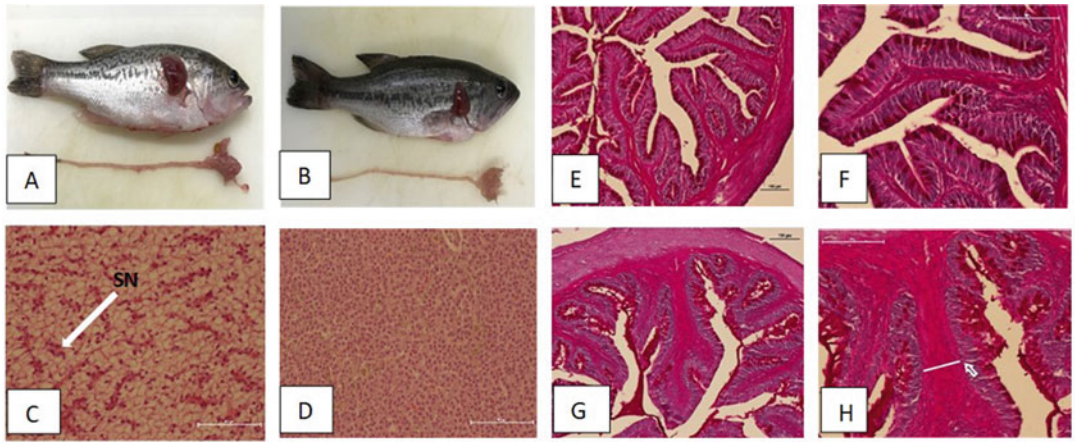

Fig. 8.7 Hepatic and intestinal morphology in largemouth bass fed low-fishmeal diets supplemented with or without methionine or taurine. The body weight of the fish was ~60 g. (**a**): Largemouth bass with a healthy liver and intestine (Li 2020); (**b**): Largemouth bass with dark skin, as well as liver and intestinal atrophies and structural abnormalities (black skin syndrome); (**c**): Largemouth bass with a healthy liver whose sinus structure is clear and well organized (white arrow); (**d**): Largemouth bass with liver atrophy and abnormal structure, as indicated by (1) reductions in the sizes of hepatocytes and their nuclei, (2) closely spaced nuclei, and (3) unclear hepatic sinus structure; (**e**) (100X) & (**f**) (200X): Morphology of the healthy intestine in largemouth bass, as stained by the periodic acid-Schiff (PAS) method and examined at different magnifications under a microscope, showing well-structured mucosae and normal lamina propria; (**g**) (100X) & (**h**) (200X): Morphology of the unhealthy intestine in largemouth bass with black skin syndrome; the gut was stained by the periodic acid-Schiff (PAS) method and examined at 100X and 200X magnifications under a microscope, showing (with an arrow) a wider lamina propria containing more inflammatory cells in comparison with normal largemouth bass. Note that the occurrence of dark skin, as well as liver and intestinal atrophies in largemouth bass fed a low (14.5%)-fishmeal diet can be reduced by dietary supplementation with either 0.5% methionine alone or 0.5% methionine plus 0.5% taurine and, to a lesser extent, with 0.5% taurine alone (Li 2020)

intestine; and (2) enhancing the secretion of pancreatic digestive enzymes. Bile acids play an important role in the emulsification of fats and increasing the surface area of fats, activating pancreatic lipase and accelerating the formation of mixed micelles. In the liver, bile acids are covalently conjugated with taurine and glycine in mammals but only with taurine in fish (Kim et al. 2007). However, recent evidence suggests that bile acids are conjugated mainly with taurine and, to a lesser extent, with glycine in the liver of fish (El-Sayed 2014). The functions of taurine in lipid digestion and the formation of bile salts have been well reviewed by Salze and Davis (2015) and El-Sayed (2014). The green liver syndrome in some fish species may be caused by the impaired conjugation of bilirubin and biliverdin in response to a dietary deficiency of taurine (Takeuchi 2014).

Fatty acids undergo β-oxidation in various tissues to produce ATP. This process involves the conversion of long-chain fatty acyl-CoA to acetyl-CoA in mitochondria (Fig. 8.8), and is enhanced in fish [e.g., Nile tilapia (Li et al. 2020e) as reported for mammals and birds (Wu 2013b). Mitochondrial carnitine palmitoyl transferase-1, which resides on the inner surface of the outer mitochondrial membrane and requires carnitine as an essential cofactor, is a major site for the regulation of mitochondrial long-chain fatty acyl-CoA transport (Wu 2018). Carnitine is derived from diets and synthesized from lysine, methionine and serine. The stimulatory effect of carnitine supplementation on the β-oxidation of fatty acids has been reported for many species, such as African catfish (Ozorio et al. 2010) and common carp (*Cyprinus carpio*; Sabzi et al. 2017). Similar results were also

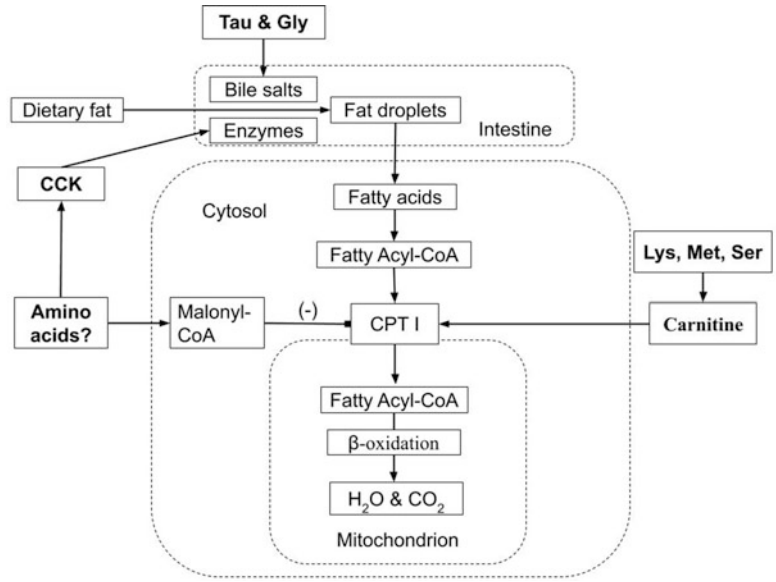

Fig. 8.8 Roles of amino acids in the regulation of fatty acid oxidation in fish. Some amino acids activate the metabolic pathways for the oxidation of long-chain fatty acids to CO_2 and water through carnitine synthesis and multiple cellular mechanisms. *CCK* cholecystokinin, *CPT 1* Carnitine palmitoyltransferase 1, *Gly* glycine, *FAs* fatty acids, *Lys* lysine, *Met* methionine, *Tau* taurine

observed in fish receiving dietary supplementation with lysine and methionine (Burtle and Liu 1994; Liao et al. 2014; Wang et al. 2016). However, there are conflicting reports that dietary carnitine supplementation either has no effect on lipid metabolism or even increases lipid deposition in fish (Dias et al. 2001; Zheng et al. 2014). Consistent with this phenotype, dietary supplementation with carnitine (331 or 3495 mg/kg diet) up-regulated mRNA levels for lipogenic genes, increased the activities of lipogenic enzymes, and reduced mRNA levels for carnitine palmitoyltransferase-1A in yellow catfish, compared with fish without carnitine supplementation (Zheng et al. 2014). In animals, β-oxidation is regulated at transcriptional and post-transcriptional levels. Transcriptional regulation involves peroxisome proliferator-activated receptors, sterol regulatory element-binding transcription factor-1, and peroxisome proliferator-activated receptor-γ coactivator-1α, whereas post-transcriptional regulation depends on the phosphorylation of acetyl-CoA carboxylase and the allosteric inhibition of carnitine palmitoyltransferase-1 by malonyl-CoA. The latter is formed from acetyl-CoA (a metabolite of AAs and glucose) and bicarbonate by acetyl-CoA carboxylase. Thus, metabolic conditions that favor lipogenesis are associated with excessive intakes of dietary AAs and starch. Based on research with mammals (e.g., Wu 2018), studies are warranted to define the mechanisms responsible for the regulation of lipid metabolism by AAs in fish.

8.6.7 Spawning and Larval Development

Newly spawned marine fish eggs have a total AA content of 40–60% of their dry mass, and this AAs pool includes proteins, peptides and free AAs (Rønnestad et al. 1999). These AAs are derived from the yolk protein (Thorsen 1995). Free AAs are an important energy source during the embryonic development of marine fishes (Fyhn 1989; Rønnestad et al. 1992, 1993), until the hatched larva has sufficiently developed its digestive system to commence exogenous feeding (Thorsen 1995; Rønnestad et al. 1993, 1999). For example, the content of free AAs in the cod (*Gadus rnorhua* L.) egg decreased from

200 nmol/egg at spawning to 25 nmol/egg or fish during the egg and yolksac larval stages (Fyhn and Serigstad 1987). As a result, an adequate supply of free AAs is necessary for successful embryonic development as they are major substrates for aerobic ATP production in eggs and yolksac larvae (Fyhn and Serigstad 1987). Some AAs, like taurine and β-alanine, are not used for protein synthesis or ATP production, but can improve the reproductive performance of fish by regulating internal osmotic pressure, neurotransmission, hormone release, anti-oxidative reactions, cellular calcium levels, and conjugation with bile acids (Matsunari et al. 2006; Pinto et al. 2010; Salze et al. 2012). A balanced provision of nutrients in broodstock feeds can increase the fecundity or egg quality by influencing the brain–pituitary–gonad–endocrine system or the availability of a substance for egg formation (Izquierdo et al. 2001). The total AA pool, fertilizability and hatchability of eggs of Nile tilapia (*Oreochromis niloticus*) can be increased by high protein intake (Gunasekera et al. 1996). Of particular note, the eggs of females were not fertilized when fish were fed a brookstock diet with only 10% crude protein (Gunasekera et al. 1996). Additional studies indicated that fertilization, hatchability or larval development were improved by the inclusion of adequate protein or AAs in broodstock feeds (El-Sayed et al. 2003; Matsunari et al. 2006; Embry et al. 2010).

Fish must initiate exogenous feeding after yolk nutrients are no longer sufficient to support the metabolic demand of the larvae. AAs are also important catabolic substrates after the onset of first feeding and may meet $\geq$ 60% of the energy requirement (Rønnestad et al. 1993). Besides, fish larvae have very high growth rates, which necessitates high requirements for dietary AAs to support protein synthesis and accretion (Rønnestad et al. 1999). In early-stage larvae, extracellular proteolytic capacity develops when they approach metamorphosis (Govoni et al. 1986). Thus, free AAs or protein hydrolysates (pre-digested protein source) are important components of the diet to initiate the feeding of marine fish larvae (Rønnestad et al. 1999, 2003, 2007; Kolkovski 2001). Larval growth, digestive system development, and metamorphosis were improved by dietary supplementation with certain AAs, such as taurine (Matsunari et al. 2005; Pinto et al. 2010), methionine (Mamauag et al. 2012), lysine (Abboudi et al. 2006; Naz and Türkmen 2009), and tryptophan (Saavedra et al. 2009). As summarized in Fig. 8.9, different kinds of protein hydrolysates have been reported to improve the quality of microdiets (Zambonino Infante et al. 1997; Cahu et al. 1999; Plascencia-Jatomea et al. 2002; Lian et al. 2008; Gisbert et al. 2012).

8.6.7.1 Other Functions of AAs

Important roles of different AAs in the growth, development, and health of fish have been well summarized by Li et al. (2009) and Andersen et al. (2016), and are briefly highlighted here. AAs are the most versatile nutrients in animals, ranging from protein structure, modifications, reactions and functions, to cell sensing and signaling, and to diverse metabolic pathways (Wu 2009; Andersen et al. 2016). Additionally, AAs contribute to the health and pigmentation of tissues in all animals, including fish. For example, abnormalities in feeding behavior and pigmentation in red sea bream fed a taurine-free diet could be ameliorated by taurine supplementation (Takeuchi 2014). Similarly, our recent studies indicated that juvenile largemouth bass fish fed diets with low fishmeal had low concentrations of taurine and methionine in serum (Li et al. 2020f), and some of the fish exhibited black skin syndrome characterized by skin darkening and retinal degeneration, as well as intestinal and liver atrophies and structural abnormalities (Fig. 8.10). Some AAs, such as arginine (Andersen et al. 2013) or glutamate (Caballero-Solares et al. 2015), are important for regulating hepatic glucose and lipid metabolism. Some AAs, like glutamate, glycine, tyrosine, and GABA, are involved in the release of pituitary hormones and could regulate

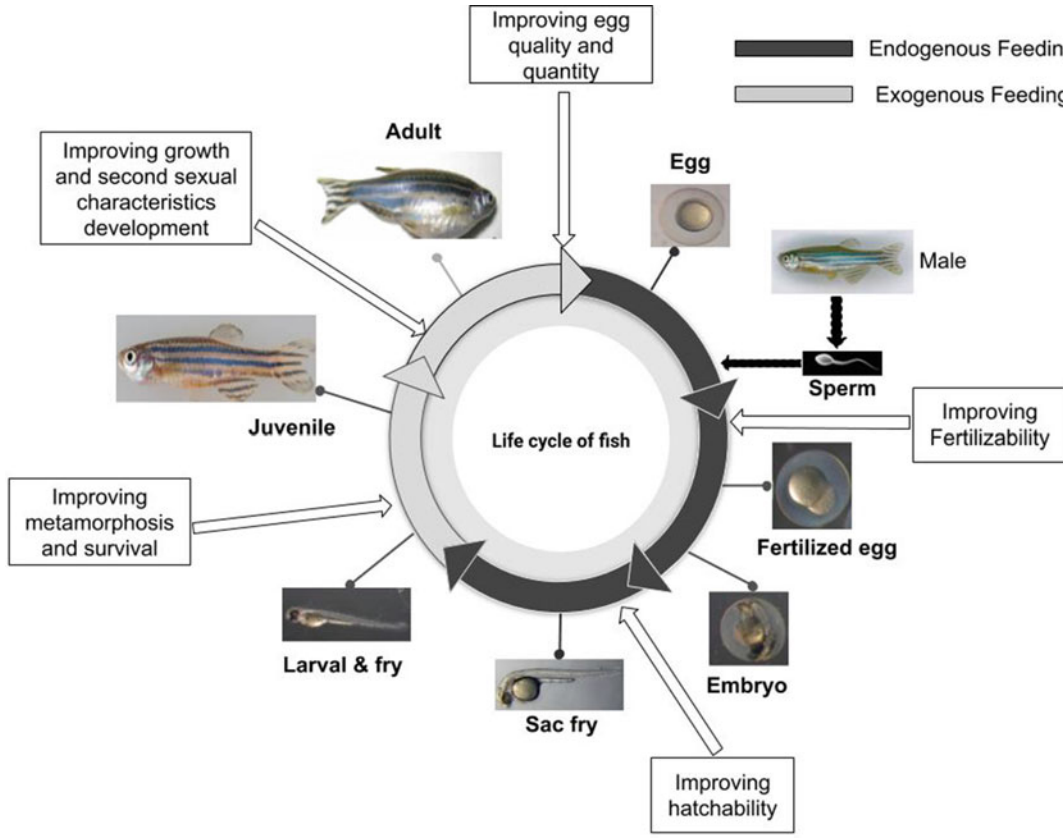

Fig. 8.9 Important roles of amino acids and protein in the development, growth and reproduction of fish. Amino acids are not only the building blocks of proteins but also signaling molecules, neurotransmitters, and regulators of metabolic pathways in animals (He and Wu 2020). These nutrients must be provided in diets to ensure optimum growth, development, health, and survival of fish

the food intake or behaviors of fish (Trudeau et al. 2000; Andersen et al. 2016). Breck et al. (2003) suggested that elevated concentrations of dietary histidine could mitigate cataract formation in the Atlantic salmon, but the underlying mechanisms are unknown. It is possible that adequate histidine is necessary for normal retinal structure and function in fish by serving as an essential precursor for the production of carnosine (a potent antioxidant) or related small peptides. Although a regulatory role for AAs in gene expression has been reported in some studies, the underlying mechanisms remain to be elucidated. Nonetheless, dietary supplementation with certain AAs can improve the growth performance and feed efficiencies in many fish species (Table 8.6), as well as their health (Andersen et al. 2016; Li et al. 2020a). Of particular note, we recently found that dietary supplementation with 0.5% methionine to a low (14.5%)-fishmeal diet reduced the incidence of black skin syndrome in largemouth bass by about 75% (Li et al. 2020g).

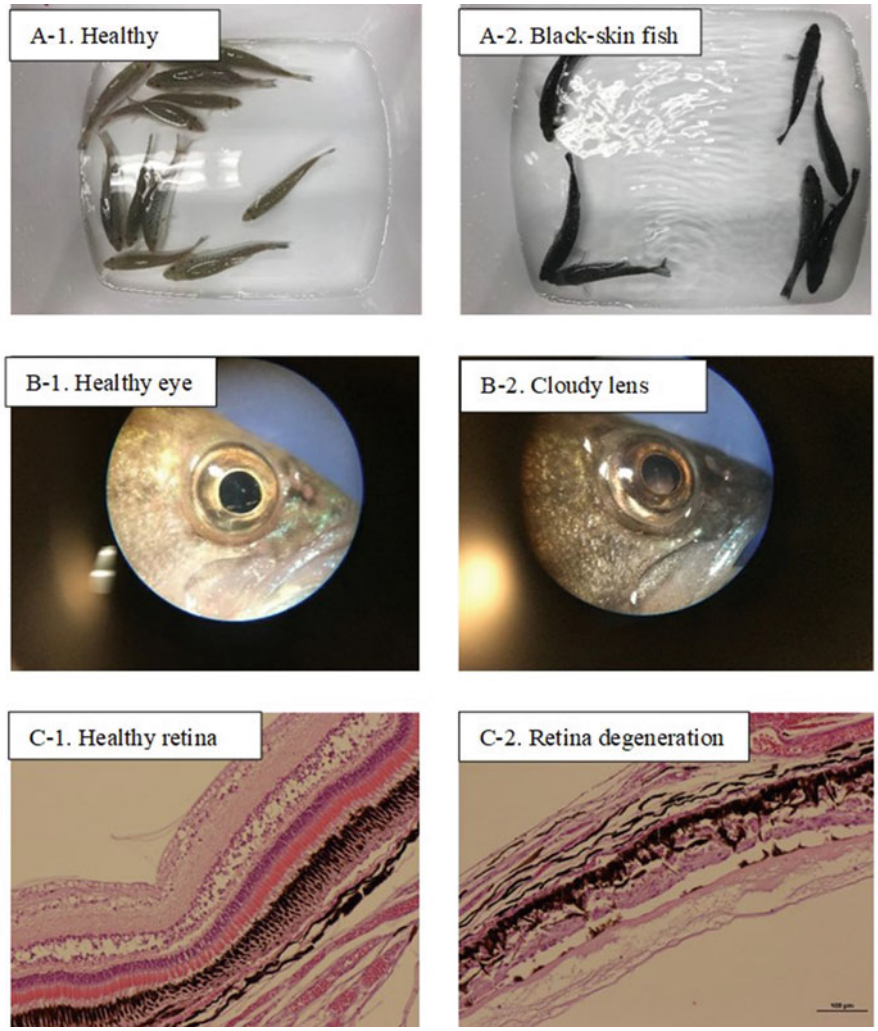

Fig. 8.10 Black skin syndrome in largemouth bass fed low (≤14.5%)-fishmeal diets (≤10% crude protein from fishmeal, dry matter basis). This syndrome is characterized by skin darkening and retinal degeneration, as well as intestinal and liver atrophies and structural abnormalities. During a 56-day experimental period, fish fed a diet containing 78.37% fishmeal do not exhibit black skin syndrome, whereas 5% of fish have the syndrome when fed a diet containing 65.3% fishmeal (Li 2020). Panels A-1, B-1 & C-1**:** Healthy juvenile largemouth bass (*Micropterus salmoides*) with normal skin pigmentation and eye morphologies (Li 2020). Panels A-2, B-2 & C-2: Fish (~ 60 g of body weight) fed low (14.5%)-fishmeal diets that exhibit dark skin and retinal degeneration after a 30-day period of feeding (Li 2020)

Table 8.6 Benenifial effects of dietary supplementation with amino acids (AAs) or protein hydrolysates on the growth and development of early-stage of fish

Species and developmental stages	AAs or protein hydrolysates	Functions	References
Gilthead seabream (*Sparus aurata*, L. 1758) larvae	Lysine	Increases in bombesin (a 14-amino acid peptide) and cholecystokinin (CCK) release	Naz and Türkmen (2009)
White seabream *(Diplodus sargus)* larvae	Tryptophan and lysine	Improvements in larval growth, survival and quality	Saavedra et al. (2009)
Cobia (*Rachycentron canadum*) larval	Taurine	Increases in specific amylase and trypsin activities	Salze et al. (2012)
Large yellow croaker (*Pseudosciaena crocea*,) larvae	Lysine	Improvements in growth and survival, as well as trypsin and leucine-aminopeptidase activities	Xie et al. (2012)
Amberjack (*Seriola dumerili*) larval	Taurine enriched rotifers	Increases in growth and survival	Matsunari et al. (2013)
California yellowtail (*Seriola lalandis*) larvae	Taurine-enriched Artemia	Increase in survival	Rotman et al. (2017)
Red Sea bream (*Pagrus major*) larvae	Methionine	Increase in the activities of digestive proteases in larvae	Mamauag et al. (2012)
Red Sea bream (*Pagrus major*) larvae	Arginine	Increase in growth	Lopez-Alvarado and Kanazawa (1994)
Sea bass (*Dicentrarchus labrax*) larvae	Fish protein hydrolysate	Increases in alkaline phosphatase and aminopeptidase N activities	Cahu et al. (1999)
		Increases in the activities of pancreatic and intestinal proteases and peptidases	Zambonino Infante et al. (1997)
Gilthead Sea bream (*Sparus aurata*) larvae	Marine protein hydrolysates	Increases in growth and innate immunity	Gisbert et al. (2012)
Nile tilapia (*Oreochromis niloticus* L) fry	Shrimp head hydrolysate	Increases in growth and feed utilization efficiency	Plascencia-Jatomea et al. (2002)
Summer flounder (*Paralichthys dentatus*) larval	Squid hydrolysate	Increases in growth and survival	Lian et al. (2008)
Asian seabass (*Lates calcarifer* Bloch) larvae	Fish muscle or squid mantle hydrolysate	Increases in digestive capacity and growth performance	Srichanun et al. (2014)

8.7 Conclusion and Perspectives

AAs play important roles in fish nutrition by serving as the building blocks of protein and precursors of low-molecular-weight substances (e.g., NO, creatine, polyamines, GABA, catecholamines, and glutathione) with enormous physiological importance, and by regulating key metabolic pathways that are vital to the growth, development, reproduction (Fig. 8.9). As in other animals, the utilization or metabolism of AAs in fish is complex and compartmentalized. Besides polypeptide synthesis, AAs participate in biological oxidation, gluconeogenesis and lipogenesis in a cell- and tissue-specific manner. Dietary protein and AAs are essential for immune responses, antioxidant reactions, metatrophosis, and adaptations to environmental changes. As a result, studies with the metabolism and functions of AAs are essential for the development of low fishmeal diets for fish by including plant-source ingredients and crystalline AAs. Furthermore, research on the environment [including air and water pollution (Wu et al. 2019b), as well as

ambient temperatures] is increasingly important in fish nutrition.

There are technical difficulties and challenges in both the industrial production of aquatic animals (e.g., fish and shellfish) and laboratory experiments (Hardy 2010; Rawles et al. 2018; Tacon and Metian 2008). First, AA requirements and functions in fish species have been determined primarily based on dose–response trials involving purified or semi-purified diets. In order to balance AA patterns in different experimental diets, different protein sources are often used among different studies. However, different feedstuffs have very different composition of AAs (Hou et al. 2019; Li and Wu 2020). Furthermore, the efficiencies of the utilization of supplemental crystalline AAs may vary considerably with diets containing different protein sources, especially at suboptimal dietary AA intakes (Gahl et al. 1994; Thu et al. 2007). This means that the same quantity and quality of crystalline AAs supplemented to diets with different protein sources will likely have different effects in fish. Second, although extruded diets have become common for many fish species in aquaculture, hard pellet feeds are used for most laboratory studies. Knowledge about nutrient losses or nutritional enhancements (e.g., improvements in the digestion of protein-bound AAs and their absorption) due to feed extrusion is still limited. It is necessary to strengthen our knowledge on how to improve protein and AA utilization by optimizing feed production processes. Third, some NEAAs (e.g., glutamate, glutamine, proline, and glycine) have long been used as an isonitrogenous control in nutritional experiments based on the unfounded belief that these AAs have no nutritional or physiological effects in fish. However, recent evidence indicated otherwise (Li et al. 2009; Andersen et al. 2016). Thus, conclusions drawn from those previous studies should be reevaluated. Furthermore, different experimental results due to the use of various fish species or strains in the published work make it difficult to propose general concepts regarding the metabolism of AAs. For example, we found that largemouth bass have an extremely high activity of arginase in their tissues to extensively degrade dietary and blood-borne arginine, but this may not be true for other species of fish (Li 2020). Therefore, it is imperative to understand the metabolic characteristics of a given fish species or a group of fish species so that we can design highly efficient, cost-effective, and sustainable aquafeeds to feed them. This, in turn, will ensure an abundant provision of high-quality animal protein (Wu 2016) and functional nutrients (e.g., taurine, creatine and glutathione; Wu 2020a) to the growing global population of humans for improving their growth, development and health, as well as resistance to metabolic and infectious diseases.

Acknowledgments We thank our students, research assistants and colleagues for helpful discussions. This work was supported by Texas A&M AgriLife Research (H-8200) and funds from Guangdong Yuehai Feeds Group Co., Ltd.

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Nutrition and Functions of Amino Acids in Aquatic Crustaceans

9

Xinyu Li, Tao Han, Shixuan Zheng, and Guoyao Wu

Abstract

Crustaceans (e.g., shrimp and crabs) are a good source of protein-rich foods for human consumption. They are the second largest aquaculture species worldwide. Understanding the digestion of dietary protein, as well as the absorption, metabolism and functions of amino acids (AAs) and small peptides is essential to produce cost-effective and sustainable aquafeeds. Hepatopancreas (the midgut gland) is the main site for the digestion of dietary protein as well as the absorption of small peptides and AAs into the hemolymph. Besides serving as the building blocks of protein, AAs (particularly aspartate, glutamate, glutamine and alanine) are the primary metabolic fuels for the gut and extra-hepatopancreas tissues (e.g., kidneys and skeletal muscle) of crustaceans. In addition, AAs are precursors for the syntheses of glucose, lipids, H_2S, and low-molecular-weight molecules (e.g., nitric oxide, glutathione, polyamines, histamine, and hormones) with enormous biological importance, such as physical barrier, immunological and antioxidant defenses. Therefore, both nutritionally essential and nonessential AAs are needed in diets to improve the growth, development, molt rate, survival, and reproduction of crustaceans. There are technical difficulties and challenges in the use of crystalline AAs for research and practical production due to the loss of free AAs during feed processing, the leaching of in-feed free AAs to the surrounding water environment, and asynchronous absorption with peptide-bounded AAs. At present, much knowledge about AA metabolism and functions in crustaceans is based on studies of mammals and fish species. Basic research in this area is necessary to lay a solid foundation for improving the balances and bioavailability of AAs in the diets for optimum growth, health and wellbeing of crustaceans, while preventing and treating their metabolic diseases. This review highlights recent advances in AA nutrition and metabolism in aquatic crustacean species at their different life stages. The new knowledge is expected to guide the development of the next generation of their improved diets.

Keywords

Amino acids · Crustaceans · Shrimp · Crabs

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1285, https://doi.org/10.1007/978-3-030-54462-1_9

Abbreviations

AA	amino acid
EAA	nutritionally essential amino acid
GABA	γ-aminobutyrate
GDH	glutamate dehydrogenase
GOT	glutamate-oxaloacetate transaminase
GPT	glutamate-pyruvate transaminase
mTOR	mechanistic target of rapamycin
NEAA	nutritionally nonessential amino acid
NO	nitric oxide
NRC	National Research Council
ROS	reactive oxygen species

9.1 Introduction

Crustaceans (including shrimp and crabs) are low-fat, good sources of high-quality protein, free amino acids (AAs), small peptides, and poly-unsaturated fatty acids for human consumption (Bhavan et al. 2010; Wu et al. 2016; Wu 2020). Therefore, they are healthy seafoods worldwide. Crustacean farming has been an economically important enterprise in either a marine or a fresh-water environment as the second largest aquaculture species (e.g., 7.86 million tons and US$ 57.1 billion in 2016; Tacon 2018). Twenty-seven (27) species of aquatic crustaceans have been reported, which include mainly shrimps, crabs, and crawfish (Tacon 2018).

Crustaceans have particularly high requirements for dietary protein, which ranges from 60% of the diet for some post-larvae to 30–50% of the diet for juvenile shrimp, crabs and lobsters (Unnikrishnan and Paulraj 2010; Jin et al. 2013; Mente 2006). High-protein diets lead to the excretion of a large amount of nitrogen and low water quality. Traditionally, fishmeal has been the major protein source for crustaceans due to its high levels of digestible protein and balanced AA profiles (Unnikrishnan and Paulraj 2010). However, fishmeal is an unsustainable protein source due to its limited source and high price (Hardy 2010). In the culture of crustaceans, the cost of feeds represents more than 50% of the production costs (Mente 2006). Therefore, continued expansion of crustaceans is not unsustainable if fishmeal is their sole or primary protein source. In addition, disease and animal health have been a major limiting factor for the culture of shrimps, crabs, and crawfish (Mente 2006; Stentiford et al. 2012). Knowledge of their optimum requirements for nutrients, particularly AAs, is key to solving this problem, because many AAs regulate key metabolic pathways that are crucial to the maintenance, growth, reproduction, and immune responses of animals (Li et al. 2007, 2009b; Wu 2010; Wu et al. 2014).

Understanding the digestion of dietary protein, as well as the absorption, metabolism and functions of small peptides and AAs are essential to manufacture environmentally-oriented aquafeeds and reduce feed costs in animal production (Li et al. 2009b). Such diets can improve the health and wellbeing of crustaceans, while preventing and treating their metabolic diseases. Although a wide range of dietary AA requirements has been reported for aquatic animals in the literature, our knowledge about AA metabolism and functions in crustaceans is limited. The crustaceans belong to the suborders of the Decapoda with different metabolic, physiological, and immunological characteristics, when compared with other animals such as fish and mammals (NRC 2011; Vazquez et al. 2009). The major objective of this article is to highlight current knowledge about AA nutrition and metabolism in shrimps, crabs, and crawfish at their different stages of lives. This will help to advance the field of protein nutrition and guide the development of future crustacean feeds.

9.2 Protein Digestion and the Absorption of Small Peptides and Free AAs in Crustaceans

The diets of crustaceans contain high concentrations of protein (NRC 2011). The digestive tract of crustaceans is essentially an internal tube and generally divided into three functional segments: foregut (a tubular esophagus and a stomach), midgut (a simple tubule with associated ceca and the hepatopancreas), and hindgut (rectum and anus; Fig. 9.1). The esophagus joins the

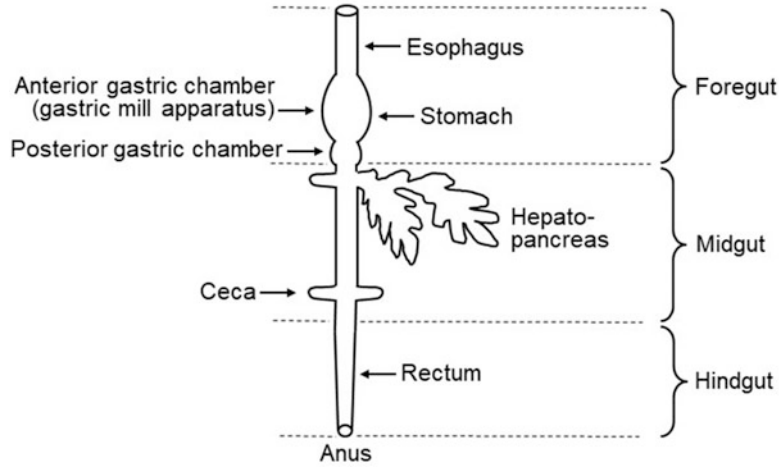

Fig. 9.1 Scheme of the digestive tract of crustaceans. The digestive tract of crustaceans consists of the foregut (a tubular esophagus and a stomach), midgut (a simple tubule with associated ceca and the hepatopancreas), and hindgut (rectum and anus). Cutting and grinding of the ingested foods, as well as their initial digestion (by digestive enzymes from the hepatopancreas) occurs in the anterior chamber of the stomach (the gastric mill apparatus). The food particles enter the anterior midgut and then the joining hepatopancreas. The hepatopancreas secretes digestive enzymes and is the major site for the extracellular digestion of foods and absorption of digestion products or simple nutrients into the hemolymph. Undigested foods and unabsorbed nutrients enter the rectum and exit the gut through the anus

mouth to the stomach [an anterior chamber (the gastric mill apparatus) and a posterior chamber]. The anterior chamber functions in mastication (cutting and grinding) of the ingested food, whereas the posterior chamber keeps food particles from leaving the stomach until the gastric mill has reduced them into a small size (McGaw et al. 2013). Secretion of HCl by gastric epithelial cells results in acidic conditions in stomach fluids (pH = ~ 4 or higher) during digestion. The food particles leave the posterior chamber of the stomach to enter the anterior midgut and then the hepatopancreas (also called the midgut gland or digestive gland; a branching array of blind-ended tubules lined with an epithelium) that connects to the anterior *midgut* via ducts (Ceccaldi and Ceccaldi 1989). The hepatopancreas secretes digestive enzymes that flow into the midgut and then retrograde into the stomach. Much extracellular digestion of foods and absorption of digestion products (free AAs, as well as di- and tri-peptides) or simple nutrients into the hemolymph occur *within* the hepatopancreas (Buarque et al. 2009, 2010; Fernández et al. 1997; Saborowski et al. 2006). The midgut plays a relatively minor role in the digestion and absorption of nutrients. Di- and tri-peptides (the major products of protein digestion) are taken up by the epithelial cells of the hepatopancreas via the apical-membrane peptide transporter-1, whereas free AAs are taken up by these cells via various sodium-dependent and independent transporters (Wu 2013). Within the absorptive cells, the small peptides are hydrolyzed by peptidases (including proline peptidases) to free AAs. AAs that are not metabolized by the hepatopancreatic cells enter the hemolymph. Undigested food particles and unabsorbed nutrients from the terminal midgut enter the rectum to form feces, which leaves the gut through the anus.

Studies with the southern brown shrimp *Farfantepenaeus subtilis* have shown the highest activity of aminopeptidase in the presence of alanine-, arginine-, lysine- or leucine-β-naphthylamide as a substrate (Buarque et al. 2010). Proteinases and peptidases activities in crustaceans are modulated by several internal and external factors (Saborowski et al. 2006). These enzymes have an optimum pH around 8 (Buarque et al. 2009; Dionysius et al. 1993). Moreover, the enzyme activities are also

influenced by ontogenetic events (Lemos et al. 2000), life stages (Lee et al. 1984), hormones (Gorell and Gilbert 1969; Thomson et al. 1971), the molting cycle (Gimenez et al. 2001, 2002), and diet composition such as protein levels and sources (Lee et al. 1984; Brito et al. 2000; Muhlia-Almazan et al. 2003). All of these results indicate that crustaceans can adapt to changes in their diets and physiological states.

Crustaceans have a high ability to digest a wide range of animal- and plant-source proteins. In whitelegs shrimp, the digestibilities of AAs are greater than 92% (Cruz-Suárez et al. 2009). Proteins from animal resources are better digested than plant proteins in several crustacean species (Forster and Gabbott 1971; Fenucci et al. 1982). A decrease in the digestibility of AAs was observed with an increase in the graded dietary level of rice protein concentrate from 0% to 100% (i.e., 25, 50, 75, and 100%) (Oujifard et al. 2012). The low digestibility of AAs in plant ingredients results from the presence of inhibitors of proteinases and peptidases (Garcia-Carreo et al. 1997; Oujifard et al. 2012). To solve this problem, heating and fermentation are the common ways to remove or reduce these anti-nutritive factors in plant-source feedstuffs (NRC 2011). Moreover, feed additives, such as organic acids and enzymes, can be added to crustacean feeds to improve the utilization of alternative dietary protein sources. In whiteleg shrimp, dietary organic acids can modify the activities of digestive enzymes and the digestibility of dietary protein possibly due to changes in gastric pH and intestinal microbes (Silva et al. 2016). Supplementation with proteases to low fishmeal diets has been reported to improve the growth or feed utilization of some shrimp (Li et al. 2016; Song et al. 2017) and crab (Chowdhury et al. 2018) species.

9.3 The Free AA pool in Crustacean Tissues

Crustaceans have an open circulatory system, where nutrients, oxygen, hormones, and cells are distributed in the hemolymph. Therefore, all of their blood is not contained within vessels, but rather blood is drawn into the heart through holes called the ostia, pumped out again to circulate through tissues, and return to the heart (Wirkner and Stefan 2013). After the hepatopancreas absorb small peptides and free AAs through its single-cell layer of epithelial cells into the hemolymph, AAs participate in metabolic pathways in the whole body as the building blocks of proteins and peptides, substrates for ATP production, and precursors for the syntheses of low-molecular-weight bioactive substances (e.g., NO, neurotransmitters, and thyroid hormones), signaling molecules (Li et al. 2007; Wu 2013). The concentrations of free AAs in most crustacean tissues are higher than those in vertebrate tissues. Table 9.1 shows the concentrations of AAs in the hemolymph of shrimp. The major free AAs in crustaceans are glycine, glutamine, alanine, arginine, and taurine, which may vary among different species (Fig. 9.2; Shinji and Wilder, 2012; Miyagawa et al., 1990). All of these AAs are abundant in animal-source feedstuffs (Li and Wu 2018; Li and Wu 2020a), whereas all plant-source feedstuffs lack taurine and contain low concentrations of glycine (Hou et al. 2019; Li and Wu 2020a; Li et al. 2011a). Of note, arginine phosphate is present in some crustaceans, such as shrimp. Concentrations of free AAs in their tissues are affected by diets and environmental factors, such as salinity (Shinji and Wilder 2012), ammonia levels (Chen et al. 1994), temperature (Rao and Ramachandra 1961), and intracellular protein turnover (Wu 2013). Free AAs in tissues are in dynamic equilibrium with the protein pool. On the molar basis, glycine is the most abundant free AA in the hemolymph (a fluid analogous to the blood in vertebrates) and the whole body of the whiteleg shrimp (*Litopenaeus vannamei*), followed by alanine, taurine, arginine, glutamine and proline in the hemolymph and by arginine, taurine, proline, glutamine, and alanine in the whole body, in descending order (Table 9.1). Of note, in the whole body of the shrimp, most of free AAs represent about 5% (g/g) of their corresponding total AAs (free plus peptide-bound), but free glycine and free arginine account for 30.5% and 23.3% of the total AAs, respectively. In the whole body of the whiteleg shrimp,

Table 9.1 Concentrations of free and peptide-bound amino acids (AAs) in the whole body of whiteleg shrimp[a]

AA	Free AAs in hemolymph (nmol/ml)	Free AAs in the whole body (mg/g of DM)	Total AAs (free plus peptide-bound) in the whole body[b]		Ratio of free AAs to total AAs in the whole body
			mg/g of DM	mg/g of protein AAs	(g/g)
Proteinogenic AAs					
Ala	958 ± 33	5.98 ± 0.26	43.5 ± 0.40	60.3 ± 0.91	0.137 ± 0.003
Arg[b]	576 ± 21	11.0 ± 0.48	47.0 ± 0.22	65.1 ± 0.43	0.233 ± 0.006
Asn	189 ± 11	1.21 ± 0.05	31.4 ± 0.46	43.5 ± 0.88	0.038 ± 0.001
Asp	80.1 ± 4.2	1.62 ± 0.06	37.7 ± 0.43	52.3 ± 0.95	0.043 ± 0.001
Cys	152 ± 11	1.10 ± 0.05	11.6 ± 0.25	16.1 ± 0.55	0.095 ± 0.002
Gln	562 ± 12	6.25 ± 0.29	40.6 ± 0.58	56.3 ± 0.97	0.154 ± 0.003
Glu	95.3 ± 6.8	2.78 ± 0.13	65.9 ± 0.44	91.3 ± 0.76	0.042 ± 0.001
Gly	1024 ± 63	15.3 ± 0.66	55.6 ± 0.32	77.1 ± 0.71	0.275 ± 0.005
His	101 ± 5.5	1.14 ± 0.05	15.0 ± 0.24	20.8 ± 0.49	0.076 ± 0.002
Ile	116 ± 7.4	1.55 ± 0.06	29.8 ± 0.29	41.3 ± 0.63	0.052 ± 0.001
Leu	162 ± 13	2.70 ± 0.11	49.8 ± 0.41	69.0 ± 0.88	0.054 ± 0.001
Lys	257 ± 15	3.92 ± 0.12	50.5 ± 0.45	70.0 ± 0.94	0.078 ± 0.002
Met	32.7 ± 1.6	0.90 ± 0.04	15.2 ± 0.23	21.1 ± 0.44	0.053 ± 0.001
Phe	70.5 ± 8.3	1.53 ± 0.05	33.1 ± 0.57	45.9 ± 0.96	0.046 ± 0.001
Pro	308 ± 19	6.60 ± 0.31	49.2 ± 0.55	68.2 ± 1.2	0.134 ± 0.004
OH-Pro	45.2 ± 3.6	0.031 ± 0.001	8.90 ± 0.26	12.3 ± 0.43	0.0035 ± 0.0002
Ser	254 ± 15	1.64 ± 0.06	37.8 ± 0.39	52.4 ± 0.86	0.043 ± 0.001
Thr	162 ± 6.9	1.89 ± 0.07	29.4 ± 0.34	40.7 ± 0.70	0.064 ± 0.001
Trp	28.3 ± 1.4	1.12 ± 0.04	8.80 ± 0.18	12.2 ± 0.40	0.127 ± 0.003
Tyr	30.5 ± 1.8	2.43 ± 0.08	27.1 ± 0.32	37.6 ± 0.68	0.090 ± 0.002
Val	224 ± 9.7	2.17 ± 0.09	33.6 ± 0.37	46.6 ± 0.75	0.065 ± 0.001
Total AAs	5428 ± 76	72.7 ± 1.0	721.5 ± 9.2	1000	–
Non-proteinogenic AAs					
β-Alanine	25.2 ± 3.8	0.009 ± 0.0003	–	–	–
Cit	0.24 ± 0.02	Trace amount[c]	–	–	–
Orn	148 ± 8.6	0.16 ± 0.01	–	–	–
P-Arg	37.4 ± 2.8	10.9 ± 0.32	–	–	–
Taurine	717 ± 55	9.06 ± 0.09	–	–	–

Cit citrulline, *DM* dry matter, *OH-Pro* 4-hydroxyproline, *Orn* ornithine, *P-Arg* phosphoarginine

[a]Values are means ± SEM, n = 8. Whiteleg shrimp (*Litopenaeus vannamei*) were fed a diet consisting of the following (as-fed basis): 20% fishmeal, 10% soybean meal, 15% wheat flour, 35% poultry by-product, 1.5% soybean oil, 1% soy lecithin, 1% cholesterol, 0.1% vitamin C, 0.13% choline chloride, 4.6% K_2HPO_4, 0.7% $MgCl_2$, 0.1% astaxanthin (5%), 0.5% vitamin-mineral premix, and 10.37% cellulose (Li and Wu 2020b). The composition of the vitamin-mineral premix (g/kg premix) was: vitamin A, 0.4; vitamin D_3, 0.04; vitamin E, 40; vitamin K_3, 2.40; vitamin B_5, 21.74; inositol, 30; vitamin B_3, 28; vitamin B_1, 6.53; biotin, 0.3; folic acid, 1.2; vitamin B_{12}, 0.04; KI, 1.06; $CuSO_4.5H_2O$, 1.10; $MnSO_4.H_2O$, 1.25; $ZnSO_4.7H_2O$, 13.68; and cellulose, 840.19. The crude-protein content of the diet was 43.0% (dry matter basis). The shrimp were raised in water (25 °C and 3–5 ppt salinity). Hemolymph (0.1 ml; a fluid that is analogous to the blood in vertebrates) was obtained from the shrimp (15 g/shrimp) at 24 h after the last feeding. Hemolymph is a fluid that is analogous to the blood in vertebrates. Free and peptide-bound AAs in the whole shrimp were analyzed as described by Li and Wu (2020a). The amounts of amino acids in the whole body were calculated on the basis of their intact molecular weights. The content of dry matter in the whole body of the shrimp was 24.2%. The true protein (calculated on the basis of the molecular weights of amino acid residues; i.e., intact molecular weight – 18) in the whole body of the shrimp was 60.9% of dry matter, whereas collagen represents 10.0% of the total true protein in the whole body. Cys is the sum of cysteine plus 1/2 cystine.

[b]Excluding phosphoarginine. The content of arginine as phosphoarginine in the whole body of the shrimp was 7.47 mg/g of dry matter, as analyzed by high-performance liquid chromatography (Wu and Meininger 2008)

[c]The value was 0.18 ± 0.01 μg/g of dry matter

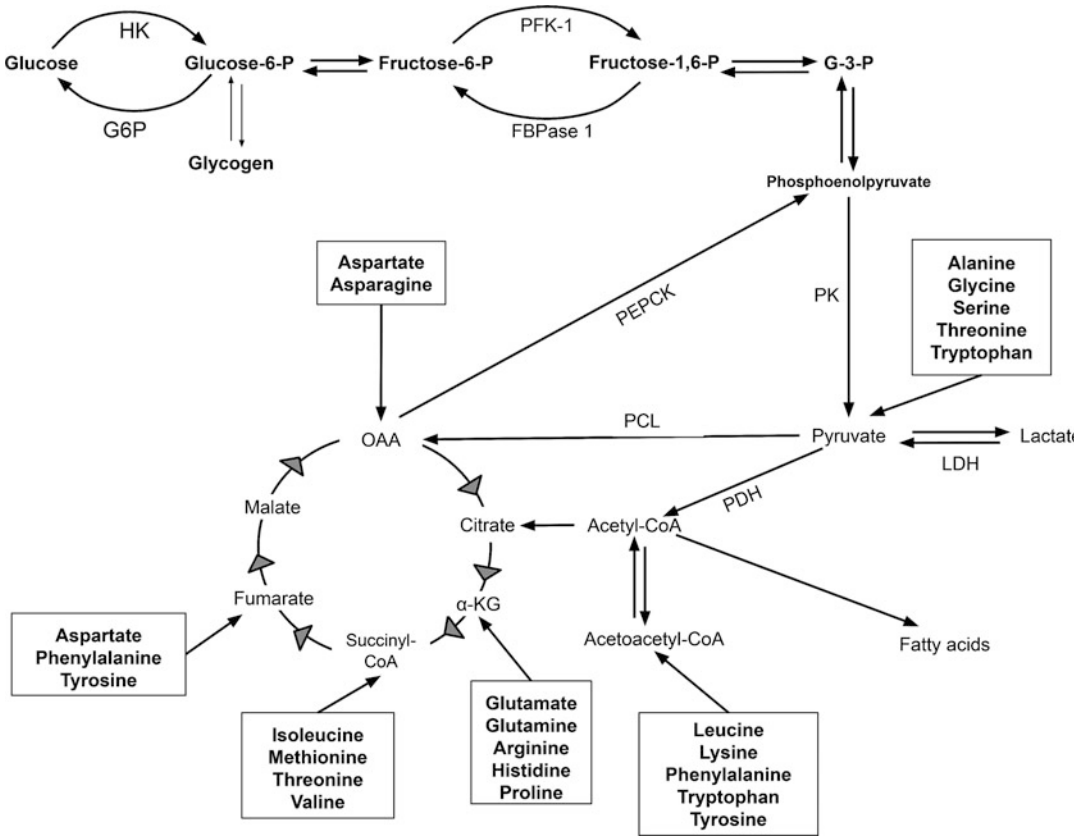

Fig. 9.2 Different metabolic pathways for the catabolism of amino acids converge to common intermediates that feed into the Krebs cycle, lipogenesis, and glucogenesis pathways in crustaceans. *G6Pase* glucose-6-phosphatase, *PCL* pyruvate carboxylase, *PEPCK* phosphoenolpyruvate carboxykinase, *PDH* pyruvate dehydrogenase, *PK* pyruvate kinase, *LDH* lactate dehydrogenase, *HK* hexokinase, *PFK-1* phospho-fructokinase-1, *G-3-P* glyceradehyde-3-phosphate, *α-KG* α-ketoglutarate, *OAA* oxaloacetate

the ratio of total free proteinogenic AAs (72.7 mg/g of dry weight) to the total proteinogenic AAs (721.5 mg/g of dry matter) is 1.0:10.0 (Table 9.1). The high abundance of free AAs is consistent with their important role in the maintenance of osmolality and metabolism in shrimp.

9.4 Protein Synthesis in Crustacean Tissues

The process of protein synthesis in both crustaceans and other animals include five steps: (1) gene transcription; (2) initiation of translation; (3) peptide elongation; (4) termination, and (5) posttranslational modifications (Wu 2013). In crustaceans, the rate of protein synthesis is generally higher in the hepatopancreas, followed by the heart, gill, tail and claw muscle in descending order (Houlihan et al. 1990; Mente et al. 2011). Among these tissues, protein synthesis in skeletal muscle is crucial for shrimp growth and production. A postecdysial increase in muscle fiber length and the associated increase in the sarcomere number are accompanied by an increase in muscle protein synthesis (Carter and Mente 2014). The rate of muscle protein synthesis (Ks, the percentage of the protein mass synthesized per day) is 1.26%/day at 27 °C in whitelegs shrimp (Mente et al. 2002), 1.15%/day at 15 °C for shore crabs (*Carcinus maenas*; El Haj and Houlihan 1987), and 0.9–1.4%/day at 30 °C

in brown tiger prawn (*Penaeus esculentus*; Hewitt 1992). For comparison, the rate of protein synthesis is lower at 0.3–0.4%/day in the claw, leg and abdominal muscles of the American lobster (*Homarus Americanus*, Haj et al. 1996). The rate of muscle protein synthesis also varies with muscle fiber type and muscle type. Slow-type tonic muscle fibers have a rate of protein synthesis that is 2.1 times greater than fast-type phasic fibers (El Haj and Houlihan 1987). Protein synthesis plays a vital role in the growth, development, health and survival of animals (Carter and Mente 2014; Li et al. 2020c). For example, vitellogenesis (synthesis of v*itellogenin* as a precursor protein of egg yolk in the blood or hemolymph of females) occurs in the ovary and hepatopancreas to support reproduction (Tseng et al. 2001). Increases in protein synthesis in the midgut gland after feeding enhance the secretions of digestive enzymes for the digestion of dietary nutrients (Houlihan et al. 1990).

The growth of crustaceans depends on ecdysis (also known as molt), which refers to the replacement of their rigid carapace with a new and larger one generated underneath the former exoskeleton that consists primarily of chitosan (Comeau and Savoie 2001). Therefore, protein synthesis is highly related to the molt cycle. The highest rate of protein synthesis occurs during the premolt stages in shore crabs (El Haj and Houlihan 1987). Moreover, protein synthesis is also influenced by several abiotic and biotic factors, such as hormones (Carter and Mente 2014), starvation and re-feeding (Pellegrino et al. 2013), dietary composition, hypoxia, hyperoxia, temperature, salinity, and other environmental factors (Intanai et al. 2009; Mente et al. 2002, 2003). For example, the rates of protein synthesis, survival, and specific growth are higher in shrimp fed diets with high quality proteins than in shrimp fed low quality proteins (Mente et al. 2002). Of note, muscle protein synthesis is substantially higher in brown tiger prawn (*Penaeus esculentus*) fed a 50%-protein diet than a 30%- or 40%-protein diet (Hewitt 1992). Similar to other animals, protein synthesis requires a large amount of energy in crustaceans and accounts 20% to 37% of oxygen consumption in the shore crab (Houlihan et al. 1990). Therefore, starch and lipids are often included in artificial diets for crustacean as an energy source to spare protein and improve protein deposition. The protein-sparing effect of dietary digestible carbohydrate has been reported in *Litopenaeus vannamei* (Wang et al. 2015). In crabs fed a high-digestible carbohydrate diet, the rate of muscle protein synthesis measured with ^{14}C-leucine has been reported to be 2.3-fold greater than that in crabs fed a high protein diet (Pellegrino et al. 2013). This conclusion, however, may not be valid because leucine is extensively catabolized by skeletal muscle and therefore, is not an appropriate tracer for the measurement of its protein synthesis (Wu 2013).

Substantial amounts of collagens are present in tissues of crustaceans, including the shell (consisting of 22–24% dry matter) and skeletal muscles of shrimp. For example, shrimp shell consists of the following (dry matter basis): 25–40% protein, 15–20% chitin, 45–50% calcium carbonate, and 15–40 mg astaxanthin/kg, with the protein comprising of 60–75% collagen, 4–5% elastine, and 20–35% keratine (Immaculada et al. 2009). Kimura and Tanaka (1986) reported that the collagen content in the skeletal muscles of three species of crustaceans (giant river prawn, fleshy prawn and spiny lobster) was 2.4% to 2.6% of total protein. The content of collagen as the percentage of total protein in the muscles of crustaceans is as follows: 1.1–2.2% in the shrimp (*Trachypenaeus curvirostris*, *Palaemon paucidens*, and *Pandalus borealis*), 2.6–2.9% in prawn (*Penaeus japonicas*), 2.5–2.7% in lobster (*Panulirus Iongipes*), 0.2–0.8% in crabs (*Charybdis japonica*, *Portunus trituberculatus*, *Chionoecetes opilio* ♂, *Chionoecetes opilio* ♀, and *Erimacrus isenbeckii*), 3.4% in crayfish (*Procambarus clarkia*), and 5.9–6.2% in squilla (*quilla Oratosquilla oratoria*) (Yoshinaka et al. 1989). For comparison, collagen represents 2% of total protein in beef skeletal muscle (Wu et al. 2016). The AA composition and solubility of the major collagen in the crustacean muscles are similar to those of Type V collagen in vertebrate skeletal muscles (Yoshinaka et al. 1989). As a major

constituent of the connective tissue, collagen supports the structure, locomotion, mechanical strength of the muscles, bones and fin in crustaceans. Based on the content of 4-hydroxyproline in the whole body of shrimp (Table 9.1), the abundance of collagen in the whole body of shrimp appears to be 66% lower than that in vertebrates (Wu 2013).

9.5 Catabolism of Energy Substrates for ATP Production in Crustacean Tissues

The requirement of crustaceans for dietary protein has been reported to be 30–60%, depending on their species, developmental stage, and production conditions (Halver and Hardy 2002; Cuzon et al. 2004; Unnikrishnan and Paulraj 2010; Jin et al. 2013; Mente 2006). However, the rate of retention of dietary nitrogen is only about 17–30%, which is even lower than that for some fish species (Bulbul et al. 2016; Panini et al. 2017; Qiu et al. 2017). In addition, the oxygen:nitrogen ratio (the ratio of oxygen consumed to nitrogen excreted; O/N, mol/mol) is often employed in energetic studies as an indicator for the use of organic substrates (i.e., lipids, carbohydrates or proteins) as metabolic fuels. An oxygen:nitrogen ratio in shrimp is < 40 (Coelho et al. 2019; Comoglio et al. 2004; Zhang et al. 2019), indicating AAs may be their predominant energy substrates. The limited utilization of glucose by penaeid shrimp has been reported in some studies, and the recommended levels of digestible carbohydrates starch in diets are generally less than 20% (Guo et al. 2006). Rosas et al. (2002) have suggested that shrimp (*Litopenaeus vannamei*) are well adapted to dietary protein as a source of energy because of its limited ability to use high carbohydrate. In crabs (*Neohelice granulate*), dietary proteins have been suggested as an important source of energy (Pellegrino et al. 2013). AAs (especially alanine) are important substrates in the gill tissue of the blue crab, and appears to play a role in both short-term cell volume regulation and long-term osmoregulatory processes (Pressley and Graves 1983).

In all animals, individual AAs have their own catabolic pathways because of their different structures (Wu 2013). However, the catabolism of many AAs shares a number of common steps to generate pyruvate, oxaloacetate (OAA), α-Ketoglutarate (α-KG), fumarate, succinyl-CoA, and acetyl-CoA (Fig. 9.2). For example, the carbon backbones of some AAs are converted to α-KG by glutamate dehydrogenase (GDH) and transaminases. Aminotransferases have been reported in the skeletal muscle, gill and hepatopancreas of crabs (*Carcinus maenas;* Chaplin et al. 1967). The catabolism of glutamine involves its deamination by phosphate-activated glutaminase to produce glutamate and ammonia. The major end product of AA metabolism in crustaceans is ammonia, which represents more than 50% of their nitrogenous wastes (Regnault 1987). Free AAs are the second most important nitrogenous waste since they account for 10–25% of the total excreted nitrogen in different species (Regnault 1987). Urea and uric acid are nitrogenous end-products but are usually excreted by crustaceans in small amounts (< 10%).

To generate ATP, the carbon backbone of glutamate, alanine, and aspartate are converted into α-KG, pyruvate, and oxaloacetate by GDH, glutamate-pyruvate transaminase (GPT), and glutamate-oxaloacetate transaminase (GOT), respectively (Wu 2013; Richard et al. 2010; Lu et al. 2015). We found that in both whiteleg shrimp (*Litopenaeus vannamei*) and blue crabs (*Callinectes sapidus*), AAs, such as aspartate, glutamine and glutamate, provide the bulk of energy but the oxidation of glucose for ATP production is very limited in their skeletal muscle and ovaries (Table 9.2 and Fig. 9.3). In both animal species, aspartate is the predominant metabolic fuel among the AAs (Fig. 9.3). Similarly, both GPT and GOT are present in different tissues (hemolymph, hepatopancreas, gills and skeletal muscle) of shrimp (*Fenneropenaeus indicus*), with the activity of GOT being 2–3 times higher than that of GPT in the same tissue (Mohankumar

Table 9.2 Rates of oxidation of energy substrates in the intestines of whiteleg shrimp and blue crabs[1]

	D-[U-^{14}C] Glucose (5 mM)	[U-^{14}C] Palmitate (2 mM)	L-[U-^{14}C] Glutamate (2 mM)	L-[U-^{14}C] Glutamine (2 mM)	L-[U-^{14}C] Alanine (2 mM)	L-[U-^{14}C] Aspartate (2 mM)	L-[U-^{14}C] Leucine (2 mM)
Oxidation of an energy substrate in tissues of whiteleg shrimp (nmol CO_2/mg tissue per 2 h)							
Midgut (n = 6)	3.89 ± 0.55^{d}	0.97 ± 0.07^{f}	7.72 ± 0.27^{b}	6.44 ± 0.28^{c}	4.39 ± 0.29^{d}	12.3 ± 0.44^{a}	1.71 ± 0.11^{e}
HP (n = 8)	1.36 ± 0.07^{b}	0.45 ± 0.01^{f}	0.82 ± 0.02^{d}	1.23 ± 0.09^{b}	0.99 ± 0.04^{c}	1.83 ± 0.07^{a}	0.64 ± 0.03^{e}
Gill (n = 8)	2.52 ± 0.13^{a}	0.49 ± 0.03^{d}	1.72 ± 0.09^{c}	1.94 ± 0.16^{bc}	0.81 ± 0.06^{d}	2.19 ± 0.11^{ab}	0.31 ± 0.03^{e}
Skeletal Muscle (n = 8)	0.19 ± 0.02^{c}	0.02 ± 0.00^{e}	0.44 ± 0.02^{b}	0.45 ± 0.02^{b}	0.09 ± 0.01^{d}	1.03 ± 0.07^{a}	0.03 ± 0.00^{e}
Oxidation of an energy substrate in tissues of blue crabs (nmol CO_2/mg tissue per 2 h)							
Midgut (n = 6)	4.38 ± 0.13^{c}	7.88 ± 0.36^{a}	2.21 ± 0.15^{d}	0.85 ± 0.07^{e}	0.84 ± 0.06^{e}	5.77 ± 0.29^{b}	0.27 ± 0.02^{f}
HP (n = 12)	1.74 ± 0.11^{b}	5.75 ± 0.27^{a}	0.41 ± 0.05^{e}	0.08 ± 0.01^{f}	0.90 ± 0.06^{d}	1.33 ± 0.13^{c}	0.32 ± 0.04^{e}
Gill (n = 9)	8.05 ± 0.31^{a}	5.35 ± 0.38^{b}	2.13 ± 0.19^{c}	2.45 ± 0.42^{c}	1.22 ± 0.10^{d}	5.00 ± 0.18^{b}	0.33 ± 0.03^{e}
Skeletal Muscle (n = 12)	0.04 ± 0.00^{f}	0.16 ± 0.01^{d}	1.75 ± 0.18^{b}	0.10 ± 0.01^{e}	0.20 ± 0.01^{c}	2.19 ± 0.16^{a}	0.06 ± 0.01^{f}
Ovary (n = 12)	0.08 ± 0.01^{ef}	0.74 ± 0.06^{c}	2.09 ± 0.18^{b}	0.06 ± 0.01^{f}	0.23 ± 0.02^{d}	5.62 ± 0.33^{a}	0.10 ± 0.01^{e}

HP Hepatopancreas

[1]Rates of oxidation of an energy substrate by the 13-g shrimp (*Litopenaeus vannamei*) or the 150-g blue crab (*Callinectes sapidus*) tissue were determined with the use of its [U-^{14}C]-labeled tracer, as described by Jia et al. (2017). A tissue was incubated in 1 ml of oxygenated Krebs-Henseleit bicarbonate buffer (pH 7.4) at 26 °C for 2 h. The incubation medium also contained a mixture of energy substrates (5 mM D-glucose, 2 mM palmitate, 2 mM L-glutamate, 2 mM L-glutamine, 2 mM L-aspartate, 2 mM L-alanine, and 2 mM L-leucine) plus either D-[U-^{14}C]glucose, [U-^{14}C]palmitate, L-[U-^{14}C]glutamate, L-[U-^{14}C]glutamine, L-[U-^{14}C]alanine, L-[U-^{14}C]aspartate, or L-[U-^{14}C]leucine. The specific radioactivity of each tracer was 100 dpm/nmol. At the end of the 2-h incubation, $^{14}CO_2$ produced by a tissue was collected into 0.2 ml of Soluene to calculate the rate of CO_2 production from a substrate. Results were analyzed by one-way analysis of variance and the Student-Newman-Keuls multiple comparison, as described by Assaad et al. (2014)

[a-f]: Within a row for shrimp or blue crabs, means not sharing the same superscript letter differ ($P < 0.05$)

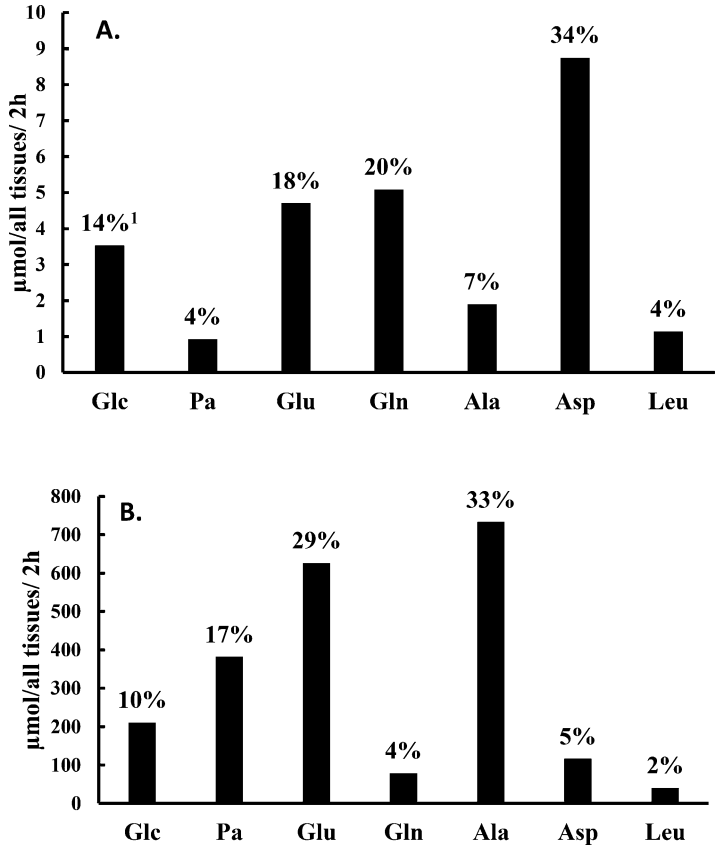

Fig. 9.3 ATP production from the oxidation of individual substrates in tissues (the midgut, hepatopancreas, gill plus skeletal muscle) of the 15-g whiteleg shrimp *Litopenaeus vannamei* (**Panel A**) and the 150-g swimming crab *Portunus trituberculatus* (**Panel B**). The rates of ATP production were calculated from the data in Table 9.1, as described by Li et al. (2020b).[1] The contribution of an individual substrate to total ATP production in tissues incubated in the presence of a mixture of substrates. *Glc* glucose, *Pa* palmitate, *Glu* glutamate, *Gln* glutamine, *Ala* alanine, *Asp* aspartate, *Leu* leucine

and Ramasamy 2006). GDH is largely responsible for the production of ammonia from AAs in crustaceans (Fernández-Urruzola et al. 2011). In whiteleg shrimp (*Litopenaeus vannamei*, the activity of GDH increases with increasing the dietary protein level from 25% to 50% (Li et al. 2011b). The measurement of GDH activity in the crude homogenates of the shrimp (*Crangon crangon*) suggests that the oxidative deamination of glutamate by GDH may account for all the ammonia excretion by this species (Batrel and Regnault, 1985). GDH transcripts are detected in most tissues of Chinese mitten crabs (*Eriocheir sinensis*; Wang et al. 2012), freshwater prawn (*Macrobrachium rosenbergii*; Chakrapani et al. 2017), whiteleg shrimp (*Litopenaeus vannamei*; Li et al. 2009a), and mud crabs (*Scylla paramamosain*; Lu et al. 2015).

Although AAs are the major energy sources for crustaceans, the rates of their oxidation to CO_2 vary among different tissues and species. For example, the specific activity of GPT in the skeletal muscle and gills of black tiger shrimp (*Penaeus monodon*) is about 3-times the value measured in the hepatopancreas (Richard et al. 2010). The activity of GDH is also relatively low in the hepatopancreas of black tiger shrimp, suggesting a minor role of this tissue in glutamate catabolism (Richard et al. 2010). Likewise, although GDH is expressed in the skeletal muscle, epithelium, eyestalk, hepatopancreas, and gill of Pacific white shrimp, its enzymatic activity in the hepatopancreas is much lower than that in the other four tissues (Li et al. 2009a). Similarly, the rate of CO_2 production from aspartate is 3–6 times higher than that from glucose in the

intestine and skeletal muscle of whiteleg shrimp, but the rate of oxidation of these two substrates is quantitatively comparable in the hepatopancreas (Table 9.2). Of particular note, in blue crabs, palmitate is the primary energy source for the midgut and hepatopancreas, with the rate of its oxidation being substantially higher than that of any AA substrates (Table 9.2). In both whiteleg shrimp and blue crabs, AAs are the most important energy substrates for ATP production in skeletal muscle. Richard et al. (2010) also reported that skeletal muscle has high activities of GPT and GDH for glutamate catabolism in black tiger shrimp.

Phosphate-activated glutaminase may be quantitatively the major enzyme for initiating glutamine catabolism in crustaceans. For example, in a fresh-water crab (*Paratelphusa hydrodrom*us), a high correlation between glutaminase activity and ammonia excretion rate has been observed at various salinity levels (Krishnamoorthy and Srihari, 1973). In whiteleg shrimp, the rate of the oxidation of glutamine is similar to or even higher than that of glutamate in various tissues (Table 9.2). However, the organs (except for the gill) of blue crabs oxidize much more glutamate than glutamine, which may be attributed to the low glutaminase activity. The gill of blue crabs oxidized both glutamate and glutamine at relatively high rates. This is in agreement with a previous report that glutaminase activity is most active in the gills of three crab species, indicating that this organ is an active site of glutamine hydrolysis and glutamate degradation (King et al. 1985). Skeletal muscles of crabs have a high activity of glutamine synthetase and may be the major site for glutamine synthesis in the body (King et al. 1985). Interestingly, the activities of GDH and glutaminase are undetectable or very low in the hepatopancreas of the three crab species studied (King et al. 1985). Similarly, our results indicated that glutamine and other AAs are not the primary energy substrates in the hepatopancreas of blue crabs. To date, our knowledge of AA catabolism in crustaceans is very limited (Table 9.3).

9.6 Glucogenesis and Lipogenesis in Crustaceans

AAs can be the precursors for glucose and lipid syntheses to provide the body with glucose and lipids (Fig. 9.2). Gluconeogenesis and its related key enzymes [e.g., phosphoenolpyruvate carboxykinase (PEPCK)] have been demonstrated in different tissues of crustacean species, such as the skeletal muscle, hepatopancreas, and gill (Reyes-Ramos et al. 2018; Vinagre and Da Silva 2002; Schein et al. 2004). The conversion of ^{14}C-alanine and ^{14}C-glycine into glucose occurred in the hepatopancreas, gill and skeletal muscle of crabs (*Chasmagnathus granulate*; Oliveira et al. 1997; 2004; Vinagre and Da Silva, 2002; Martins et al. 2011). The in vitro experiments also showed that these tissues were able to incorporate ^{14}C-glycine to lipids (Vinagre and Da Silva 2002; Martins et al. 2011). The presence of gluconeogenesis from AAs in the skeletal muscle of crabs is interesting, because such a biochemical pathway is absent from terrestrial mammals and birds (Wu 2018).

Glucose and lipids are important energy sources for crustaceans under certain physiological conditions or stresses (Reyes-Ramos et al. 2018). For example, intramuscular lipids are used for ATP production in crabs in the fall and winter (Kucharski and Da Silva 1991). Dietary AAs are converted into lipids in skeletal muscle when crabs (*N. granulate*) are fed diets with high protein content, and the intramuscular lipids serve as an important energy reserve for the animals during osmoregulation and in the winter (Pellegrino et al. 2013). Moreover, gluconeogenesis and lipogenesis contribute to the adjustment of the intracellular concentration of nitrogenous compounds to withstand changes in the salinity of the surrounding water (Martins et al. 2011). Therefore, both gluconeogenesis and lipogenesis from AAs are important for the growth and health of crustaceans exposed to different levels of salinity. Previous experiments indicated that the incorporation of [^{14}C]alanine into glucose in the jaw muscles of crabs submitted to a

Table 9.3 Nutritional and physiological functions of amino acids and their metabolites in crustaceans

Metabolites	Amino acids	Reported functions in crustaceans	References
NO, polyamines	Arginine	Improves antioxidant and immune systems in shrimps *Fenneropenaeus chinensis* and *Marsupenaeus japonicus*	Jiang et al. (2006)
Phosphoarginine	Arginine	Storage of biological energy, controlling osmoregulation in crustaceans, such as the shrimp (*Litopenaeus vannamei*), the blue crab (*Callinectes sapidus*), and the common littoral crab (*Carcinus maenas*)	Holt and Kinsey (2002) and Kotlyar et al. (2000)
NO, polyamines, taurine, phosphoarginine	Arginine, ornithine, and methionine	Regulation of osmotic and ionic homeostasis in crustances, such as blue crabs (*Callinectes sapidus* Rathbun)	Lovett and Watts (1995)
Nucleotides, ATP	Glutamine, glycine and aspartate	Improves the growth of black tiger shrimp (*Penaeus monodon*)	Do Huu et al. (2012), (2013)
Carnitine, hydroxylysine, taurine, polyamines	Lysine and methionine	Improves immune functions, antioxidant defense systems, and energy metabolism in whilteleg shrimp (*Litopenaeus vannamei)* and in narrow clawed crayfish (*Astacus leptodactylus leptodactylus* Eschscholtz, 1823)	Safari et al. (2015) and Zhou et al. (2017)
Glucosamine, glutamate, ATP	Glutamine	Serves as a substrate for glycoprotein synthesis and as a female signal (i.e. contact sex pheromone) in mate recognition [e.g., in caridean shrimp (*Palaemonetes pugio*)]; improves wound healing, pathogen encapsulation, and maintenance of normal crustacean connective tissues in custaceans	Caskey et al. (2009) and Martin et al. (2003).
Catecholamines, melanin	Phenylalanine and tyrosine	As components of primary stress responses in whilteleg shrimp (*Litopenaeus vannamei)*	Aparicio-Simón et al. (2010)
Glutathione	Cysteine, glutamate and glycine	Improves growth, antioxidant system and stress resistance in whilteleg shrimp (*Litopenaeus vannamei)*	Xia et al. (2018)
Glutathione	Glycine	Improves growth, antioxidant and immune system in whilteleg shrimp (*Litopenaeus vannamei)*	Xie et al. (2014)
Pyrroline-5-carboxylate	Proline	Improves growth, antioxidant system and stress resistance in whilteleg shrimp (*Litopenaeus vannamei)*	Xie et al. (2015a)
GABA, ATP	Glutamate	Improves growth, antioxidant system and stress resistance in whilteleg shrimp (*Litopenaeus vannamei)*	Xie et al. (2015b)
Serotonin, melatonin	Tryptophan	Regulator of growth, reproductive function and agonistic behavior in the black tiger shrimp (*Penaeus monodon*) and the mud crab (*Scylla serrata*)	Wongprasert et al. (2006)and Laranjia et al. (2010)

GABA γ-aminobutyric acid, *NO* nitric oxide

hyperosmotic shock increased by 77% over the control group (Schein et al. 2004). In the posterior gills of *N. granulata* subjected to hyper- and hypo-osmotic stresses, the formation of ^{14}C-lipids from ^{14}C-glycine increased at 72 h after the treatment, but the activity of PEPCK (a rate-controlling enzyme for glucose synthesis) decreased (Martins et al. 2011). Similarly, the rate of lipid synthesis in shrimp exposed to both hypo- or hyper-osmotic conditions was slightly enhanced with an increase in FAS activity, when compared with a normo-osmotic condition (Chen et al. 2014). Thus, the partition of AAs toward the synthesis of either lipids or glucose in crustaceans, depending on nutritional, physiological and environmental factors.

9.7 Syntheses of Bioactive Metabolites in Crustaceans

In addition to the syntheses of proteins, lipids and glucose, AAs are the precursors of many low-molecular-weight substances with important and diverse biological roles in animals (Wu 2013, 2018). These products of AAs include NO, billirubin, carnosine and related dipeptides, carnitine, catecholamines, neurotransmitters, creatine, glucosamine, glutathione, heme, histamine, polyamines (putrescine, spermidine and spermine), purines, and pyrimidines, and are produced in a tissue-specific manner (He and Wu 2020; Wu 2013). Polyamines, which are synthesized from methionine and arginine, play vital roles in chromatin structure, gene transcription and translation, DNA stabilization, signal transduction, cell growth, and proliferation in animals. Polyamines are also involved in the regulation of osmotic and ionic homeostasis by interacting directly with the Na^+, K^+-ATPase enzyme in crabs (Lovett and Watts 1995). GSH is formed from cysteine, glutamate, and glycine via two ATP-dependent enzymes in the cytosol: γ-glutamyl-cysteine synthetase and glutathione synthetase (Wu 2013). Glutathione exerts both growth-promoting and immunostimulatory effects in *Litopenaeus vannamei* (Xia and Wu 2018). L-Phosphoarginine (arginine phosphate), which is generated from arginine and ATP by arginase kinase, exists in skeletal muscles from various invertebrate animals. Of particular note, concentrations of L-phosphoarginine in the skeletal muscles of some crustacean species (e.g., crayfish) can be up to 83 to 100 mM (Ennor et al. 1956; Marcus and Morrison 1964). We found that the concentration of phosphoarginine in the hemolymph of *Litopenaeus vannamei* was about 40 nmol/ml. The main function of phosphoarginine is to store biological energy like phosphocreatine in animals (Wu 2013). Phosphoarginine also plays a role in the metabolic support of the gill's function to regulate osmoregulation in crustaceans (Holt and Kinsey 2002; Kotlyar et al. 2000). However, knowledge about the metabolism and functions of these AA metabolites in most crustacean species is limited.

9.8 Functions of AAs in the Culture of Crustacean Species

9.8.1 Molt and Survival

The growth of crustaceans occurs through the shedding of an old exoskeleton (shell) and the formation of a new exoskeleton, and is greatly influenced by the extended intermolt period (molt frequency) and the molt increment (carapace and body weight growth at molt). Moreover, the survival of some crustacean species is highly dependent on the molting processes. For example, many deaths are due to the presence of calcium deposits embedded on and in the inner surface of the exuvial exoskeleton, which is known as the molt death syndrome (Bowser and Renée 1981; Wang et al. 2016). The molting process is under the control of several regulatory hormones, environmental factors (Hosamani et al. 2017), and diets (Kibria 1993; Millikin 1980). The cumulative molts in crabs are strongly affected by voluntary feed and protein intakes, indicating that AAs are required for tissue growth especially during the postmolt period (Nguyen et al. 2014). AAs have been suggested as important factors for molting processes through energy provision for ecdysis, osmoregulation, collagen synthesis, and the removal of the exokeleton (Dooley et al. 2002), as well as the regulation of hormone release (Qi et al. 2019). For example, free proline and glycine may be used as metabolic fuels during ecdysis (Claybrook 1983) and substrates for the synthesis of the new exoskeleton in the later premoult (Yamaoka and Skinner 1976). Concentrations of a molt hormone, ecdysterone, are increased in the serum of crab (*Eriocheir sinensis*) receiving dietary supplementation with arginine (Qi et al. 2019). The same species have higher survival rates and molt frequency when fed diets containing adequate lysine and arginine (Jiang et al. 2005; Qi et al. 2019). More details about the functions of AAs in the molting of shrimp are presented in Table 9.4.

Osmoregulation is an essential physiological process for the majority of aquatic crustaceans since many of them have been widely farmed in

Table 9.4 Main functions of amino acids at different stages of the molt cycle in shrimp

	Stage	Duration	Exoskeleton	Feeding	Main functions of AAs
Postmolt	A	1–2 h	Soft exoskeleton	None	Osmoregulation regulation, protein synthesis for tissue growth
	B	2–5 h	Little hardened exoskeleton	Weak	
Intermolt	C	8–10 days	Hard exoskeleton	Maximal	Energy sources; and protein and peptide syntheses
	D0	1–2 days	Epidermis starts apolysis	Maximal	
	D1	1–2 days	No new cuticle	Decrease	
Premolt	D2	2 days	New cuticle appears	Decrease	
	D3	1 day	Interval between the old and the new cuticle	Decrease	Collagen synthesis, osmoregulation regulation, and hormone release
	D4	1 day	Water absorption and old exoskeleton splits	None	
Molt	E	15 min	Old cuticle is shed, body expanded		Energy source and osmoregulation regulation

The molt stage is adapted from (Rao et al. 2008)

inland and oceans with different environmental conditions (Romano and Zeng 2012). As a result, the crustaceans usually are faced with numerous stresses such as low or high salinity, high density, and hot or cold temperatures. Free AAs in the hemolymph appear to play important roles in ATP production (Pressley and Graves 1983). Their levels generally increase in the hemolymph under various stress conditions (Shinji and Wilder 2012). Of particular note, some free AAs (e.g., glutamate, proline, glycine, alanine, taurine and arginine) are known to be involved in the active adjustment of intracellular osmoregulation in marine invertebrates (Tan et al. 1981; Chen and Chen 2000; Liu et al. 2012; 2018; Chakrapani et al. 2017). A recent review has indicated that an increase in protein levels in the diet of *Litopenaeus vannamei* is a practical method of nutritional modulation to increase their production at extreme high and low salinities (Li et al. 2015). After an acute salinity change, the survival of whiteleg shrimp is increased with increasing the dietary glycine level from 2.26% to 2.70% (Xie et al. 2014).

AAs play an important role in controlling osmoregulation in crustaceans because their metabolic enzymes such as transaminase (Koyama et al. 2018), GDH (Lu et al. 2015) and arginine kinase (Holt and Kinsey, 2002; Kotlyar et al. 2000) are regulated by salinity levels. In the abdominal muscle of the kuruma shrimp, the concentrations of alanine and glutamine are elevated in response to increased salinity in association with a decrease in GPT gene expression and an increase in GDH gene expression (Koyama et al. 2018). Acute salinity stress increases GDH expression, as well as the syntheses of glutamate, proline and alanine in the muscle of the Chinese mitten Crab (*Eriocheir Sinensis*) to meet the demand for osmoregulation at hyperosmotic conditions (Wang et al. 2012). Consistent with this finding, a reduction in ^{14}C-alanine oxidation appears to be one of the mechanisms responsible for the increase of the free AA pool in the hepatopancreas of crabs (*Chasmagnathus granulate*; Schein et al. 2005) during hyperosmotic stress. A hyperosmotic stimulus also induces proline synthesis from glutamate in *Tigriopus californicus* (Burton 1991).

Much evidence shows that AAs play a central adaptive role in crustaceans during exposure to cold, starvation and ammonia (Chen et al. 1994, 2000; Zhou et al. 2011). For example, the accumulation of proline and alanine in the hepatopancreas seems to be a common response to cold stress in some invertebrates (Hanzal and Jegorov 1991; Fields et al. 1998; Liu et al. 2018). Increasing the content of proline from 2.02% to 2.6% in low (15%) fishmeal diets improved the tolerance of *Litopenaeus vannamei* to ammonia stress (Xie et al. 2015a, b). Moreover, shrimp fed diets with a deficiency of lysine had the greatest incidence and severity of neural lesions when they were challenged with subsequent stress exercises

(Katzen et al. 1984). Clearly, it is imperative to study the functions of specific AAs in crustaceans exposed to different stresses.

9.8.2 Growth and skeletal muscle development

AAs have been traditionally classified as essential (EAAs) or nonessential (NEAAs) for animals, including crustaceans. The diets of crustacean species must contain ten EAAs for survival and growth: arginine, methionine, valine, threonine, isoleucine, leucine, lysine, histidine, phenylalanine, and tryptophan, all of which are not synthesized de novo by eukaryotic cells (NRC, 2011). These AAs are considered as limiting nutrients in commercial feed formulas and are indispensable for the growth, development and survival of the animals. If one of the EAAs is deficient, it will limit the use of all AAs for intracellular protein synthesis, therefore increasing their oxidation to CO_2. For example, a low rate of retention of dietary protein in the *Litopenaeus vannamei* results from a deficiency of lysine (Xie et al. 2012) or threonine (Zhou et al. 2013) in their diets. Purified or semi-purified diets have been employed to determine both qualitative and quantitative requirements of crustaceans for dietary EAAs. Lysine, arginine, and methionine are regarded as the most limiting factors for whole-body growth. Most of these studies were based on the growth performance of select crustaceans as shown in Table 9.5. To date, NEAAs have been recommended to be included in the diets of all animals (Wu 2013). This revises the classical "ideal protein" concept to formulate balanced diets for improving protein accretion, feed efficiency, and health in animals (Wu 2018). A recent study indicated that weight gains and specific growth rates were increased in juvenile Pacific white shrimp receiving dietary supplementation with glycine (Xie et al. 2014). Many factors, such as feeding regime, stocking density, water quality, and other rearing conditions, may affect the requirements of aquatic organisms for dietary AAs (Façanha et al. 2016; Zhang et al. 2018).

AAs can promote muscle development and protein synthesis by either providing the building blocks or stimulating signaling pathways. In mammals, dietary supplements with branched-chain amino acid (BCAAs) alone elicits an anabolic response (e.g., muscle protein synthesis; Wolfe 2017; Wu 2013). An evolutionally conserved protein kinase, mechanistic target of rapamycin (mTOR), is the master regulator of protein synthesis and cytoskeleton remodeling, as well as intracellular protein degradation via autophagy (Wu 2013). AAs, such as leucine, arginine, glutamine, glycine, tryptophan and valine, activate the mTOR cell signaling to initiate protein synthesis in skeletal muscle and intestine (Li et al. 2011c; Wu 2018). The mTOR plays an important role in the regulation of growth, molting, cell differentiation, and nutrient metabolism in crustacean species (Abuhagr et al. 2014; Shyamal et al. 2018; Wu et al. 2019). In the Chinese white shrimp (*Fenneropenaeus chinensis*), intraperitoneal administration of leucine and arginine stimulated the expression of fch-TOR and activated the mTOR signaling pathway in skeletal muscle (Sun et al. 2015a). Functional AAs are expected to enhance the growth, survival, and productivity of crustaceans, as reported for terrestrial mammals and birds (Wu 2018).

9.8.3 Release of Hormones

Similar to terrestrial animals, hormones in crustaceans are messengers that help to regulate their physiological states and functions, such as temperature, satiety, nutrient and energy metabolism, growth, development, and reproduction. For example, AAs regulate muscle growth not only through direct actions on myogenic regulatory factors and mTOR signaling, but also indirectly via the growth hormone/insulin-like growth factor (IGF) axis. Growth hormone in serum and the expression of IGF2 in the hepatopancreas of the Chinese mitten crab *(Eriocheir sinensis)* were significantly enhanced by dietary supplementation with arginine (Qi et al. 2019). In addition, the concentrations of insulin and neuropeptide Y in the blood of *Litopenaeus vannamei* were increased in response to dietary supplementation

Table 9.5 Reported requirements of crustacean species for dietary lysine, arginine and methionine

Species	Initial body weight	Dietary crude protein, % (sources)	Isonitrogenous control	Requirement (% of the diet, model)	Variables	References
Lysine requirements						
Whiteleg shrimp (*Litopenaeus vannamei*)	0.52 g	40 (FM and WGM)	Asp/Gly (1:1)	1.64 (BL)	SGR	Xie et al. (2012)
	3.62 g	38 (FM and CGM)	Arg[a]	2.11 (Anova)	WG, SGR	Feng et al. (2013)
Giant tiger prawn (*Penaeus monodon*)	21 mg	40 (casein and gelatin)	Asp and Glu	2.08 (BL)	WG	Millamena et al. (1998)
Atlantic ditch shrimp (*Palaemonetes varians*)	17 mg	45 (FM and WGM)	AAs mix[b]	2.42 (BL), 2.63 (EX)	WG	Palma et al. (2015)
Swimming crab (*Portunus trituberculatus*)	7.86 g	50 (FM and SBM)	Asp/Gly (1:1)	2.17 (BL)	SGR	Jin et al. (2015a)
Chinese mitten crab (*Eriocheir sinensis*)	2.05 g	38 (casein, FM, SBM)	Glu	2.34 (Qua)	WG	Ye et al. (2010)
	6.86 mg	60 (casein and gelatin)	AA mix[c]	2.55	ML, survival	Jiang et al. (2005)
Arginine requirements						
Whiteleg shrimp (*Litopenaeus vannamei*)	0.5 g	41 (FM and WGM)	Asp/Gly (1:1)	1.96 (BL)	SGR	Zhou et al. (2012)
	3.62 g	38 (FM and CGM)	Lys[a]	1.80 (ANOVA)	WG, SGR	Feng et al. (2013)
Giant tiger prawn (*Penaeus monodon*)	21 mg	35 (casein and gelatin)	Asp and Glu	1.85 (BL)	WG	Millamena et al. (1998)
	1.19 g	45 (casein)	Casein	2.5 (BL)	WG	Chen et al. (1992)
Kuruma shrimp (*Marsupenaeus japonicus*)	0.25 g	50 (casein and gelatin)	Glu	2.66 (BL)	WG	Alam et al. (2004a)
Atlantic ditch shrimp (*Palaemonetes varians*)	17 mg	45 (FM and WGM)	AAs mix[b]	2.05 (BL), 2.39 (EX)	WG	Palma et al. (2015)
Chinese mitten crab (*Eriocheir sinensis*)	2.03 g	38 (casein, FM, SBM)	Glu	3.62 (Qua)	WG	Ye et al. (2010)
	6.86 mg	60 (casein and gelatin)	AA mix[c]	2.0 (Anova)	ML, survival	Jiang et al. (2005)
Swimming crab (*Portunus trituberculatus*)	4.72 g	50 (FM and SBM)	Asp/Gly (1:1)	2.77 (BL)	SGR	Jin et al. (2016)
Methionine requirements						
Whiteleg shrimp (*Litopenaeus vannamei*)	0.55 g	40 (FM, SBM)	Asp/Gly (1:1)	0.91 (Qua)	WG	Lin et al. (2015)

(continued)

Table 9.5 (continued)

Species	Initial body weight	Dietary crude protein, % (sources)	Isonitrogenous control	Requirement (% of the diet, model)	Variables	References
	4.18 g	38 (FM, SBM)	Asp/Gly (1:1)	0.67 (Qua)	WG	Lin et al. (2015)
	9.77 g	34 (FM, SBM)	Asp/Gly (1:1)	0.66 (BL)	WG	Lin et al. (2015)
Atlantic ditch shrimp (*Palaemonetes varians*)	17 mg	45 (FM and WGM)	AAs mix[b]	0.96 (BL), 0.99 (EX)	WG	Palma et al. (2015)
Chinese mitten crab (*Eriocheir sinensis*)	2.05 g	38 (Casein, FM, SBM)	Glu	1.12 (Qua)	WG	Ye et al. (2010)
Giant tiger prawn (*Penaeus monodon*)	21 mg	37 (casein and gelatin)	Asp and Glu	0.89 (BL)	WG	Millamena et al. (1996)
Swimming crab (*Portunus trituberculatus*)	11.3 g	50 (FM and SBM)	Asp/Gly (1:1)	0.96 (BL)	SGR	Jin et al. (2015b)

Regression model: *BL* broken line, *Qua* quadratic, *EX* exponential
Parameters: *IGR* instantaneous growth coefficient, *WG* weight gain, *SGR* specific growth rate, *ML* Molt
Protein sources: *FM* fishmeal, *SBM* soybean meal, *SPC* soybean protein concentrate, *WGM* wheat gluten meal, *CGM* corn gluten meal
[a]The study is about the optimal ratio and content of lysine to arginine in diet for shrimp
[b]Premix of amino acids (g/100 g): cystine, 5; tryptophan, 3; threonine, 11; isoleucine, 9; histidine, 12; valine, 12; leucine, 15; phenylalanine, 20; tyrosine, 13
[c]Amino acid mixture (g/100 g): leucine, 8.53; isoleucine, 5.01; lysine, 7.06; methionine, 1.27; phenylalanine, 5.17; threonine, 5.21; tryptophan, 2.76; valine, 2.37; histidine, 2.29; aspartic acid, 11.31; serine, 4.74; glutamic acid, 16.74; proline, 5.62; glycine, 7.25; alanine, 7.07; tyrosine, 6.96; cysteine, 0.63

with GABA (Xie et al. 2015b). Tryptophan is the precursor of the monoaminergic neurotransmitter serotonin (5-hydroxytryptamine). In mud crabs, tryptophan supplementation contributed to a significant increase of serotonin in the hemolymph, thus suppressing the agonistic behavior of mud crabs during aggressive encounters and improving their survival (Laranjia et al. 2010). In the Chinese mitten crab *(Eriocheir sinensis)*, dietary supplementation of tryptophan can promote limb regeneration by regulating regeneration-related gene expression and the digestion of foods within the hepatopancreas, which may be related to the enhanced levels of melatonin and the binding of serotonin and dopamine to their corresponding receptors (Zhang et al. 2019). In the juvenile *Litopenaeus vannamei*, dietary supplementation with tryptophan was beneficial to improve its growth performance possibly by mediating serotonin and GABA signaling pathways (Sun et al. 2015b).

9.8.4 Immune and Antioxidant Responses

Proper nutrition is critical not only to achieve optimal growth rates but also to maintain the health of cultured aquatic animals (Pohlenz and Gatlin 2014). AAs are essential components of the cells and tissues of the immune system, and play a vital role in the immunity of mammals, fish and crustacean species (Trichet 2010; Li et al. 2007). Like other invertebrates, crustaceans lack adaptive immune systems and depend solely on the innate immune system to defend against infectious pathogens *(*Vazquez et al. 2009). The prophenoloxidase activating system (the proPO-system) and associated factors are important mediators of immunity in crustaceans. The proPO is activated by substances of microbial origins (e.g., β-1,3-glucans, lipopolysaccharides, and peptidoglycans) to stimulate the circulating

hemocytes (large granular hemocytes, small granular hemocytes, and hyaline cells). These cells play important roles not only through direct sequestration and killing of infectious agents but also by synthesis and exocytosis of a battery of bioactive molecules (Söderhäll and Cerenius 1992). Along with hemocytes, crustaceans possess plasma proteins or humoral factors, such as lectin, α-2 macroglobulin responsible for clotting, lipopolysaccharide-binding protein, β-glucan-binding protein, antimicrobial peptides, and lysosomes (Trichet, 2010; Vazquez et al. 2009). As the nitrogenous precursor for NO, arginine has a beneficial effect on tissue oxygenation and immune function for animals (Wu et al. 2009), including crustaceans (Qi et al. 2019; Zhu et al. 2009). Thus, increasing the dietary arginine content from 1.72% to 3.72% improved the growth, feed efficiency survival, immunity, and disease resistance to *Aeromonas hydrophila* in the juvenile Chinese mitten crab (Qi et al. 2019). Similarly, dietary supplementation with tryptophan to Chinese mitten crabs increases their survival after a challenge with pathogens (Yang et al. 2019).

Reactive oxygen species (ROS) are highly reactive molecules that may contribute to radiation-induced cytotoxicity (e.g., chromosome aberrations, protein oxidation, and muscle injury), as well as metabolic and morphologic changes (e.g., increased muscle proteolysis and dysfunction of the central nervous system) in animals (Fang et al. 2002). Endogenous antioxidant defenses are crucial for the control of ROS production and the prevention of oxidative damage in cells. The principal defense systems against oxygen free radicals are superoxide dismutase, glutathione, glutathione peroxidases, glutathione reductase, catalase (a heme-containing enzyme), and antioxidant nutrients (Fig. 9.4). AAs and their derivatives are important antioxidant nutrients for crustacean species, as for

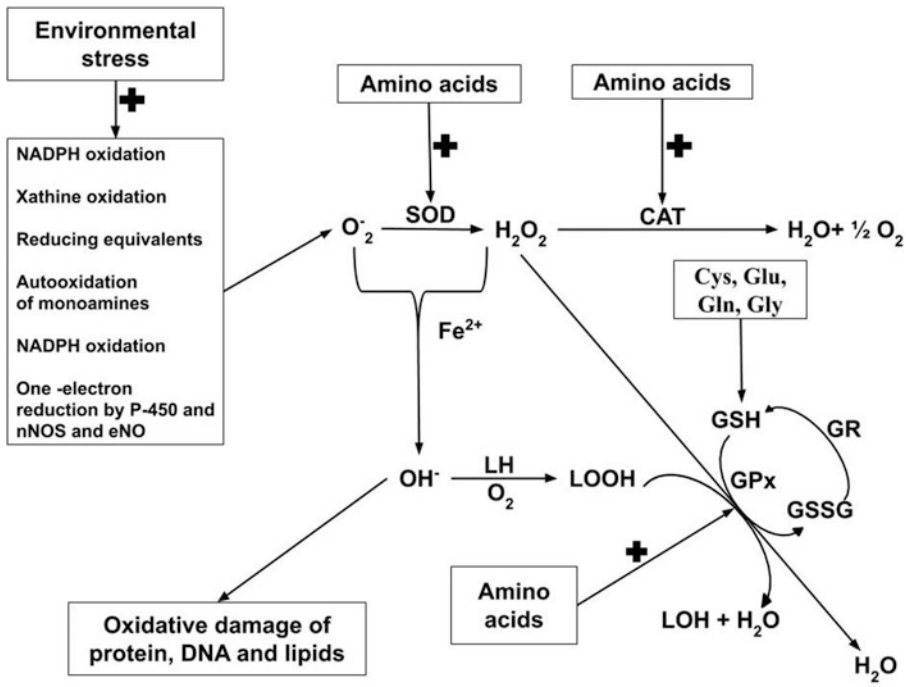

Fig. 9.4 Roles of amino acids and their metabolites as antioxidants in crustaceans. *Cys* cystine, *CAT* catalase, *Gln* glutamine, *Glu* glutamate, *Gly* glycine, *GPx* glutathione peroxidase, *GR* glutathione reductase, *GSH* glutahione, *GSSG* oxidized glutathione, *Lys* lysine, *Leu* leucine, *LH* lipids (unsaturated fatty acids), *LOOH* lipid hydroperoxide, *Met* methionine, *SOD* superoxide dismutase. (Adapted from Fang et al. 2002)

terrestrial animals (Fang et al. 2002). For example, glutathione, which is the most abundant thiol-containing substance of low molecular weight in cells, is synthesized from glutamate, cysteine, and glycine. Dietary supplementation with glutathione to *Litopenaeus vannamei* enhances immunity and antioxidant defenses (Xia and Wu 2018). Glycine supplementation also improves the resistance of the shrimp to acute salinity challenge (Xie et al. 2014). More details about the functions of AAs and their derivatives in immunity and antioxidant responses are summarized in Table 9.6. Adequate AA nutrition plays a crucial role in protecting crustaceans from infectious and metabolic diseases, such as the white spot syndrome caused by viral infection (Corteel 2013), bacterial infection (Zhang et al. 2018), and oxidant-induced tissue damage (Dong et al. 2018; Li et al. 2020a,c).

9.8.5 Spawning and Larval Development in Crustaceans

Most crustaceans have separate sexes. The weight of the gonad of maturing shrimp or crabs increase during their reproductive development, which prepares sufficient nutrients needed for the formation of egg yolk or spermatogenesis. This process is important to sustain the normal development of the embryos and the production of pre-feeding larvae in crustacean species (Islam et al. 2010). Optimum development of ovaries is necessary for maximum crab production as they are a popular edible tissue (Wu et al. 2020). Vitellogenesis is the process of yolk formation, which plays the central role in ovarian development and reproduction (Subramoniam 2011). Vitellogenin is an egg yolk precursor protein and is synthesized in the hepatopancreas and gonad tissues in decapod crustaceans (Tsukimura 2001). Its synthesis is under the control of estradiol-17β and other neuropeptidic precursors from the nervous system (Fig. 9.5). Furthermore, the hepatopancreas is an important site for the syntheses of vitellogenin and sex steroid hormones. Therefore, the crustacean hepatopancreas is crucial for maximum growth and optimum maturation of ovaries. An unbalanced or incomplete diet causes poor reproductive performance or may even stop animals from reproducing (Woulters et al. 2001). As noted previously, the release of some hormones can be influenced by dietary AA intake. By augmenting the syntheses of egg yolk proteins, hormone peptides and enzymes during maturation and reproduction, AAs are also essential to ovarian development. Indeed, we found that AAs, particularly aspartate and glutamate, are important metabolic fuels in the ovaries of blue crabs (Table 9.2). Thus, increasing dietary provision of AAs (particularly aspartate and glutamate) may beneficially improve reproduction in crustaceans.

Protein and AAs are the main components of dry matter in invertebrate eggs, and support embryonic survival, growth and development (Heras et al. 2000; Xu et al. 2013). Moreover, broodstock nutrition can significantly affect the biochemical profiles of embryos and, therefore,

Table 9.6 Primary roles of amino acids during the life cycle of shrimp and crab species

Life cycle			
Shrimp	Crab	Feeding	The main functions of AAs
Fertilized egg	Fertilized egg	–	Improves the quality of fertilized eggs
Nauplius	–	Yolk reserves	Improves survival and development
Zoae	Zoae	Microalgae	
Mysis	Megalopa	Algae and zooplankton	
Post-larvae	–	Zooplankton and micro-diets	
Juvenile	Juvenile	Pellet diets	Improves survival and growth
Adult	Adult	Pellet diets	Improves the development of gonads and sperm; enhances egg production

The life cycle is adapted from Tuan (2016) and Mcleady et al. (2015)

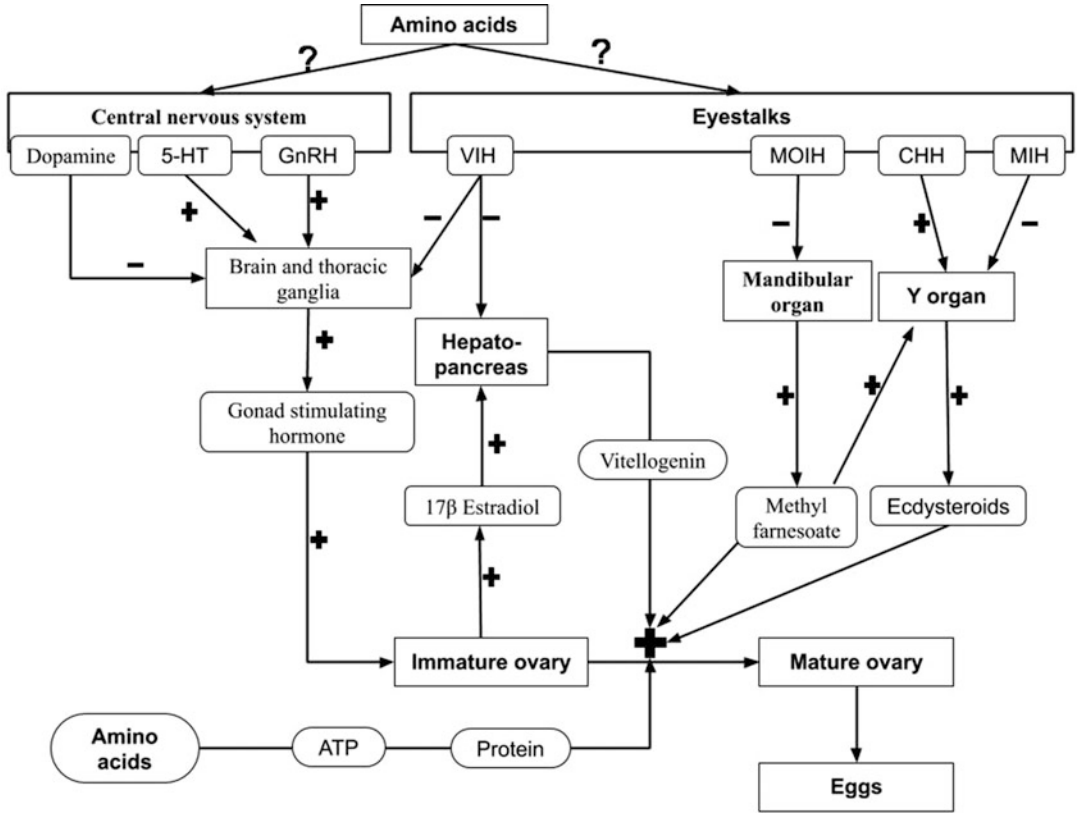

Fig. 9.5 Roles of internal and external factors in the regulation of reproduction in female crustaceans. *DA* Dopamine, *5-HT* 5-hydroxytryptamine (serotonin), *VIH* vitellogenesis-inhibiting hormone, *MOIH* Mandibular organ-inhibiting hormone, *CHH* crustacean hyperglycemic hormone, *MIH* molt-inhibiting hormone, *B & TG* brain and thoracic ganglia, *GOSH* gonad stimulating hormone, *MO* mandibular organ, *MF* methyl farnesoate, *E2*, 17β-estradiol, *Vg* vitellogenin '+' and '-' denotes activation and inhibition, respectively. (Adapted from Pamuru (2019) and Subramoniam (2011), (2017))

embryogenesis, the quality of larvae and post-larvae (Calado et al. 2005; Harrison 1990). Shrimp (*Litopenaeus setiferus*) fed a 35%-protein diet had a lower sperm quality than shrimp fed a 45%-protein diet, indicating that dietary AAs are important for its reproductive performance (Gonimier et al. 2006). There are suggestions that a deficiency of dietary protein or certain AAs can induce *Daphnia pulex* to enter a resting, non-reproductive state (Koch et al. 2009, 2011). Arginine and histidine can enhance not only the number of eggs, but also the development of subitaneous eggs in *Daphnia pulex* (Fink et al. 2011).

Crabs and shrimp must initiate exogenous feeding after yolk nutrients are no longer sufficient to support the metabolic demand of their larvae. The diet for the larvae relies on either live food (algae and zooplankton) or artificial micro-diets, depending on the life stage. Free AAs are important for the metamorphosis of crustacean larvae by providing them with energy, enhancing protein synthesis in their tissues, and promoting their rapid growth (Bahabadi et al. 2018; Rønnestad et al. 2000). For example, feeding *Litopenaeus vannamei* larvae taurine-enriched rotifers improved their survival and development (Jusadi et al. 2011). Likewise, the enrichment of *Artemia* with lysine increased the survival, growth performance, and stress resistance capacity of *Litopenaeus vannamei* post-larvae (Bahabadi et al. 2018).

9.9 Conclusion and Perspectives

Both EAAs and NEAAs play vital roles in the production of aquatic crustacean species. AAs are substrates for ATP production, as well as the syntheses of lipids, glucose, protein and other bioactive molecules (e.g., NO, creatine, polyamines, GABA, catecholamines, and glutathione). In addition, AAs increase the ability of crustaceans to resist various adverse factors (such as hyperosmotic, ammonia, hot and cold stresses), improve their immune and antioxidant defense systems, and regulate their hormone release, metabolic pathways and osmotic homeostasis. Thus, dietary AAs are vital to the growth, development, reproduction, health, and survival of these aquatic animals. Dietary protein and AAs may also play important roles in spawning and larval development, although traditional studies have focused on the nutrition of lipids.

Based on the recent advances in our understanding of AA metabolism and nutrition in shrimp and crabs, considerations should be given on the use of crystalline AAs (particularly aspartate, glutamate, glutamine, leucine, and glycine) and their alternative sources to feed crustaceans for enhancing their survival and productivity (Huo et al. 2017; Wu 2018). At present, there are several technical difficulties and challenges in the use of crystalline AAs to formulate diets for crustacean species. We would also like to propose solutions to solve the problems.

First, although heating can increase the digestibility of native proteins in plants by unfolding the polypeptide chains and removing the intrinsic protease inhibitors, overheated meals or feeds are undesirable because they have reduced biological values in animals (Wu 2018). The Maillard reaction during the feedstuff heating process damages protein and AAs, leading to reductions in the digestibility of dietary protein and the bioavailability of AAs in feeds (Deng et al. 2005). Animal-source feedstuffs, which contain large amounts and proper balances of AAs (Li and Wu 2020a), can be used as the major source of dietary AAs to reduce the inclusion of plant-source ingredients and fishmeal in the diets of crustaceans.

Second, leaching can lead to the loss of nutrients (including protein and AAs) from the diets fed to crustacean species such as shrimp and crabs, particularly because most of them are slow and continuous eaters. These animals can pick up a feed pellet, cradle it with their maxillipeds (an appendage modified for feeding in crustaceans that is situated in pairs behind the maxillae), and begin to tear and crush the end of a pellet with their mandibles (Obaldo et al. 2006). Therefore, nutritional studies with shrimp and crabs have met with the difficulties of enhancing feeding efficiencies due to the leaching of nutrients before feed pellets are consumed by the animals. If crustaceans are fed an experimental diet with a high leaching rate, their estimated requirements for dietary AAs may be inaccurate. To optimize the utilization of crystalline AAs, a possible approach is to coat AAs with lipids (Alam et al. 2004b; Gu et al. 2013).

Third, crystalline AAs in diets enter the systemic circulation of crustaceans more rapidly than the protein-bound AAs, possibly resulting in the asynchronous absorption of dietary AAs and a suboptimal efficiency of utilization of dietary AAs (Lovell 1991; Guo et al. 2020). For example, there are higher percentages of AAs lost in the urine (e.g., 13.6% for His; 17.6% for phenylalanine; and 8–10% for isoleucine, leucine, lysine and valine) when shrimp fed diets with crystalline AAs in comparison with diets with proteins (Liou et al. 2005). Similarly, a previous study showed that shrimp fed diets with coated crystalline methionine grew more rapidly than those fed diets with uncoated crystalline methionine (Chi et al. 2011). Therefore, it is necessary to systematically evaluate the efficiency of utilization of different free AAs (either coated vs crystalline) to define an appropriate replacement level of protein-bound AAs by crystalline AAs. Some studies with pigs (Gahl et al. 1994) and rainbow trout (Tran et al. 2007) demonstrated that the efficiencies of utilization of supplemented crystalline AAs varied with diets, depending on protein sources especially at suboptimal dietary levels of AAs. This

means that AAs with the same quantity and quality may yield different effects on the growth of animals when they are supplemented to diets with various feedstuff ingredients.

Fourth, there are no standardized diets or AAs as isonitrogenous controls for nutritional research in crustaceans. Due to the inadequate understanding of NEAAs in the past decades, glutamate, glycine and aspartate have long been used as an isonitrogenous control in nutritional experiments. This is inappropriate based on recent studies with terrestrial animals (Hou and Wu 2018; Wu 2018), fish (Li et al. 2020a), and crustaceans (Xie et al. 2014, Xie et al. 2015a, b) indicating that these AAs have nutritional or physiological effects in the animals. We suggest that L-alanine be used as the isonitrogenous control in nutritional studies with crustaceans where it is not a test AA.

Fifth, there is limited knowledge about the cell- and tissue-specific metabolism of AAs in different aquatic crustaceans (e.g. crabs and shrimp). For example, GPT and GOT are abundant in both the mitochondria and the cytoplasm of hepatocytes of many animal species (Wu 2013). Thus, the activities of these two enzymes in serum are often determined to assess hepatic integrity in human medicine. Similarly, both enzymes in the hemolymph of giant tiger prawn and Pacific white shrimp have been regarded as important indicators of the hepatopancreatic injury (Pan et al. 2003; Liu et al. 2019). This, however, it may be not valid for all species of shrimp and crabs. For example, the activity of GPT and GDH in the hepatopancreas of black tiger shrimp (*Penaeus monodon*) is either very low or undetectable (Richard et al. 2010). Furthermore, in blue crabs, the hepatopancreas is not a main site for the catabolism of AAs (Table 9.2).

Finally, although there has been active research to determine the dietary requirements of crustaceans for crude protein over the past 50 years (Table 9.5), much emphasis should be directed to studies of the dietary requirements of these animals for NEAAs. Nutritionists should move away from the traditional concept of crude protein toward all AAs with nutritional and physiological functions in the animals. The composition of AAs in the diets with various protein sources for crustaceans may differ substantially even though the diets have the same crude-protein level. Dietary requirements of crustaceans for all AAs (including AAs that are synthesized in animal cells, such as glutamate, glutamine and glycine) should be defined to optimize dietary formulations for both health and growth performance. Research on the metabolism and functions of AAs is fundamental to achieve this goal so as to manufacture future environment-friendly aquafeeds and reduce feed costs in crustacean production. The new nutritional concepts of "dietary requirements of animals for NEAAs" and "functional AAs", which were originally proposed on the basis of basic and applied studies with terrestrial animals (Wu 2010), are expected to transform nutritional studies with shrimp and crabs, as well as feeding practices in the global crustacean production (Xie et al. 2014, 2015a,b).

Acknowledgments We thank our students, research assistants and colleagues for helpful discussions. This work was supported by Texas A&M AgriLife Research (H-8200) and funds from Guangdong Yuehai Feeds Group Co., Ltd.

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Amino Acids in Dog Nutrition and Health 10

Anita M. Oberbauer and Jennifer A. Larsen

Abstract

The dog has assumed a prominent role in human society. Associated with that status, diet choices for companion dogs have begun to reflect the personal preferences of the owners, with greater emphasis on specialty diets such as organic, vegan/vegetarian, and omission or inclusion of specific ingredients. Despite consumer preferences and many marketing strategies employed, the diets must ensure nutritional adequacy for the dog; if not, health becomes compromised, sometimes severely. The most frequent consideration of consumers and dog food manufacturers is protein source and concentration with a growing emphasis on amino acid composition and bioavailability. Amino acids in general play diverse and critical roles in the dog, with specific amino acids being essential. This review covers what is known regarding amino acids in dog nutrition.

Keywords

Amino acids · Dog · Protein · Nutrition

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Abbreviations

AAFCO	Association of American Feed Control Officials
DCM	dilated cardiomyopathy
mTOR	mechanistic target of rapamycin
NRC	National Research Council
SND	Superficial necrolytic dermatitis

10.1 Introduction

Much of the canine nutrition work addressing protein and amino acid requirements was done in the mid-1900's with a resurgence of interest and characterization of dog diets in the last 20–25 years. Similarly, in the most recent decades, within human society dogs have moved from a more utilitarian relationship into the role of family members (Power 2008) and with that evolution, pet humanization has become a driving force in consumer product purchasing (Kumcu and Woolverton 2015) and food in the human societal context is complex, symbolic, and cultural. Recent dietary formulations for the dog target consumer preferences with emphases on premium, exotic or novel, natural, organic, and/or sustainable ingredients. Despite the anthropomorphizing, and regardless of marketing category, all balanced diets must reflect the physiological needs of the dog.

G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1285, https://doi.org/10.1007/978-3-030-54462-1_10

Dietary provision of protein must provide the essential amino acids necessary for structural protein synthesis, both for growth and maintenance. Additionally, dietary amino acids serve as precursors for dispensable (non-essential) amino acids that are synthesized by the body (Wu 2013). These serve as a source of energy and provide components necessary for key metabolic functions. As such, an adequate supply of digestible nitrogen must be provided. Both non-essential and essential amino acids are also used in the synthesis of neurotransmitters, hormones, and purine and pyrimidine nucleotides. Thus, it is critical to match the amino acid profile (concentrations and proportions) of a dietary formulation with a dog's physiological state. Failure to do so results in deficiencies that translate into preventable health conditions. Knowledge of the role of amino acids in the canine diet is essential to enable continued improvements in canine nutrition.

10.2 Overview of Amino Acids

The amino acids in circulation are derived from meal digestion, protein degradation, and de novo synthesis. Dietary amino acids have different metabolic fates that include incorporation into structural proteins, synthesis of dispensable amino acids, conversion into signaling molecules such as hormones and neurotransmitters, and use as an energy source either as glucose or fat storage (Wu 2013). The physical structure of the amino acids plays a role in their function; this is particularly true based upon whether the side chains confer hydrophobic, polar, or neutral configurations.

Most proteins contain alpha amino acids with alpha carboxyl groups, except for proline, and there are generally 22 alpha-amino acids in proteins (Case et al. 2010). Physiologically, cells synthesize the L-isomer of amino acids and only that form is incorporated into proteins. However the D-isomers of amino acids have been isolated from bacterial cell walls and recent evidence shows the D-isomer form is found in microorganisms, plants, insects, and mammals and they are proposed to have distinct biological functions in diverse tissues such as neurological development and transmission and endocrine systems (reviewed by Genchi 2017). Mammals have measurable proportions of D-isomer amino acids predominantly D-serine and D-aspartate (Ohide et al. 2011; Fujii 2002). Some evidence also suggests a role for D-isomer amino acids as osmolytes in aquatic invertebrates (Abe et al. 2005) and in host defense mechanisms and neurotransmitters for mammals (Sasabe and Suzuki 2018; Fujii 2002; Snyder and Kim 2000). Despite these findings, when fed to dogs, D-isomer tryptophan resulted in over one-third of the ingested tryptophan being excreted in the urine (Czarnecki and Baker 1982). In general, D-isomers are thought to impair the digestion of proteins and reduce bioavailability of other amino acids (reviewed in Man and Bada 1987) resulting in impaired growth (Friedman and Levin 2012), although other studies have suggested a potential role as antimicrobials (reviewed in Friedman and Levin 2012)). Whereas D-isomer amino acids are naturally synthesized, they can also be the result of artificial processing or thermal and environmental insult (Genchi 2017); the latter, racemisation, being an important consideration in diet preparations given the putative role of D-isomers in reducing amino acid availability (Tran et al. 2008).

Ten amino acids are categorized as indispensable, or essential, for the dog because the rate of synthesis is insufficient to meet the physiological demands of the body and thus must be supplied by the diet (Table 10.1). Dispensable, or non-essential, amino acids can be synthesized from indispensable amino acids. Some authors

Table 10.1 Indispensable/essential amino acids for dogs

Arginine	Methionine
Histidine	Phenylalanine
Isoleucine	Threonine
Leucine	Tryptophan
Lysine	Valine

have suggested that the description of some amino acids as nutritionally non-essential is incorrect or otherwise inappropriate (Hou and Wu 2017). Similarly, arguments in support of this concept have been addressed with knowledge of species-specific essential amino acids as well as the widely accepted principle of conditionally essential amino acids for example (Morris et al. 2017; Lourenco and Camilo 2002).

Amino acids differ in their overall structure, side chain structure and general chemical characteristics and they are often categorized based upon these properties. The aliphatic class includes glycine, alanine, valine, leucine, and isoleucine. The sulfur-containing class includes cysteine and methionine. Proline is the lone amino acid classified as an imino acid. The aromatic class includes phenylalanine, tyrosine, and tryptophan. The basic classification includes histidine, lysine, and arginine and the acid amino acid classification includes aspartate and glutamate. Histidine may also be considered an aromatic amino acid.

Additionally, amino acids may be classed based upon their side chain configuration. The branched-chain amino acids have an aliphatic side chain that branches, and this group includes leucine, isoleucine, and valine. The branched-chain amino acids represent the majority of amino acids found in muscle proteins. They also promote muscle protein synthesis through the mechanistic target of rapamycin (mTOR) pathway and translational activation (Kimball and Jefferson 2006) while reducing protein catabolism (Fulks et al. 1975). The mTOR pathway is involved in many physiological processes including protein and lipid synthesis and energy metabolism. Amino acids, especially leucine and arginine, are essential for the activation of this pathway, even mediating the action of growth factors and integrating the levels of energy, stress, and oxygen (reviewed in (Laplante and Sabatini 2012). Sancak et al. (2008) demonstrated that the activation of the mTORC1 growth promotion pathway by amino acids is mediated by the Rag family of GTPAses and that when activated by branched-chain amino acids permit mTOR to relocate within the cell thereby enabling interaction with the key downstream enzymes for each synthetic pathway.

The hydrophobicity of branched-chain amino acids also contributes to their role in phospholipid bilayer signaling molecules (Brosnan and Brosnan 2006). Catabolism of branched-chain amino acids has been associated with obesity in humans (Newgard et al. 2009). Branched-chain amino acids are unique in that amounts beyond that needed for protein synthesis are metabolized in peripheral tissues rather than the liver, especially skeletal muscle.

The aromatic ring amino acid configuration is synthesized by plants and microorganisms but not animals, which lack the shikimate pathway necessary for the synthesis of these amino acids. All but tyrosine of the aromatic amino acids are indispensable if the diet provides phenylalanine; tyrosine can be formed from phenylalanine through the action of phenylalanine hydrolase. Aromatic amino acids play a role in protein-protein interactions conferring specificity to the binding process (Moreira et al. 2013) and both phenylalanine and tryptophan are precursors to the crucial metabolic hormones of dopamine, epinephrine, norepinephrine, serotonin, and thyroxine.

In the formation of energy, amino acids are further classified as ketogenic or glucogenic, reflective of the fate of their carbon skeleton during protein catabolism. To contribute to energy supply, the carbon backbone of the amino acid is used for gluconeogenesis. Amino acids that when broken down form acetyl CoA or acetoacetyl CoA are ketogenic, so named because they form ketone bodies that are ultimately used in the citric acid cycle to generate ATP. Ketogenic amino acids are leucine and lysine. Glucogenic amino acids, the majority of amino acids, are those whose carbon skeletons are converted into glucose and include alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, methionine, proline, serine, and valine. Five amino acids, phenylalanine, isoleucine, threonine, tryptophan, and tyrosine, share both ketogenic and glucogenic properties.

Early research confirmed the role of amino acids in stimulating glucagon response in the dog (Rocha et al. 1972). When exogenously

administered intravascularly, the majority of amino acids stimulated glucagon secretion except for leucine which is strictly nonglucogenic (Rocha et al. 1972), as well as isoleucine which much more strongly stimulated insulin release, and valine which was among the weakest to stimulate both glucagon and insulin. Since the first step of branched-chain amino acid catabolism is primarily in muscle, with slow removal of the keto-acids, the concentration of these in plasma rises faster after a meal than for other amino acids. As such, the reduced to absent stimulation of glucagon release, and greater effect on insulin at least for leucine and isoleucine, may be an adaptive response to the normal physiological milieu that occurs in the post-absorptive state.

10.3 Digestion of Dietary Protein and Amino Acids

The dog is a meal feeder having a monogastric digestive system. Although in the phylogenetic order of Carnivora, dogs are more appropriately classified as omnivorous (D'Mello 2003). The dog, to a greater degree than their wolf ancestors, consume and utilize a wider variety of food sources including starches, perhaps reflecting their long evolutionary relationship with humans (Axelsson et al. 2013). Despite this ability to efficiently digest and utilize starch as an energy source, the dog was considered to rely primarily on pancreatic amylase due to a lack of salivary α-amylase enzyme (Chauncey et al. 1963) although recent studies using newer techniques have detected measurable quantities in dog saliva (de Sousa-Pereira et al. 2015; Contreras-Aguilar et al. 2017).

To accommodate the meal feeding pattern, the dog digestive system has adapted to cope with large meals followed by long periods of fasting. Some modifications are seen in the ability to synthesize dispensable amino acids such as taurine (Fig. 10.1). In addition, amino acid catabolism can be slowed during period of reduced food availability (Bosch et al. 2015). When compared to the cat, the dog shows higher apparent digestibility of not just crude protein but also fat, carbohydrate by difference, and gross energy from a range of processed commercial diets and fresh foods (Kendall et al. 1982).

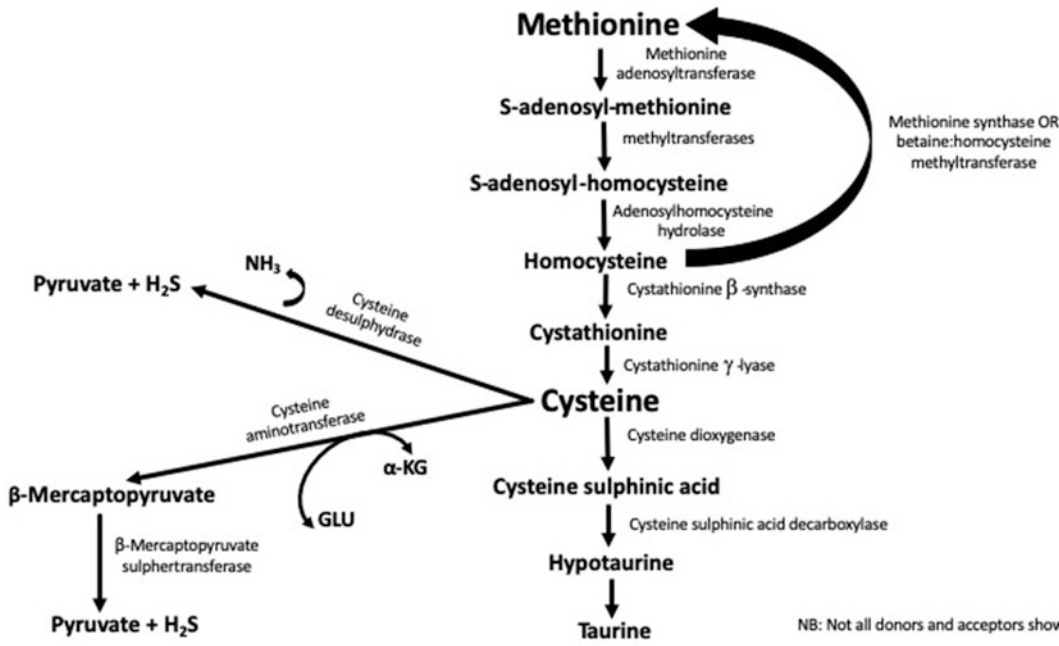

Fig. 10.1 Taurine biosynthetic pathway

As in other species, the canine stomach secretes pepsin that functions in proteolysis. Pepsin optimally coordinates with collagen intake, thus is important in the digestion of consumed animal-based tissue (meat). Meat and other protein sources that contain certain amino acids promote digestive function such that gastric secretion is proportional to protein consumed. The small intestine is the site of the majority of the dog meal digestion. Peptides containing phenylalanine enhance cholecystokinin release by the duodenal gastrointestinal endocrine cells, which in turn promotes fat and protein digestion in the small intestine. Component amino acids derived from digestion are absorbed from the diet in the small intestine.

The dog, similar to the cat (Che et al. 2020), has an obligatory requirement to utilize solely taurine for bile acid conjugation. The conjugating enzyme in dogs and cats is taurine-specific, while other species use both glycine and taurine (Czuba and Vessey 1981). Once the chyme reaches the distal small intestine, the conjugated bile acids are reabsorbed via the ileum, resulting in an efficient recycling mechanism of taurine in the dog, assuming the absence of medications, dietary factors, or other reasons for accelerated bile losses through bacterial degradation or fecal excretion.

Colonic/post ileal absorption contributes only a small proportion to the overall digestion of foods and contributes greatest for diets having high starch or legume content (Zentek and Meyer 1995; Meyer et al. 1989). Diet is known to affect the microbial composition of the gastrointestinal tract (Hooda et al. 2012) and conversely, metabolism of certain dietary nutrients are enhanced by particular microbial populations. For example, amino acid utilization is in part dependent upon the composition of the microbial communities within the gut (Dai et al. 2011). Thus, diet, and in particular the profile of amino acids, shape the diversity of the microbial communities and those communities in turn influence the utilization and metabolism of dietary components. Characterization of this interplay is in its infancy and caution must be applied to studies assessing dietary influences on the dog microbiome composition because the high within animal variability in microbiome signatures may confound conclusions (Cuscó et al. 2017). It has been hypothesized that the microbial population in the distal colon ferments any remaining undigested protein (Council 2006) and Stevens and Hume (Stevens and Hume 2004) reported that absorption of ammonia in the distal colon conserves the nitrogen associated with secreted enzymes necessary for the digestive processes.

The fermentation of undigested amino acids in the colon contribute to pungent and unpleasant odors associated with flatulence and feces in the dog. The addition of Yucca schidigera extract can reduce fecal odor even when the dog has high dietary protein inclusion (Dos Reis et al. 2016; Lowe et al. 1997). The modulating effect is likely due to the interactions with the microbiota through the impact on composition or metabolism (Pinna et al. 2017). In addition, dietary supplementation of prebiotics, such as oligofructose, added to diets serve as nutritional substrates for the microflora that inhabit the gastrointestinal tract. Optimizing the microflora community and types and numbers of species has health benefits including reduced risk of colon cancer as observed in rodent models (Pool-Zobel et al. 2002). Increasing the diversity in general and the abundance of Bifidobacterium in particular are considered especially beneficial. Of course, the production of short-chain fatty acids resulting from the fermentation of various fibers constitute an important energy source for colonocytes in addition to likely beneficial systemic effects even in carnivores adapted to consuming diets very low in this dietary component (Verbrugghe et al. 2012).

Interestingly, circulating amino acids, both indispensable and dispensable, differ based upon the size of the dog leading many commercial pet food manufacturers to develop diets tailored to size or even breed. (Middleton et al. 2017). Dogs categorized as "small" based upon weight (6.1–15.6 kg) have elevated arginine levels when compared to that observed for dogs classified as "large" (18.4–54.4 kg); the situation is reversed for phenylalanine, tyrosine, lysine, glutamine, hydroxyproline, and prolylhydroxyproline, despite the higher digestibilities in the

"small" dogs for a common diet fed to all dogs. The fecal microbiome varied between the two size categories leading the investigators to hypothesize that the resident microflora of the dogs differentially used the amino acids. Furthermore, the authors speculate that genetic selection for phenotype in breed development altered metabolism beyond just that related to physical size.

10.4 Diet

10.4.1 Food Intake and Role of Dietary Factors

If permitted free choice dogs will selectively consume ~25–30% of calories as protein. (Romsos and Ferguson 1983; Tôrres et al. 2003). The literature also suggests that dogs offered a choice will avoid diets deficient in essential amino acids (Leung and Rogers 1987). Of course, in the dog, palatability plays an important role in diet selection and preference and is difficult to differentiate from metabolic effects of nutrient composition. Overall protein amount and type as well as some individual amino acids (alanine, proline, lysine, histidine, and leucine) will increase preference, palatability, and satiety in cat and dog diets (White and Boudreau 1975; Weber et al. 2007; Hargrove et al. 1994). Purified proteins are virtually flavorless while specific amino acids or isolated peptides can be bitter, sweet, or sour. Furthermore, the D-isomer amino acid form may present as a different and/or more potent flavor. For example L-tryptophan is detected as a bitter flavor yet D-tryptophan is detected as even sweeter than sucrose (Bachmanov et al. 2016).

Both energy and protein intake are regulated by satiety and behavioral feeding mechanisms. Protein regulation is known to be influenced by the amino acid composition of the protein source. Anderson posited that the presence of certain amino acids, particularly those that serve as precursors for neurotransmitters, could account for the mechanism of protein and amino acid regulation of feeding behavior (reviewed in Anderson 1979). Of course, other dietary factors including fiber content have been shown to influence food intake to a variable degree depending on the study design. Fiber is used to dilute energy in diets formulated for weight control, but the impact of fiber on satiety is a challenging issue to define, and study design or other dietary features may influence findings. Dogs fed high fiber diets ad lib showed voluntary reduction in food intake in one study, while another study demonstrated that dogs on a weight loss plan lost similar amounts of weight while consuming either a low or high fiber diet, with no apparent effects on satiety associated with either diet (Jewell and Toll 1999; Butterwick and Markwell 1997; Jackson et al. 1997).

10.4.2 Amino Acid Composition and Availability

Dietary protein is the sole dietary component for which an objective definition of nutritional "quality" is established. Protein quality is determined by the overall digestibility as well as the amino acid quantity, pattern, and bioavailability. Dietary sources of amino acids vary in these characteristics. Certain amino acids are present at sufficient levels in both animal and plant protein sources (Hou et al. 2019; Li and Wu 2020). These include leucine, isoleucine, and valine. Other protein sources are more limited in their amino acid profiles. For instance, legumes are limiting in sulfur amino acids, some cereal crops such as wheat and corn have low lysine content, and corn is also limiting in tryptophan.

Assessment of novel protein ingredients that are being incorporated into dog diet formulations, such as calamari meal, alligator, venison, and duck, revealed that differences in protein composition are accompanied by variable digestibility of the amino acids within those proteins. Specifically, digestibilities differed for indispensable amino acids dependent upon the protein source. For instance, calamari meal had more highly digestible indispensable amino acids when compared to lamb meal, indicating a critical need for fully analyzing dietary components prior to incorporating them into a diet (Deng et al.

2016). It appears that the amino acid composition as well as bioavailability must be considered when using both uncommon and more typical protein sources. Novel protein sources may have different levels of essential and dispensable amino acids (McCusker et al. 2014), and this should be defined when considering use for food. In general, animal-sourced proteins of high quality have better amino acid profiles compared to those derived from plants. However, interestingly, even rabbits, a common prey species as well as one sometimes used in pet foods, contains taurine concentrations below the known requirements for cats (Owens 2016) and exclusive feeding has been associated with the development of taurine deficiency and dilated cardiomyopathy in cats (Glasgow et al. 2002).

Well formulated diets provide adequate protein and amino acid content in dietary concentrations which meet the needs of the target dog population. However, comprehensive in vivo testing is necessary to fully assess adequacy in the long term. This is particularly true for vegetarian/vegan pet food diets in which concentrations and/or bioavailability of some amino acids may be inadequate to meet needs for growth or maintenance (Kanakubo et al. 2015). In a study of beagles, body weight was maintained along with baseline general health with a diet providing the minimal protein (from egg) requirement (Sanderson et al. 2001). Interestingly one beagle on that study developed dilated cardiomyopathy and the condition was fully reversed with taurine supplementation. This underscores that amino acid adequacy cannot be predicted by measures of energy and protein status; more targeted and precise assessments are needed.

Bioavailability of amino acids from plant and animal protein sources can differ. In general, animal proteins have higher digestibilities than plant based proteins (Neirinck et al. 1991; NRC 2006). However even highly digestible proteins can be negatively impacted by other factors (Bednar et al. 2000), including processing factors (Dust et al. 2005; Backus et al. 1998), whereas digestibility of other proteins may be enhanced (Morgan et al. 1951); however, the impact of other dietary components and their interactions with protein must also be considered.

The negative impact of high dietary fiber inclusion on protein digestibly is well known. Protein digestibility will decrease 6% if alpha cellulose is added to diets (Burrows et al. 1982; Burkhalter et al. 2001) reducing availability of amino acids for essential bodily functions. Fiber also dilutes energy density, which is a benefit or a detriment depending on the individual dog (obese-prone vs. small breed puppy, for example). Yet, depending upon the fiber added, it can exert an amino acid sparing effect (Wambacq et al. 2016). Thus incorporating high amounts of fiber into diets must consider the life stage and other characteristics of the dog. Practically speaking, fecal water content, transit rate, protein concentration, and protein digestibility all impact stool quality which differs by body size (Nery et al. 2012). Furthermore, protein digestibility differs across dog breeds (Hannah et al. 1995) and sizes (Nery et al. 2012).

Provision of certain dietary components exert an anti-nutritional effect. Some cereal grains contain trypsin inhibitors or lectins that alter the intestinal epithelium, reduce protein digestibility, and diminish of essential amino acids (Lajolo and Genovese 2002). Dietary oligofructose, a fermentable fiber additive to diets, alters nutrient digestibility when added to dog diets. Specifically, the level of fecal ammonia was proportionally decreased with increasing concentrations of oligofructose whereas bifidobacteria were elevated, indicating alterations in amino acid use (Flickinger et al. 2003; Hussein et al. 1999). Amino acid sparing is also observed when soluble fiber (sugar beet pulp mixed with guar gum) is added to the diet; in that situation, the use of the short-chain fatty acid proprionate is preferentially utilized for gluconeogenesis over the use of amino acids (Wambacq et al. 2016).

Soyabean meal is added to some dog diets as a supplemental or primary source of protein as well as to include isoflavones which may assist with weight management (Pan 2007). However, soy is associated with reduced small intestine digestibility likely due to the presence of tannins, lectins, trypsin inhibitors, phytates, oligosaccharides (e.g., raffinose and stachyose), and β-mannans (Sarwar Gilani et al. 2012). If the soyabean meal

is not properly processed to reduce or inactivate anti-nutritional factors, digestibility of specific amino acids is profoundly impaired (lysine, methionine, cystine, threonine; Willis 2003). In addition, methionine is usually the first or second limiting amino acid in dog diets formulated with soybean meal and rendered meats (NRC 2006) and methionine is particularly susceptible to heat processing damage with subsequent reduction in bioavailability (Marshall et al. 1982; Hurrell et al. 1983).

It is clear that thermal processing can improve protein digestibility and both global and specific amino acid availability due to destruction of anti-nutritional factors or protein denaturing; however, heat damage can also have direct negative effects on protein and amino acid adequacy in certain matrices. It has been reported that much of the variation in quality of rendered animal meals as it relates to amino acid digestibility is related to processing effects (Hendriks et al. 2004; Johnson et al. 1998). Poultry by product meal has reduced essential amino acid digestibility compared to other meat sources (especially cysteine and lysine but also to some extent threonine) (Johnson et al. 1998) underscoring the importance of processing in amino acid adequacy in canine diets. Amino acids present in foods can be affected by several aspects of processing (heat, pH, moisture) in various ways including oxidation, desulfuration, and isomerization. In addition, although naturally occurring amino acid cross-linking is noted (such as in keratin and collagen), similar reactions can also occur as a result of processing, which result in decreased amino acid availability. Individual amino acid destruction and formation of new (non-nutritional) amino acids may also occur, as well as Maillard-type reactions between reducing sugars and the free amine groups of lysine. Maillard-type reactions result in the obvious loss of lysine, but also reduce global protein digestibility and therefore most other essential amino acids as well (van Rooijen et al. 2013; Cheftel 1976).

The recent publication by Hendriks et al. (2015) indicates that the recommendations of amino acid requirements is still a work in progress. The authors suggest that the recommendations by National Research Council (NRC), Association of American Feed Control Officials (AAFCO), and the European Pet Food Industry Federation underestimate the amino acid requirements for many of the essential amino acids when true digestibility and bioavailability are taken into account (Table 10.2). The authors caution that for especially lysine but also other

Table 10.2 Published recommended allowances for essential amino acids for adult canine maintenance per the National Research Council (NRC 2006) and recommendations for minimum concentrations of essential amino acids in diets for adult dogs per the Association of American Feed Control Officials (AAFCO 2019) and the European Pet Food Industry Federation (FEDIAF 2018[a])

			FEDIAF 2018	
			based on MER of	
	NRC 2006	AAFCO 2019	95 kcal/kg$^{0.75}$	110 kcal/kg$^{0.75}$
Amino acid	g/1000 kcal[b]			
Arginine	0.88	1.28	1.51	1.3
Histidine	0.48	0.48	0.67	0.58
Isoleucine	0.95	.i95	1.33	1.15
Leucine	0.83	1.7	2.37	2.05
Lysine	1.63	1.58	1.22	1.05
Methionine	1.7	0.83	1.16	1
Methionine+cystine	0.88	1.63	2.21	1.91
Phenylalanine	1.13	1.13	1.56	1.35
Phenylalanine+tyrosine	1.85	1.85	2.58	2.23
Threonine	1.08	1.2	1.51	1.3
Tryptophan	0.35	0.4	0.49	0.43
Valine	1.23	1.23	1.71	1.48

[a]Fédération européenne de l'industrie des aliments pour animaux familiers
[b]Assumes energy density of 4000 kcal/kg dry matter as indicated

amino acids, such as methionine and cystine, may be present in processed foods and even absorbed yet remain unavailable for metabolic utilization due to thermal and chemical destruction or transformation.

10.5 Role of Amino Acids

Within the body, amino acids have diverse roles with specific amino acids being important in different physiological contexts. Amino acids have a direct role in regulating genes involved in amino acid metabolism such as arginine's downregulation of argininosuccinate synthase mRNA (Haines et al. 2010) or lysine's regulation of ornithine decarboxylase, although a frequent observation is the down regulation of genes, such as those involved in fatty acid synthesis or those in the mTOR pathway, when amino acids are limiting (Fafournoux et al. 2000; Jousse et al. 2004). In addition to their role in developing and maintaining muscle mass, dietary provision of branched-chain amino acids to dogs improves cognitive function in active and aged dogs (Fretwell et al. 2006) which suggests a dual role in ameliorating signs of both cognitive aging and sarcopenia.

The dispensable amino acid glutamic acid can form the potent neurotransmitter, glutamate, upon protonation in physiological fluid. Glutamate is the most abundant neurotransmitter in the brain (Lipton and Rosenberg 1994) and regulates key neurological functions including cognition, learning, memory, and neuronal developmental plasticity; excess activation has been associated with neurological injury. Excess glutamate activation, or "excitotoxicity", has been implicated in idiopathic epilepsy in the dog (Podell and Hadjiconstantinou 1997; Platt 2007). Anxiety disorders in dogs are also correlated with elevated levels of plasma glutamine and γ-glutamyl glutamine (Puurunen et al. 2018). In neurological conditions, the normal cycling between glutamine and glutamate (or the neurotransmitter γ-aminobutyric acid is disrupted with altered pools of each. Glutamine is the most abundant free amino acid and its elevation in states of fear is intriguing yet the implication is unknown. Whether that deviation correlates with release from tissue stores creating a greater demand for dietary provision of glutamine has yet to be determined (Puurunen et al. 2018).

Protein content of the diet may be associated with expression of certain behaviors such as dominance aggression; low protein diets were associated with higher aggressive behavioral assessments whereas high protein diets, or low protein diets supplemented with the amino acid tryptophan, were associated with reduced aggressive tendencies (DeNapoli et al. 2000). It is well recognized that neuronal and endocrine signaling act in behavioral control. Tryptophan serves as a precursor for a number of neurotransmitters and hormones including serotonin, that when secreted in the brain impacts mood and is associated with inhibition and modulation of aggressive behaviors (Bosch et al. 2007). Higher concentrations of tryptophan have been incorporated into diets marketed for canine anxiety. Tyrosine is a precursor for the catecholamines adrenalin and nor-adrenaline and thus plays a significant role in stress reactions; studies in dogs are lacking.

As noted above, dietary amino acids and the gastrointestinal microflora show complex and mostly uncharacterized interactions. Additionally, although studied in the pig, arginine, glutamine, glycine, cysteine, and proline have been shown to protect the gut, improve the integrity of the mucosal barrier of the intestine, and promote immune capacity through regulation of cytokine secretion (Liu et al. 2017b; Ruth and Field 2013; Li et al. 2016; Liu et al. 2017a).

Leucine can repress proteolysis, to an even greater degree than insulin, suggesting that provision of particular amino acids can conserve lean body mass. One study showed that a 90 minute intravenous infusion of pharmacological doses of leucine to adult dogs resulted in 75–80% reduction in the rate of global proteolysis (Frexes-Steed

et al. 1992). Under long term dietary caloric restriction, serum levels of valine, leucine, lysine, and phenylalanine are possibly reflecting an altered energy metabolism (Richards et al. 2013)

A recent study of young Labrador retrievers and Newfoundlands targeted phenylalanine plus tyrosine to determine if supplementation would result in more intense, darker coat colors. Though this was done in puppies, it was likely reflecting the adequate synthesis of eumelanin in the hair; no other physiological parameters were assessed (Watson et al. 2015). It is also well established that in black cats, the dietary requirement for phenylalanine plus tyrosine in order to maintain black coats is almost twice that established for optimal growth (Anderson et al. 2002) demonstrating a vital role of tyrosine in pigment synthesis.

10.6 Deficiencies

Protein adequacy in the dog is most practically assessed over the long term by confirmation of maintenance of normal serum albumin concentration and normal lean body mass. Clinical signs of deficiency of specific amino acids may be preceded by, or confirmed with, decreases in plasma concentrations. Plasma amino acids measurements are an accessible and noninvasive clinical tool for assessment of global or individual amino acid status (Table 10.3). However, most

Table 10.3 Plasma amino acid concentrations in adult dogs, and puppies where available, fed diets with adequate amino acid concentrations

	Adult[a]	Puppy#
Amino acid	Mean ± SEM (nmol/mL)	(nmol/mL)
Alanine	388 ± 9.6	
Arginine	102 ± 2.6	72
Asparagine	40 ± 1.1	
Aspartate	7 ± 0.2	
Citruline	41 ± 1.9	
Cysteine	46 ± 1.3	
Glutamate	23 ± 1.2	
Glutamine	495 ± 9.4	
Glycine	268 ± 8.4	
Histidine	71 ± 1.6	
Hydroxyproline	67 ± 4.1	
Isoleucine	51 ± 1.3	
Leucine	120 ± 3.2	100
Lysine	132 ± 5.0	85
Methionine	57 ± 1.6	
Ornithine	35 ± 1.5	
Phenylalanine	45 ± 0.9	85
Proline	246 ± 8.2	
Serine	107 ± 2.6	
Taurine	77 ± 2.1	
Threonine	178 ± 5.0	
Trptophan	60 ± 1.7	
Tyrosine	39 ± 1.1	12
Valine	157 ± 4.1	

(Council 2006)

[a]source: (Delaney et al. 2003) and confirmed in (Chan et al. 2009)

unbalanced diets are unlikely to be lacking only one essential nutrient, and with regard to protein and amino acid adequacy, it may be a challenge to differentiate the effects of a single nutrient in the face of multiple deficiencies. Furthermore, the interrelationship of energy and protein intake means that both factors must be considered when interpreting both published data as well as when undertaking animal assessments in real time.

Humbert et al. (2001) characterized the metabolic adaptations in the dog to diets that were energy equivalent yet either deficient in overall protein or protein deficient diets that were also lysine and tryptophan deficient. The dogs fed the protein deficient diet reduced protein degradation 11% compared to dogs fed a control diet and the effect was more pronounced for the diets that were lysine and tryptophan deficient which exhibited a 25% reduction. This conservation mechanism is due to downregulation of catabolic pathways although protein turnover rates can only be reduced to a limited extent. As such, some ongoing losses are unavoidable. Similarly, the dogs showed decreased protein synthesis with the greatest effect observed in the diet with specific amino acid deficiencies with reductions of approximately 25% for the overall protein deficient diet and 30% for the lysine and tryptophan deficient diet. In contrast, oxidation of proteins was unaffected. Thus, the dog can modify to a limited extent its metabolic use of proteins in the face of dietary deficiencies (Humbert et al. 2001).

In humans, and thus, could possibly be extrapolated to dogs, generalized chronic dietary protein deficiency causes reduction in circulating essential amino acids with a concomitant increase in serum dispensable amino acids (Holt Jr et al. 1963). The metabolism of an animal will change to accommodate alterations in the abundance of certain amino acids. For example amino acid catabolism will be altered in the face of reduced dietary protein content. The flux of alanine, aspartic acid, and glutamic acid through the urea cycle is changed reflective of abundance or deficiency. Arginine is essential for proper metabolism and homeostasis of amino acids as is the regulation of nitrogen catabolic enzymes. Arginine deficiency in dogs, as in cats, results in hyperammonemia. This is due to the resultant low hepatic ornithine concentration derived from the reduced arginine substrate that precludes ornithine serving as an carbamoyl phosphate acceptor, thereby slowing down synthesis of urea and disposal of excess nitrogen/ammonia (Milner et al. 1975; Dimski 1994).

It is postulated that although dogs are omnivores, they retain metabolic specializations reflective of their evolutionary ancestry. This specialization can be seen in the physiological dysfunction from a deficiency in arginine (D'Mello 2003). As a consequence of hyperammonemia, dogs fed a diet lacking in arginine for as little as 1 week, will exhibit muscle tremors and gastric upset (Burns et al. 1981). This is less dramatic than the outcome in cats, where a single meal lacking arginine can cause signs as severe as coma and death (Morris and Rogers 1978).

As noted above, generally reduced dietary protein intake can be reflected in potential deficiencies of critical amino acids. Those amino acids are important in all body processes ranging from maintenance to growth to repair. Both essential amino acids as well as nitrogen provision are important. Feeding to meet an overall nitrogen content in the diet is adequate provided the essential amino acids are present at required levels. The overall dietary protein intake, if adequate, does not appear to influence the essential amino acid requirements for either dogs or cats (Delaney et al. 2001; Strieker et al. 2006).

Along with other essential nutrients, adequate amino acids are responsible for normal skin structure and function. This is demonstrated by studies in many species including dogs and cats which show dermatological lesions related to amino acid deficiencies (Council 2006). Furthermore, the disease process referred to as hepatocutanous syndrome or superficial necrolytic dermatitis (SND), is characterized by severe skin lesions together with profound hepatic disease or less commonly, glucagon-secreting tumor. The plasma amino acid profiles of affected dogs differ from those with hepatitis, and are notably reduced compared to normal (Outerbridge et al. 2002). Interestingly, higher branched-chain to aromatic amino acid

ratio is correlated with SND but not other types of hepatopathy (Outerbridge et al. 2002). Together with the finding that intravenous infusions of amino acids typically yield better clinical response compared to oral supplementation, this suggests that aberrant hepatic catabolism of amino acids may be occurring. More research to further characterize this disease is needed.

Provision of adequate arginine and phenylalanine is essential to prevent cataracts in dogs (Ranz et al. 2002) and arginine is key for urea cycle as stated above (Milner et al. 1975). Restriction of histidine in adult dogs results in reduced hematocrit, hemoglobin, body weight and circulating histidine, carnosine, albumin, zinc, and copper; behaviorally dogs exhibited lethargy and meal avoidance (Cianciaruso et al. 1981). A recent study assessed the dietary phenylalanine requirements as a function of breed size and found no difference; that is, the phenylalanine requirements for small breed dogs did not differ from that for large breeds (Mansilla et al. 2018)

Dispensable amino acids when absent from the diet results in reduced plasma levels of certain essential amino acids, notably proline and asparagine in kittens (Taylor et al. 1997) though not studied to date in puppies. Furthermore, in kittens fed diets with 9 and 12% glutamic acid showed signs of thiamin deficiency compared to those fed diets with 6% glutamic acid or less, even when thiamin was provided in excess (Deady et al. 1981). Presumably the same effects would be seen in the dog.

Sulfur containing amino acids are quite important and specific imbalances of these amino acids in the diet causes alterations in weight gain in growing dogs (for example, Bressani 1963; Czarnecki et al. 1985; Hirakawa and Baker 1985). The sulfur containing amino acids, methionine and cysteine, are considered necessary for normal hair development because of their abundance in the keratin, the main protein found in hair. Provision of methionine will facilitate retention of body nitrogen stores even in an overall state of protein deficiency creating a nitrogen sparing situation (Allison et al. 1947). Furthermore, methionine is necessary for the synthesis of insulin-like growth factor I (IGF-I) a key mediator of the actions of growth hormone and a potent stimulator of cell proliferation in its own right (Stubbs et al. 2002). The metabolism of sulfur amino acids results in the excess sulfur eliminated in the urine; diets high in sulfur amino acids acidifies the urine. This is the mechanism of the urine of carnivorous animals having more acidic urine than those that are herbivorous.

The B vitamin niacin comes from both dietary sources and the conversion of tryptophan and therefore a deficiency in tryptophan can result in a niacin deficiency. Dietary lysine can compensate for a niacin deficiency due to the convergence with the tryptophan pathway during lysine catabolism (Baker 2005). Recent research has shown a correlation between reduced circulating tryptophan and the expression of protein-losing enteropathy, a subset of canine bowel diseases (Kathrani et al. 2018). Whether a tryptophan deficiency is causal or a consequence of the disease remains unknown at this time. In addition to its role in protein synthesis, tryptophan is an important precursor for kynurenine, serotonin, and melatonin (Richard et al. 2009). Kynurenine is a metabolite produced in the tryptophan to niacin pathway and has wide-ranging roles in humans including cancer, immune function, and neurological disorders (Stone et al. 2013) although little studied in the dog. Administration of the neurotransmitters/neurohormones of serotonin and melatonin is recommended to reduce anxiety and other behavioral conditions.

Dogs are often utilized as models to characterize human protein dietary allergens (Dearman and Kimber 2009) which may or may not be ideal for human purposes but does serve canine dietary development. Although most proteins evoke some degree of allergic response as evidenced by elevated IgG, the vast majority do not reach the degree of allergenic sensitization characterized by elevated IgE. Interestingly it is the protein as whole and its configuration that appears to confer allergenicity (e.g. the specific epitope structure of the peptide) and not particular amino acids that cause the immune system to mount a response. Because adverse food reactions are seen in a measurable proportion of pet dogs, the development of diets with limited

and often uncommon ingredients to reduce allergenicity have been designed although many of the diets purported to be limited actually are contaminated with other protein sources (Fossati et al. 2019). The additional concern with some diets is that the composition and bioavailability of the indispensable amino acids may be lacking (Kanakubo et al. 2015). In fact, this may be contributing to the recent cases of nutritionally medicated dilated cardiomyopathy (DCM) in dogs that are fed grain-free diets. As previously noted, deficiencies in taurine are causal for DCM in cats. A taurine deficiency in dogs likewise was also associated with DCM (Fascetti et al. 2003), and currently ongoing cases are likely also due to the same problem (Kaplan et al. 2018). Whereas taurine deficiency in cats manifests as retinal degeneration, reproductive abnormalities, and impaired growth as well as DCM, in the dog, taurine deficiency appears mostly limited to DCM.

10.7 Life Stages

Although it is well known that protein and amino acid quantity requirements vary during growth relative to pre or post weaning stages, this information is more global. There have been few studies targeting specific amino acids during the growth and development stage and what is known about particular amino acids predominantly comes from laboratory rodent models and some work with kittens. This is the case despite that need being articulated in 1994 (Morris and Rogers 1994). Specific controlled experiments to evaluate the role of particular amino acids during the growth phase in dogs are limited. Milner and colleagues undertook studies limiting particular amino acids in immature (14–15 weeks old) male beagles to define dietary requirements in the dog (Milner 1979a; Milner 1979b; Burns and Milner 1982; Milner 1981). In all cases, removing the essential amino acids resulted in reduced food intake, growth, nitrogen balance, plasma transaminase activities, and elevated plasma urea. With the restriction of methionine and threonine, plasma ammonia and dermatitis were also seen. Provision of higher than required threonine (e.g., at 0.8%) tended to reduce food intake and depressed growth (Burns and Milner 1982). An early study of sulfur containing amino acids found breed specific differences in dietary levels of methionine and cysteine that conferred adequacy for growing puppies (Blaza et al. 1982)

A recent study used a multipronged approach to assess the impact of dietary supplements on growth and development during the neonatal stage (Wang et al. 2017) Although multiple components were in the supplement including d*ocosahexaenoic acid* (DHA), carotenoids, and vitamins, taurine was also included at 2.5 times the level fed to the control group. Thus, although changes in growth rate, body composition, and circulating growth factors were observed, the direct impact of the taurine supplementation could not be assessed.

It is known that diets during pregnancy and lactation require provision of carbohydrate to spare the diversion of proteins for energy and maintenance use in the bitch. In the absence of carbohydrate, protein use for conceptus growth and development is prevented, leading to fetal and neonatal mortality (Kienzle et al. 1985). Beyond these studies, research targeting diets and dogs are limited.

Animal factors such as life stage may also influences protein and amino acid requirements. Senior dogs appear to require higher amounts of crude protein in their diets to sustain a reserve level of readily available protein (Wannemacher Jr and McCoy 1966). However, this increased protein requirement does not appear to be an effect of decreased digestibility, as no studies have reported a decreased digestive efficiency in geriatric dogs. Although there are potentially important physiological effects of aging on the digestive process, most studies report no differences in nutrient absorption when comparing young adult to geriatric dogs. One study reported higher inter-individual variability in older vs. younger dogs (Buffington 1989), so this may partly explain conflicting results. Regardless, adequate amounts of high quality protein are indicated in aged dogs, given the age-related decline in protein synthesis (Rattan 1996) and increased protein turnover in older animals (Wannemacher Jr and McCoy 1966).

Since older pets have a higher requirement for dietary protein, a reduction in intake further widens the gap between need and provision and is likely to be even more detrimental than in younger animals. In addition, if caloric intake is decreased to manage weight gain, the proportion of energy provided as protein should be increased (Laflamme 2005, 2012).

Another aspect to be considered is that the gut microbiota and its metabolism has been shown to change with age as well as under conditions of dietary restriction (Wang et al. 2007) which has overarching implications for amino acid metabolism although the extent of impact is yet to be determined.

10.8 Summary

Amino acids play a pivotal role in the health and well-being of dogs. Provision of a balanced diet that contains the appropriate quantities of amino acids each with adequate bioavailability is necessary for optimal health of the dog throughout its life. Although indispensable amino acids are typically those of concern in deficiency states, overall amino acid profile and the impact of many other dietary factors must also be considered. Deficiency syndromes are often clinically nonspecific and less commonly may be characterized by precise diseases such as DCM secondary to taurine deficiency. The roles of life stage, age, and other animal factors including the microbiome will likely prove to have major influences on amino acid nutrition for the individual. The involvement of amino acids in all physiological processes of the dog from muscle growth to energy metabolism to neurological function and behavioral states argue for a greater and more comprehensive understanding of their nutrition in the dog.

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11 Amino Acids in the Nutrition, Metabolism, and Health of Domestic Cats

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Abstract

Domestic cats (carnivores) require high amounts of dietary amino acids (AAs) for normal growth, development, and reproduction. Amino acids had been traditionally categorised as nutritionally essential (EAAs) or nonessential (NEAAs), depending on whether they are synthesized de novo in the body. This review will focus on AA nutrition and metabolism in cats. Like other mammals, cats do not synthesize the carbon skeletons of twelve proteinogenic AAs: Arg, Cys, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Tyr, and Val. Like other feline carnivores but unlike many mammals, cats do not synthesize citrulline and have a very limited ability to produce taurine from Cys. Except for Leu and Lys that are strictly ketogenic AAs, most EAAs are both glucogenic and ketogenic AAs. All the EAAs (including taurine) must be provided in diets for cats. These animals are sensitive to dietary deficiencies of Arg and taurine, which rapidly result in life-threatening hyperammonemia and retinal damage, respectively. Although the National Research Council (NCR, Nutrient requirements of dogs and cats. National Academies Press, Washington, DC, 2006) does not recommend dietary requirements of cats for NEAAs, much attention should be directed to this critical issue of nutrition. Cats can synthesize de novo eight proteinogenic AAs: Ala, Asn, Asp, Gln, Glu, Gly, Pro, and Ser, as well as some nonproteinogenic AAs, such as γ-aminobutyrate, ornithine, and β-alanine with important physiological functions. Some of these AAs (e.g., Gln, Glu, Pro, and Gly) are crucial for intestinal integrity and health. Except for Gln, AAs in the arterial blood of cats may not be available to the mucosa of the small intestine. Plant-source foodstuffs lack taurine and generally contain inadequate Met and Cys and, therefore, should not be fed to cats in any age group. Besides meat, animal-source foodstuffs (including ruminant meat & bone meal, poultry by-product meal, porcine mucosal protein, and chicken visceral digest) are good sources of proteinogenic AAs and taurine for cats. Meeting dietary requirements for both EAAs

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and NEAAs in proper amounts and balances is crucial for improving the health, wellbeing, longevity, and reproduction of cats.

Keywords

Cats · Amino acids · Nutritional requirements · Protein deficiency

Abbreviations

AA	amino acid
BCAA	branched-chain amino acid
BCKAD	branched chain α-ketoacid dehydrogenease
CP	crude protein
DM	dry matter
FHL	feline hepatic lipidosis
IDO	indoleamine 2,3-dioxygenase
MAT	methionine adenosyltranferase
NO	nitric oxide
SAA	sulfur-containing amino acid
SAM	*S*-adenosylmethionine

11.1 Introduction

Domestic cats (*Felis silvestris*) are obligate carnivores (Zoran 2002). The word "obligate", which means "by necessity", is used to emphasize the fact that they are somewhat different than many other meat-eating predators. The cats eat "prey" or depend on nutrients [such as amino acids (AAs)] in animal tissues as their foods, and are also known as hypercarnivores (Adronie et al. 2013). Thus, the cats have evolved to lose an ability of synthesizing taurine (Sturman and Hayes 1980), which is an abundant AA in animals-source feedstuffs but absent from plant-source feedstuffs (Hou et al. 2019; Li and Wu 2020). Hypercarnivores require more dietary protein than omnivorous mammals (Holliday and Steppan 2004). Verbrugghe and Bakovic (2013) have suggested that cats have many physical and metabolic variations due to evolution pressure that includes the metabolism of one-carbon molecules and fatty acids. The requirements of carnivores for dietary protein are higher than omnivores and herbivores, because the former need AAs [e.g., Glu, Gln, Asp, Ala, and branched-chain AAs (BCAAs)] for ATP production by major tissues. The carnivorous mammals may be just like carnivorous fish in using Glu, Gln and Asp as the major metabolic fuels (Jia et al. 2017; Li et al. 2020a). In addition, AAs are used for glucose synthesis in all carnivores. During the deprivation of food, the gluconeogenic capacity of cats is maximized with the high expression of the needed enzymes in the liver (Rogers et al. 1977; Verbrugghe and Bakovic 2013).

Protein metabolism in domestic cats is different than that in omnivores (Wortinger 2010). This includes dietary requirements of cats for arginine and taurine (Wester et al. 2015; Wu 2018). Protein in the body cats consists of 20 proteinogenic AAs and other AA derivatives, including 4-hydroxyproline, 3-hydroxyproline, hydroxylysine, 3-methylhistidine, and methylarginines (Wu 2013). Like other mammals, cats do not synthesize the carbon skeletons of 12 proteinogenic AAs: Arg, Cys, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Tyr, and Val (Jungnickel et al. 2018). These AAs have been traditionally classified as nutritionally essential AAs (EAAs) and must be included in diets for the cats of all age groups (Hou and Wu 2018a). Like other feline carnivores but unlike many mammals, cats do not synthesize citrulline de novo and have a limited ability to produce taurine. Taurine has a plethora of physiological functions (Wu 2020b) and must also be provided in their diets to prevent disorders, such as retinal, cardiovascular, muscular, and reproductive disorders. However, cats can synthesize de novo eight proteinogenic AAs: Ala, Asn, Asp, Gln, Glu, Gly, Pro, and Ser, as well as some nonproteinogenic AAs, such as γ-aminobutyrate, ornithine, and β-alanine with important physiological functions (Rogers et al. 1998). The biosynthesizable proteinogenic AAs had been historically classified as nutritionally nonessential AAs (NEAAs; see Hou et al. 2015 for review),

but this term has now been recognized as a misnomer in nutritional sciences and should not be used in nutrition research or practices (Hou and Wu 2017). Studies with pigs, rats, chickens, and fish have shown that these animals have dietary requirements for at least some of the NEAAs (Hou et al. 2015, 2016; Li et al. 2020a). This may also be true for cats (e.g., Gln and Gly), particularly those with cancers and intestinal damage (Morrison 2002).

Based on their metabolic fates, AAs are classified as glucogenic, ketogenic, or both glucogenic and ketogenic (Wu 2013). Glucogenic AAs are: Ala, Arg, Asp, Asn, Cys, Gln, Glu, Gly, His, Met, Pro, Ser, Thr, and Val that can produce pyruvate or an intermediate of the Krebs cycle (Burns et al. 1981; D'Mello 2003; NRC 2006; Saxton et al. 2016). Ketogenic AAs are Leu and Lys that produce acetyl-CoA and ketone bodies but no glucose (Harris et al. 2004; NRC 2006; Zhao et al. 2010). Amino acids that serve as both glucogenic and ketogenic are Ile, Phe, Thr, Trp and Tyr that can generate pyruvate or an intermediate of the Krebs cycle (substrates of glucose), as well as acetyl-CoA and ketone bodies (Hendriks 1996; Yu et al. 2001; NRC 2006). Furthermore, in domestic cats, cysteine, glycine, and glutamate [derived from branched-chain AAs (BCAAs)] participate in the syntheses of three unique sulfur-containing AAs (felinine, isovalthine, and isobuteine) through inter-organ metabolism that involves the liver and kidneys (Brosnan and Brosnan 2006; NRC 2006; Hand et al. 2010). The major objective of this article is to highlight the important roles of AAs in the nutrition, metabolism, and health of these companion animals.

11.2 Requirements of Protein and AAs for Growing and Adult Cats

Dietary AAs are required by cats for the growth and maintenance of body tissues and also for the production of nitrogen-containing organic compounds, including purines, pyrimidines, serotonin, creatine, polyamines, nitric oxide (NO), and glutathione (Hendriks 1996; Wu 2013). In practice, dietary protein is the primary source of AAs for the animals. Cats use a large amount of dietary protein for ATP production (Zoran 2002). The minimum requirement of growing and reproductive cats for dietary crude protein (CP) is 30% of the dietary dry matter and the minimum maintenance requirement of adult cats for dietary CP is 26% of the dietary dry matter (AAFCO 2014). Both EAAs and NEAAs are needed in the diets of animals (including cats) for their optimum health, growth and development (Wu 2018). Because some of the free AAs confer bitter, salty or unpleasant tastes and because it is expensive to prepare free AA-based purified diets, the cats that can eat and have a healthy digestive tract are generally provided with intact protein. Fully developed cats need dietary protein for the maintenance of digestive enzymes and proteins in tissues, such as those in blood, skeletal muscle, gastrointestinal mucosae, skin, hair, liver, and brain (Laflamme 2008). Growing cats and kittens need dietary protein for maintenance (just like adult cats), as well as the growth and development of tissues.

Cats can adapt to changes in dietary protein intake from 14% to 56% CP (Green et al. 2008; Rogers et al. 1998). This likely involves alterations in the activities of AA-metabolic enzymes and the rates of whole-body protein turnover (protein synthesis and degradation). Thus, cats fed a low-protein diet produce less ammonia, urea, and creatine than those fed a normal-protein diet (Zoran 2002). Animals can utilize excess protein as the source of energy if they are fed low-energy diets that contain relatively low levels of lipids and digestible carbohydrates (e.g., starch/glycogen). If dietary energy intake by animals is adequate, excessive dietary protein will be converted into lipids and glycogen, with nitrogen being excreted primarily as ammonia and urea in the urine (Wu 2013). The content of protein in meat is relatively constant. Of note, cats fed meats that naturally contain 70–75% CP [dry matter basis; about twice their minimum requirement for dietary CP (NRC 2006)] do not exhibit any adverse response. When their arginine intake is adequate and their

liver functions normally, healthy cats that consume meat do not exhibit ammonia toxicity. This indicates a high capacity of young and adult cats to catabolize dietary AAs.

Because Cys is formed from Met in the liver and Tyr is produced from Phe in both the liver and kidneys (Hou et al. 2020; Li et al. 2020d; Wu 2013), Cys and Tyr are generally not considered by some authors as EAAs (Verbrugghe and Bakovic 2013). However, a great dependence on Met for Cys provision will reduce the availability of Met as a methyl group donor for critical biochemical reactions (e.g., creatine synthesis and protein methylation) in the body. In addition, because the conversion of Phe into Tyr requires tetrahydrobiopterin (Wu 2013), which is readily oxidized and can be depleted under conditions of oxidative stress and disease (Shi et al. 2004), the degradation of Phe may not provide sufficient Tyr in a catabolic state. Cats that have genetic defects in Cys synthesis and Phe hydroxylation must obtain both Cys and Tyr from diets. To meet metabolic needs and reduce metabolic burdens on AA synthesis, all proteinogenic AAs should be provided to young and adult cats, just like livestock mammals and poultry (Wu 2014). In addition, cats of all age groups have a dietary requirement for taurine, as noted previously.

There are differences in the recommended requirement values of some EAAs for growing and reproductive cats between the 2006 and 2014 versions (Table 11.1). The requirements for His, Ile, Leu, Phe (+ Tyr), Phe and Val in the 2014 version are greater than those in the 2006 version. However, the recommended requirement values of most EAAs in the 2014 version are the essentially the same as those in the 2006 version. Interestingly, the recommended requirement value for Arg in the 2014 version is slightly lower by a 0.01% unit than that in the 2006 version. Of particular note, the recommended requirement values for Phe and Tyr in the 2014 version is substantially greater than those in the 2006 version to maintain the black hair color of the cats. Adequate intakes of Cys, Met and taurine are of exceptional concern in cat nutrition (Case et al. 2011). Deficiencies of

Table 11.1 Recommended requirements of cats for dietary protein and nutritionally essential amino acids

Nutrient (% of dry matter in diet)	AAFCO (2007); National Research Council (NRC 2006)			AAFCO (2014)		
	Minimum requirement for growth and reproduction	Minimum requirement for maintenance in adults	Maximum requirement	Minimum requirement for growth and reproduction	Minimum requirement for adult maintenance	Maximum requirement
Crude protein	30	26	–	30	26	–
Arginine	1.25	1.04	–	1.24	1.04	–
Histidine	0.31	0.31	–	0.33	0.31	–
Isoleucine	0.52	0.52	–	0.56	0.52	–
Leucine	1.25	1.25	–	1.28	1.24	–
Lysine	1.20	0.83	–	1.20	0.83	–
Methionine	0.62	0.62	1.5	0.62	0.20	1.5
Methionine (+ cysteine)	1.10	1.10	–	1.10	0.40	–
Phenylalanine (+ tyrosine)	0.88	0.88	–	1.92	1.53	–
Phenylalanine	0.42	0.42	–	0.52	0.42	–
Taurine	0.20	0.20	–			–
Threonine	0.73	0.73	–	0.73	0.73	–
Tryptophan	0.25	0.16	–	0.25	0.16	1.7
Valine	0.62	0.62	–	0.64	0.62	–
Total EAAs	8.11	7.44		9.25	7.38	

– Data are not available

EAAs nutritionally essential amino acids (including Cys and Tyr)

these AAs result in protein malnutrition in cats, leading to weight and lean tissue losses, poor work and reproductive performance, and insulin resistance (Case et al. 2011; Verbrugghe et al. 2012). The poor health of the animals may be caused by a deficiency of NO, which is a metabolite of Arg (Wu and Meininger 2009).

Compelling evidence shows that cats have dietary requirements for NEAAs (Verbrugghe and Bakovic 2013; Rogers et al. 1998). For example, growing kittens fed a 14% CP diet with all EAAs [1X NRC (1986) requirements] but without any NEAA lost body weight during a 10-day experimental period (Table 11.2). Additionally, kittens fed a 21% CP diet with all EAAs [2.8X NRC (1986) requirements] but without any NEAA grew poorly. Furthermore, kittens fed a 35% CP diet with all EAAs [4.7X NRC (1986) requirements] but without any NEAA grew at a suboptimal rate, as compared with the animals fed a 25% CP containing both EAAs and NEAAs. Disappointingly, the mixture of NEAAs used in the previous studies did not contain serine, and the ratios of NEAAs to EAAs were not consistent with those in meat (Wu et al. 2016) or the animal body (Wu 2013).

Unfortunately, nutritionists have generally considered only EAAs for cats (Table 11.1). However, the sum of these EAAs is less than 31% CP of the diet. Feeding only these EAAs to cats in any age group will not support their maintenance needs. Clearly, NEAAs should be included in the diets of cats at all of their developmental stages. At present, such data are not available. Based on the content (on the basis of dry matter) of true proteins, small peptides, and free AAs in the beef loin meat (Wu et al. 2016), as well as a lower metabolic rate in the adult than in

Table 11.2 Growth of kittens fed purified diets containing various rations of EAAs to NEAAs for 10 days[a]

CP content %	EAAs[b] : NEAAs[c]	(X) EAA requirement[d]	Number of animals	Weight gain (g/day)	AA in plasma (nmol/ml)		
					Glu	Arg	Pro
35	0.27 : 0.73	1.5	36	24.4	–	–	–
14[e]	1.00 : 0.00	1.9	12	– 4.7	60	136	75
14	0.47 : 0.53	1.0	12	14.7	102	107	199
21[e]	1.00 : 0.00	2.8	12	10.8	72	344	70
21	0.31 : 0.69	1.0	12	16.9	124	106	513
21	0.61 : 0.39	2.0	8	19.1	100	301	207
35[e]	1.00 : 0.00	4.7	12	21.5	72	290	67
35	0.18 : 0.82	1.0	12	13.3	182	78	1062
35	0.55 : 0.45	3.0	10	29.0	77	262	257
42	0.23 : 0.77	1.5	10	28.8	188	121	801
42	0.45 : 0.55	3.0	10	18.2	105	348	633
56	0.11 : 0.89	1.0	12	1.3	413	68	2165
56	0.17 : 0.83	1.5	10	16.5	228	98	1165
56	0.23 : 0.77	2.0	8	18.2	157	119	890
56	0.34 : 0.66	3.0	10	24.3	143	227	742

AA amino acid

[a]Adapted from Rogers et al. (1998). Cats (8 to 12 weeks of age; the initial body weights = 1.02 to 1.30 kg) were used for the experiments. Crude protein (CP) = nitrogen in the diet x 6.25. All diets contained 0.15% taurine

[b]Nutritionally essential amino acids (EAAs; L-isoform) used in the study are Arg, His, Ile, Leu, Lys, Met, Cys, Phe, Tyr, Thr, Trp, and Val

[c]The mixture of nutritionally nonessential amino acids (NEAAs) used in the study contained the following (%): was L-Ala, 17.5; Gly, 17.5; L-Gln, 17.5; L-Glu, 7.5; L-Asn, 15; L-Asp, 10; and L-Pro, 15. Note that: (1) the NEAA mixture did not provide Ser and therefore was incomplete; and (2) the proportion of NEAAs in the mixture was very different than that in meat or the animal body

[d]National Research Council (NRC 1986). The 1X EAA requirements (% of diet) are: Arg, 1.0; His, 0.3; Ile, 0.5; Leu, 1.2; Lys, 0.8; Met, 0.4; Cys, 0.35; Phe, 0.4; Tyr, 0.45; Thr, 0.7; Trp, 0.15; and Val, 0.6

[e]The diet contained only EAAs as the source of nitrogen

the young (Wu 2018), we recommend the minimum and maximum dietary requirements of cats for protein, NEAAs, and EAAs (Table 11.2) as references for feeding and a framework for future studies. The CP content (on the basis of dry matter) of the beef loin meat is 73.4% (Wu et al. 2016). The minimum and maximum requirements for dietary AAs are based on those for dietary protein (i.e., the minimum dietary requirements of young and adult cats for 30% and 26% CP, respectively, and the maximum dietary requirements of both young and adult cats for 73.4% CP; dry matter basis). To prevent or alleviate the loss of skeletal muscle in aging cats through enhancing NO synthesis, protein synthesis, and anti-oxidative reactions, as well as reducing white fat accretion, we recommend that elderly cats have higher minimum dietary requirements for Arg, Glu, Gly, and Trp than young adult cats. This is mainly because of the following considerations. First, Arg (Yao et al. 2008), Gly (Sun et al. 2016), and Trp (Cortamira et al. 1991; Dukes et al. 2015; Lin et al. 1988) enhance protein synthesis in skeletal muscle (Lin et al. 1988; Sun et al. 2016; Yao et al. 2008). Second, both Arg and Gly increase glutathione synthesis to protect cells from oxidative stress (Jobgen et al. 2009; Wang et al. 2014). Third, Arg, Gly and Trp improve intestinal immune function and health (Liang et al. 2018, 2019; Wang et al. 2014, 2015; Wu 2014). Fourth, Glu is a major energy substrate for the small intestine of animals (He et al. 2018; Hou and Wu 2018b; Jia et al. 2017; Li et al. 2020a) and plays an important role in maintaining intestinal integrity (Hou and Wu 2018a; Jiao et al. 2015) (Table 11.3).

Table 11.3 Recommended requirements of cats for dietary amino acids[a]

Crude protein and amino acid	Minimum dietary requirements of cats for amino acids			Maximum dietary requirements of young, adult, and elderly adult cats for amino acids
	Young cats	Young adult cats	Elderly adult cats	
Crude protein	30	26	30	73.4
Taurine	0.2	0.2	0.2	0.29
Proteinogenic amino acids that are not synthesized de novo by cats				
Arg	2.14	1.86	2.33	5.24
Cys	0.50	0.50	0.50	1.12
His	1.30	1.12	1.12	3.17
Ile	1.68	1.46	1.46	4.11
Leu	2.73	2.36	2.36	6.67
Lys	2.94	2.55	2.56	7.20
Met	1.03	0.90	0.90	2.53
Phe	1.37	1.19	1.19	3.35
Thr	1.51	1.31	1.31	3.70
Trp	0.41	0.35	0.44	1.00
Tyr	1.23	1.07	1.07	3.01
Val	1.94	1.68	1.68	4.74
Proteinogenic amino acids that are synthesized de novo by cats				
Ala	1.86	1.61	1.61	4.54
Asn	1.37	1.18	1.18	3.34
Asp	1.68	1.46	1.46	4.11
Glu	3.07	2.66	3.33	7.51
Gln	2.04	1.77	1.77	4.99
Gly	1.38	1.19	1.49	3.37
Pro[b]	1.42	1.23	1.23	3.47
Ser	1.45	1.25	1.25	3.54

[a]Values are % of dry matter in the diet

[b]Proline + 4-hydroxyproline (the ratio of proline to 4-hydroxyproline = 18.6:1.0; g/g)

11.3 Protein Deficiency in Cats

Protein deficiency occurs in cats when their dietary protein intake is less than their minimum protein requirement. Inadequate intake of protein can result in an insufficient provision of both EAAs and NEAAs (Agnew and Korman 2014). As noted previously, EAAs must be provided in the diet simply because they are not formed de novo in the animal body. Therefore, like other mammals (e.g., rats; Anonymous 1975), when a diet lacking protein is consumed by cats, there is a decrease in enzyme activity for EAA catabolism to conserve the AAs (Morris 2002). Clinical signs of protein deficiency in cats are: reduced lean body mass, hindered growth in young cats, loss of body weight, impaired reproduction, and reduced work performance (Case et al. 2011). This is because dietary protein is particularly important for not only "feline health", but also the prevention of various metabolic and infectious diseases (Backlund et al. 2011; Kantorosinski and Morrison 1988; Wu 2020a). If dietary protein deficiency happens with sufficient energy intake, plasma AA and albumin concentrations decrease, leading to edema or ascites (Agnew and Korman 2014; Case et al. 2011; Wester et al. 2015; Zoran 2002). Because cats depend on dietary protein for gluconeogenesis when their typical diets contain a small amount of digestible carbohydrate, low dietary AA intake may affect glucose provision and therefore, the function of the brain, red blood cells, retina, and kidney medulla (Verbrugghe and Bakovic 2013).

11.4 Glucogenic Amino Acids

As a carnivore, the domestic cat consumes diets rich in protein and fats. Thus, there are differences in glucose metabolism between cats and non-carnivorous mammals (Schermerhorn 2013). For example, healthy cats lack salivary amylase (for glycogen and starch hydrolysis), as well as hepatic glucokinase (for glycolysis and glucose sensing) and hepatic glucokinase regulatory protein, and are prone to periods of fasting hyperglycemia (Schermerhorn 2013). Glucogenic AAs, which are derived primarily from net protein degradation in skeletal muscle, can be converted into glucose through the biochemical pathway of gluconeogenesis (Brosnan 2003). Among them, Ala, Arg, Asp, Asn, Gln, Glu, Ile, Pro, Ser, Thr, and Val are quantitatively the most important glucogenic substrates in post-prandial and post-absorptive cats. The synthesis of glucose from AAs occurs in the liver and kidneys, and involves the degradation of AAs to their α-ketoacids and an intermediate of the Krebs cycle. This process is quantitatively substantial for AA catabolism and physiologically vital in cats under catabolic conditions, such as fasting and hunger (Young and Ajami 2001). Gluconeogenesis is used for the disposal of excess AA carbons (Case et al. 2011).

11.4.1 Arginine

Arginine is an EAA for cats (NRC 2006), because their small intestine has a very low activity of pyrroline-5-carboxylate synthase (Rogers and Phang 1985). This enzyme converts Glu into pyrroline-5-carboxylate, an intermediate in the formation of Arg from Gln, Glu, and Pro. There is likely little or no synthesis of citrulline from glutamine and glutamate in the enterocytes of the feline small intestine under physiological conditions. It is also possible that Pro oxidase, which generates pyrroline-5-carboxylate from Pro, is negligible or absent from the feline gut. Of note, Arg contains a positively charged nitrogen side chain as a binding site for negatively charged molecules (Burns et al. 1981). Cats have a high requirement for Arg to maintain the hepatic urea cycle in an active state and the whole-body nitrogen balance (Baker and Czarnecki-Maulden 1991). In the urea cycle (also known as the ornithine cycle), Arg is an allosteric activator of *N*-acetylglutamate synthase, which generates N-acetylglutamate to stimulate

carbamoylphosphate synthase-I (Wu and Morris 1998). The latter converts NH_3 and bicarbonate into carbamoylphosphate. In addition, Arg stimulates the secretion of some hormones (e.g., insulin, glucagon and gastrin) (D'Mello 2003) and the synthesis of NO in endothelial cells (Shi et al. 2004). Furthermore, Arg activates the mTORC1 cell signalling pathway to promote protein synthesis in skeletal muscle (Yao et al. 2008; Saxton et al. 2016), placenta (Kong et al. 2012), brown adipocytes (Ma et al. 2017), and mammary epithelial cells (Ma et al. 2018). Cats rapidly display hyperammonaemia within 2 to 5 h after consuming an arginine-free diet (Baker and Czarnecki-Maulden 1991), and the clinical syndromes of ammonia toxicity include vomiting, nausea, tremors, seizures and even death (Morris 1985).

Morris et al. (1979) reported that the inclusion of ornithine in the Arg-free diets could prevent the onset of hyperammonaemia in cats but could not restore their weight gains. Therefore, blood-borne ornithine can facilitate ammonia detoxification but is not a substrate for Arg synthesis in the body. This is explained by the complex compartmentation of ornithine metabolism in the small intestine to favour Pro production (Wu and Morris 1998). Note that there is no net synthesis of Arg via the hepatic urea cycle because Arg is rapidly hydrolyzed by arginase into urea plus ornithine. In contrast to ornithine, both extracellular and intracellularly generated citrulline are readily used for Arg synthesis by argininosuccinate synthase and lyase in cats (Baker and Czarnecki-Maulden 1991). Thus, citrulline can fully replace Arg in the diets for cats. This is important for those cats that genetically lack intestinal transporters for cationic AAs.

11.4.2 Threonine, Histidine and Valine

Threonine contains a hydroxyl group that is chemically reactive for phosphorylation by protein kinase (Wu 2018). This is an important mechanism for the regulation of enzyme or protein activity. In cats, neutral AA transporters are responsible for the absorption of threonine by the small intestine and the proximal tubules of the kidneys in Na^+-dependent and independent mechanisms. In addition, Thr may play a role not only in hepatic glucose synthesis but also insulin secretion or cell apoptosis (Depaoli-Bug et al. 1994).

Histidine contains a positively charged imidazole side chain. Basic AA transporters are essential for absorbing histidine by the small intestine, and the proximal tubule of the kidneys actively reabsorb plasma histidine in the Na^+-independent manner. Histidine is a structural component of proteins that plays a crucial part in oxygen exchange and is the precursor of biologically active compounds, such as histamine and carnosine (NRC 2006). Haemoglobin is present at a high concentration in the blood; the positive charge on the imidazole side chain of histidine facilitates oxygen exchange in the lungs and other tissues (Cianciaruso et al. 1981). As a neuroactive molecule, histamine plays a role in immune function and vasodilation. As a histidine-derived dipeptide, carnosine acts as a cellular antioxidant and a chelator of copper and zinc in animal cells (Boldyrev et al. 2013). Meat is rich in histidine (Wu et al. 2016).

Valine is a BCAA. It is catabolised in the body through the cooperation of multiple organs, including in the skeletal muscle, adipose tissue, kidneys, brain, and liver (Wu 2013). This AA is an abundant AA in both animal- and plant-source proteins (Hou et al. 2019; Li and Wu 2020). The carbon skeleton of Val is either oxidized for ATP production or used for hepatic glucose synthesis in cats, depending on their physiological states (Garlick and Grant 1988; Radford 2004). An intermediate of Val may be used as a precursor for the synthesis of a unique AA (isobuteine) in cats.

11.5 Ketogenic Amino Acids

Leucine and Lys are two strict ketogenic AAs that produce acetyl-CoA and acetoacetyl-CoA in the liver (D'Mello 2003). These two intermediates are metabolized to form acetoacetate and β-hydroxybutyrate in the liver, the ketone bodies that are major metabolic fuels in the extra-hepatic tissues, such as the brain, heart, skeletal muscle,

and kidneys (Eisert 2011). Ketogenic AAs cannot be converted into glucose in animals due to the absence of the glyoxylate cycle, and are oxidized to CO_2 plus water (Wu 2018). Hydroxylation of certain Lys residues in collagen is essential for its structure, whereas an intermediate of Leu is used as a precursor for the synthesis of a unique AA (isovalthine) in cats. Leucine is an abundant AA in both animal- and plant-source proteins (Hou et al. 2019; Li and Wu 2020). In contrast, Lys is abundant in animal-source proteins but is deficient in most of the plant-source proteins.

Leucine is metabolized through transamination in cats to form Glu, Gln, Ala and Asp (Baker and Czarnecki-Maulden 1991). Because of its large mass, skeletal muscle is the primary site for initiating Leu degradation to form α-ketoisocaproic acid via BCAA transaminase in animals (Wu 2013). In lactating mammals, BCAA transaminase is also highly active in their mammary tissues (Li et al. 2009), which helps to explain why the milk of mammals (including cats and sows) is highly abundant in Gln and Glu (Davis et al. 1994). The activity of this enzyme is nearly absent in the feline liver under physiological conditions. α-Ketoisocaproic acid is decarboxylated by branched-chain α-ketoacid (BCKA) dehydrogenease, which is highly active in the liver (Harris et al. 2004) and mammary tissue (Li et al. 2009; Zhang et al. 2019). In addition, Leu has been reported to enhance protein synthesis by increasing plasma insulin concentration (Anthony et al. 2002; Balage et al. 2001) and activating the MTOR cell signalling in skeletal muscle (Manjarín et al. 2018). Furthermore, Leu and α-ketoisocaproic acid inhibit protein degradation in skeletal muscle (Nagasawa et al. 2002). Therefore, dietary Leu exerts an anabolic effect in animals after absorption.

Lysine is degraded primarily in the liver of animals (Wu 2013). Caution should be taken to avoid an imbalance among basic AAs in diets, blood and cells, because these AAs share the same transporters in the plasma membrane. As a positively charged AA, Lys plays an important role in the methylation and acetylation of proteins, which contribute to the modulation of certain cytoskeleton-associated proteins (e.g., actin, tubulin, and small GTPases) and epigenetic regulation of gene expression (Ali et al. 2018; Wang et al. 2012; Zhao et al. 2010). Genetic defects in basic AA transporters can cause the poor absorption of Lys, as well as ornithine, Arg and His by the small intestine and the renal tubules, leading to Lys deficiency in animals (Hoppe et al. 1993).

11.6 Glucogenic and Ketogenic Amino Acids

11.6.1 Phenylalanine and Tyrosine

Phenylalanine and Tyr are the precursors for the syntheses of dopamine, noradrenaline and adrenaline in neurons, whereas Trp is the substrate for the production of serotonin, N-acetylserotonin, melatonin, and indoles in a cell-specific manner (Hendriks 1996; Wu 2013). Thus, the availability of these three aromatic AAs influences the health and behaviour of cats. Of note, Phe and Tyr are particularly important for cats to maintain their hair color (Rogers and Morris 1979). Phenylalanine is degraded by the tetrahydrobiopterin-dependent Phe hydroxylase to yield Tyr (Wu 2013). Tyrosine is also the precursor of thyroid hormones, melanin, and catecholamine neurotransmitters (dopamine, norepinephrine and epinephrine). Dietary restriction of Phe along with excess tyrosine results in decreased weight gain and negative nitrogen balance, compared with cats fed a Phe-adequate diet (Rogers and Morris 1979; Williams et al. 1987). About half of the requirement for aromatic AAs may be met by Tyr (Williams et al. 1987). A deficiency of dietary Tyr decreases the production of pigment substances (e.g., dopaquinone, trichochromes, eumelanin, and pheomelanin) in the skin (Anderson et al. 2002; Yu et al. 2001), and this phenomenon is reversed by dietary supplementation with Tyr (Anderson et al. 2002).

11.6.2 Tryptophan

Tryptophan is a large neutral AA. It shares the same transmembrane transporters with other

large neutral AAs, such as Leu, Val, Met, Ile, Tyr and Phe for uptake into cells (Hawkins et al. 2006). In the gastrointestinal tissue and brain, Trp is metabolized via the tetrahydrobiopterin-dependent Trp hydroxylase to generate serotonin and N-acetylserotonin. This pathway regulates the response of cats to environmental stress challenges and their behaviours (Da Graça Pereira and Fragoso 2010). In lymphocytes and macrophages, Trp is metabolized by indoleamine 2,3,-dioxygenase to form kynurenine, and this pathway plays an important role in intestinal and whole-body anti-inflammatory responses (Kato et al. 2012; Oxenkrug 2010). Furthermore, animals (including cats) can synthesize niacin from Trp (Baker and Czarnecki-Maulden 1991). However, Trp cannot fully substitute nicotinic acid in the diet of cats. Thus, these animals will die after they are fed a diet with adequate Trp level but a low level of nicotinic acid (NRC 2006). Of note, Trp is deficient in most of the plant-source proteins but abundant in animal-source proteins (Hou et al. 2019; Li and Wu 2020).

11.7 Carnitine

Carnitine is an AA derivative that is synthesized from Lys, Met and Ser in the presence of vitamin B_6, vitamin C, α-ketoglutarate, and iron (Wu 2013). Over the past two decades, there has been much interest in the role of carnitine in preventing and treating feline hepatic lipidosis (FHL), as well as enhancing white-fat loss in cats through stimulating fatty acid oxidation in the liver and other tissues such as skeletal muscle and white adipose tissue (Blanchard et al. 2002; Center et al. 2000). The FHL, also known as feline fatty liver syndrome, is one of the most common forms of liver disease in cats that are often obese. The clinical signs of this disease include dramatic weight loss, lethargy, vomiting, hepatomegaly, jaundice, and gastroparesis (Wills and Simpson 1994). Although carnitine is present in meat, dietary supplementation with this nutrient may be beneficial for mitigating the FHL in cats, which generally consume meat with a relatively high content of lipids.

11.8 Sulfur-Containing Amino Acids

Cats have high requirements for dietary Met and Cys (Burger and Smith 1987; Hendriks 1996) to maintain their dense hair and metabolic activities (MacDonald et al. 1984). Nutritional insufficiencies of Met and Cys occur in cats fed home-made vegetable-based diets, leading to reduced growth and crusting dermatitis in the mucocutaneous skin of the mouth and nose (Hoppe et al. 1993). Among the following four sulfur-containing AAs (i.e., Met, Cys, homocysteine, and taurine), only Met and Cys are precursors for protein synthesis (Brosnan and Brosnan 2006). Methionine is the initial AA for the formation of proteins in eukaryotic cells, whereas N-formyl methionine serves the same function in prokaryotes. In the liver of cats, Met is degraded via the transsulfuration pathway to generate Cys, with methionine adenosyltransferase (MAT) catalysing the initial step to form S-adenosylmethionine (SAM) (Teeter et al. 1978; Wu 2013). SAM is the major donor of the methyl group for protein and DNA methylation reactions in the body. Cys is either oxidized to CO_2 plus water or used for the synthesis of glutathione, a potent antioxidant (Stead et al. 2006). In addition, Cys contributes to disulfide linkages in proteins, thereby influencing their structure and biological activities. The formation of Cys from Met can substitute 50% of dietary Met requirement in cats (Hendriks et al. 1995). As an intermediate of Met catabolism, homocysteine (a potent oxidant) can be recycled into Met in the liver via the vitamin B_6-dependent Met synthase. Partial catabolism of Met may occur at a low rate in extrahepatic tissues, but generates little or no CO_2. Excessive intakes of Met and Cys are highly toxic to animals due to the production of their metabolites, such as H_2S, SO_2, and H_2SO_4 (Hou and Wu 2018a), and therefore must be avoided at all times.

11.8.1 Taurine

Taurine is a crucial nutrient for cats (Knopf et al. 1978; Morris et al. 1990). It is a sulfur-containing β-AA that is abundant in meat, fish and crustaceans (Li et al. 2020b,c) but is absent from proteins (Wu et al. 2016). In the liver of cats and dogs (Oberbauer and Larsen 2020), taurine is the only AA that conjugates with bile acid to yield bile salts, which are essential for the digestion and absorption of dietary lipids. Moreover, as an abundant antioxidant AA, taurine protects the eyes, brain, heart, skeletal muscle, reproductive tract, and immune organs from damage (Hand et al. 2010; Morris et al. 1990; Sturman and Lu 1997). In contrast to most species of dogs, cats have a very limited ability to produce taurine from Cys because of a low activity of cysteine dioxygenase and cysteinesulfinate decarboxylase, and therefore taurine must be included in the feline diets (Case et al. 2011; Knopf et al. 1978; Morris and Rogers 1992). Clinical syndromes of taurine deficiency in cats include retinal degeneration, poor reproductive performance, fetal and post-natal developmental abnormalities, and dilated cardiomyopathy (Hall et al. 2018; Hand et al. 2010; Markwell and Earle 1995). The recommended intake of cats for dietary taurine is 0.2% (NRC 2006), which is below taurine content in meat (0.23% to 0.29%) (Wu et al. 2016).

11.8.2 Production of Three Unique Sulfur-Containing AAs (Felinine, Isovalthine, and Isobuteine) by Domestic Cats

Domestic cats synthesize three unique sulfur-containing AAs (felinine, isovalthine, and isobuteine; Kodama et al. 1980; Kuwaki et al. 1963; Mizuhara and Oomori 1961; Oomori and Mizuhara 1962). The sources of the cysteine moiety and the remaining portion in these AAs are glutathione (formed from Glu, Gly and Cys) and an appropriate fatty acid, respectively. The latter is isopentenyl pyrophosphate (an intermediate of cholesterol biosynthesis) in felinine (Rutherfurd et al. 2002), isovaleric acid (a metabolite of leucine) in isovalthine (Rutherfurd-Markwick et al. 2005), and possibly isobutyric acid (a metabolite of valine) in isobuteine (Herring et al. 2020). In the liver of cats, glutathione is conjugated with isopentenyl pyrophosphate, isovaleric acid, and isobutyric acid to yield respective derivatives, which are transported in the blood to the kidneys. In the proximal renal tubules of the kidneys, the glutathione conjugates are metabolized via cauxin (a carboxylesterase), γ-glutamyl transferase and dipeptidases (e.g., aminopeptidase M) to release felinine, isovalthine, and isobuteine for excretion in the urine (Miyazaki et al. 2008). Because isopentenyl pyrophosphate is generated from acetyl-CoA from the oxidation of AAs, glucose and fatty acids, and because the skeletal muscle is the major site for initiating BCAA catabolism and therefore the production of isovaleric acid isobutyric acid, the inter-organ metabolism of macronutrients is crucial for the production of felinine, isovalthine, and isobuteine in cats.

Male cats produce more felinine than female cats (Hendriks et al. 1995; Rutherfurd-Markwick et al. 2005), but there is no gender-specific for the urinary excretion of isovalthine (Hendriks et al. 2004). There are reports that in both male and female cats, increasing dietary intake of Met or Cys enhances the synthesis of felinine and isovalthine (Hendriks et al. 1995; Hendriks et al. 2004). The biological significance of felinine, isovalthine, and isobuteine, as well as their derivatives remains largely elusive. These sulfur-containing AAs and metabolites may serve as non-toxic, non-reactive, and relatively stable end-products of Met and Cys to prevent excessive formation of toxic and highly toxic substances (e.g., H_2S, SO_2, and H_2SO_4) from Met and Cys (Herring et al. 2020). There are also suggestions that felinine is a territorial marker for intra-species communications and is a putative precursor of a pheromone that serves as a chemical signal to attract females (Miyazaki et al. 2008).

11.9 Summary

Dietary protein provides both EAAs and NEAAs for domestic cats to synthesize tissue proteins, peptides, neurotransmitters, and other AA derivatives (e.g., NO, GABA, polyamines, thyroid hormones, melanin, melatonin, and felinine) with enormous biological importance. Glutamate and Gln may be the major metabolic fuels for the feline small intestine to maintain its integrity and health. All of the proteinogenic AAs are nutritionally and physiologically essential for the growth, development, health, and survival of the animals. Because of an inability to synthesize Arg from Gln, Glu and Pro, cats are very sensitive to a deficiency of dietary Arg with very rapid onset of life-threatening hyperammonemia. Although dietary EAAs have been recommended to young and adult cats, little data are available on the dietary requirements of these animals for NEAAs. The present article fills this important gap of the knowledge to guide feeding practices and future studies. In addition, cats have a very limited ability to synthesize taurine (a non-proteinogenic AA), which must be included in their diets to prevent the eyes, brain, heart, skeletal muscle, reproductive tract, and other tissues from damage. Plant-based foods with inadequate or no taurine should not be fed to cats in any age group. Besides meat, animal-source foodstuffs (including ruminant meat & bone meal, poultry by-product meal, porcine mucosal protein, and chicken visceral digest) are excellent sources of proteinogenic AAs (in both amounts and balances) and taurine. New advances in AA nutrition and metabolism are expected to improve the health and wellbeing of cats in their life cycle.

Acknowledgments Research in our laboratories was supported by funds from Jilin Agricultural University (to G. Che) and Texas A&M AgriLife Research H-8200 (to G. Wu). We thanks our students and colleagues for helpful discussions.

Conflicts of Interest The authors declare no conflict of interest.

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Amino Acid Nutrition for Optimum Growth, Development, Reproduction, and Health of Zoo Animals

12

Cassandra M. Herring, Fuller W. Bazer, and Guoyao Wu

Abstract

Proteins are large polymers of amino acids (AAs) linked via peptide bonds, and major components for the growth and development of tissues in zoo animals (including mammals, birds, and fish). The proteinogenic AAs are alanine, arginine, aspartate, asparagine, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. Except for glycine, they are all present in the L-isoform. Some carnivores may also need taurine (a nonproteinogenic AA) in their diet. Adequate dietary intakes of AAs are necessary for the growth, development, reproduction, health and longevity of zoo animals. Extensive research has established dietary nutrient requirements for humans, domestic livestock and companion animals. However, this is not true for many exotic or endangered species found in zoos due to the obstacles that accompany working with these species. Information on diets and nutrient profiles of free-ranging animals is needed. Even with adequate dietary intake of crude protein, dietary AAs may still be unbalanced, which can lead to nutrition-related diseases and disorders commonly observed in captive zoo species, such as dilated cardiomyopathy, urolithiasis, gut dysbiosis, and hormonal imbalances. There are differences in AA metabolism among carnivores, herbivores and omnivores. It is imperative to consider these idiosyncrasies when formulating diets based on established nutritional requirements of domestic species. With optimal health, populations of zoo animals will have a vastly greater chance of thriving in captivity. For endangered species especially, maintaining stable captive populations is crucial for conservation. Thus, adequate provision of AAs in diets plays a crucial role in the management, sustainability and expansion of healthy zoo animals.

Keywords

Amino acids · Protein · Nutrition · Zoo animals · Captivity

Abbreviations

AA	amino acid
AAFCO	Association of American Feed Control Officials
BCAA	branched-chain amino acid
CP	crude protein
DM	dry matter
GABA	γ-aminobutyrate
NRC	National Research Council

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1285, https://doi.org/10.1007/978-3-030-54462-1_12

12.1 Introduction

Amino acids (AA) are nitrogenous, organic compounds consisting of both an amino group and an acid group (Wu 2018). All proteinogenic AAs have a carboxylic acid group, and non-proteinogenic AAs may contain a carboxylic acid [e.g., citrulline, ornithine, β-alanine, and γ-aminobutyrate (GABA)] or a sulfonic acid (e.g., taurine) group. Twenty proteinogenic AAs are precursors for protein synthesis (Wu et al. 2016), namely alanine, arginine, aspartate, asparagine, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. Some of them (e.g., glutamate, glycine and tryptophan) play an important role in chemical sensing in tissues [including the skin and digestive tract (Solano 2020; Wu 2020c)], as well as in intestinal and pulmonary immune and antioxidative responses (Beaumont and Blachier 2020; Chen et al. 2020; Ren et al. 2020). Although non-proteinogenic AAs are not required for protein synthesis, they (e.g., taurine and GABA) have important physiological functions and their deficiencies can result in multi-organ abnormalities (Bazer et al. 2015; Wu 2020a, b). Furthermore, some end products of AA metabolism, such as felinine, isovalthine, and isobuteine (Fig. 12.1) produced by certain members of the Felidae species, may serve as territorial marks and intra-species communication signals in animals (Che et al. 2020; Miyazaki et al. 2008).

Protein or AA requirements for zoo animals (Allen and Ullrey 2004), like livestock and poultry (Wu 2018), vary among different stages of their growth and development and in response to alterations in nutritional, environmental, and pathological conditions. For example, the mink (a carnivore) is not able to synthesize arginine de novo (NRC 1982), whereas tigers and cheetahs (carnivores) do not produce taurine just like domestic cats (Gelatt 2014). In addition, mammals (Hou and Wu 2018) and birds (Wu 2009), as well as carnivorous and omnivorous fish (Jia et al. 2017; Li et al. 2020a) need large amounts of glutamate and glutamine for the growth and health of their small intestine. Much work has been done in recent years to establish optimal AA requirements for domestic livestock species, birds, fish, and humans (Wu 2009, 2018). Traditionally, AAs have been classified as nutritionally essential (EAA) or non-essential (NEAA; Wu 2010). The carbon skeletons of EAAs cannot be synthesized *de novo* by the body or cannot be synthesized in an adequate amount; therefore, these AA must be provided in diets (Wu 2009). Even though the body is able to synthesize NEAAs, their formation may not be adequate for maximal growth and optimal health, especially at certain physiological stages, such as pregnancy, lactation, and growth after weaning

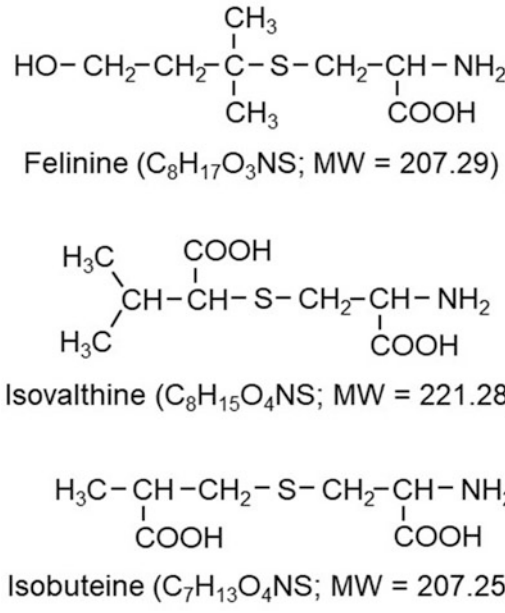

Fig. 12.1 Chemical structures of felinine (2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid; (2*R*)-2-amino-3-[(3-hydroxy-1,1-dimethylpropyl)thio]propanoic acid]), isovalthine (2-amino-5-carboxy-6-methyl-4-thiaheptanoic acid), and isobuteine [2-amino-6-carboxy-4-thiaheptanoic acid; *S*-carboxyisopropylcysteine; *S*-(2-methyl-2-carboxyethyl)cysteine]. Certain members of the Felidae family (e.g., cats) synthesize and excrete these three unique sulfur-containing amino acids.
In addition, humans with hypothyroidism and hypercholesterolemia, as well as other select mammals (e.g., the rat, rabbit, guinea pig, and dog) are known to produce isovalthine

(Hou and Wu 2017; Hou et al. 2015; Wu et al. 2017, 2018). For this reason, dietary requirements of zoo animals for NEAAs must be established.

For zoo and endangered animals, it is difficult to determine exact dietary nutritional requirements due to the invasive nature of the methods used (Schmidt et al. 2007). Therefore, domestic animals are often employed to estimate dietary nutritional requirements for captive carnivores, herbivores, and omnivores (Schmidt et al. 2007). However, these estimations may not be completely accurate considering unique biochemical and physiological differences among species. Even when analyzing nutrient concentrations in the serum of a captive exotic animal is possible, the results may be vastly different from those in a free-ranging animal because differences in nutrient intakes [e.g., dry matter (DM), AAs, carbohydrates, vitamins, and minerals] and blood hormone levels (Schmidt et al. 2007).

The major objective of this article is to highlight unique features of AA nutrition and metabolism in zoo animal species based on the limited data available. Due to the complicated processes necessary to define nutritional requirements of zoo animals, it is important to use the information established for domestic species (e.g., sheep, cattle, pigs, chickens, and farmed fish) and make adjustments based on observations to best formulate adequate diets for zoo animals.

12.2 Carnivores

Carnivores, by definition, eat animals or animal products and have unique physiological features that support the consumption and digestion of prey. Their diets are rich in protein and fats, but contain a very small amount of carbohydrate. Thus, carnivores must synthesize a large amount of glucose from AAs (Ala, Arg, Asp, Asn, Cys, Gln, Glu, Gly, His, Met, Pro, Ser, Thr, and Val that can produce pyruvate and an intermediate of the Krebs cycle) in the liver and kidneys to support the metabolic needs of their brain, red blood cells, retina, and kidney medulla (Wu 2018). Based on studies with cats and dolphins, carnivores lack hepatic glucokinase (for glycolysis and glucose sensing) and hepatic glucokinase regulatory protein, and are prone to periods of fasting hyperglycemia, contrary to monogastric mammals (Schermerhorn 2013). This class of animals includes mammal obligate carnivores [i.e. felids (e.g., domestic cats, tigers, and lions), giant anteaters, otters, hyenas, sea lions, mink, tarsiers, dolphins, seals, and walruses] and non-mammal obligate carnivores (e.g., largemouth bass, rainbow trout, salmon, hawks, eagles, crocodilians, many snakes and lizards, and most amphibians]. Obligate carnivores must eat animals or animal products because they lack the enzymes to synthesize or metabolize certain nutrients that cannot be obtained from plants and bacteria (Kleiman et al. 2010). As an example, felids do not synthesize either ornithine, citrulline and arginine from glutamic acid or taurine from cysteine (MacDonald et al. 1984).

Ornithine serves as an intermediate for urea synthesis in mammals by stimulating the conversion of ammonia, a product of protein metabolism, into urea for excretion (Wu 2013). Ornithine can also be used for proline synthesis or converted into polyamines (putrescine, spermidine and spermine), which are important regulators of DNA and protein synthesis (Wu 2013). As an allosteric activator of *N*-acetylglutamate synthase, arginine is also a crucial AA for urea-cycle function and ammonia detoxification as urea in mammals (Wu and Morris 1998). Thus, cats (which cannot synthesize arginine due to an intestinal deficiency of pyrroline-5-carboxylate synthase) develop severe hyperammonemia after consuming an arginine-free diet, which often quickly leads to death (Baker 2007). Severe hyperammonemia occurs in cats since they cannot synthesize ornithine, and therefore citrulline, which limits renal arginine synthesis (Ball et al. 2007). As obligate carnivores, cats eat high levels of protein, and therefore, need high levels of dietary arginine for urea-cycle function and nitrogen excretion. Likewise, mink grow very poorly and die when fed an arginine-free or deficient diet (NRC 1982).

Taurine is critical for regulating intracellular osmolality and retinal photoreceptor activity, modulating the digestion and absorption of dietary fats and lipid-soluble vitamins, as well as the nervous, muscular and reproductive systems, and

it is also a major antioxidant (Wu 2018). As in domestic cats (Che et al. 2020), the concentrations of taurine in the plasma and whole blood of zoo felids [the fishing cat (*Prionailurus viverrinus*), lion (*Panthera leo*), Bengal tiger (*Panthera tigris tigris*), Siberian tiger (*Panthera tigris altaicia*); cheetah, leopard (*P. pardus*), cougar (*Puma concolor*), and serval (*Leptailurus serval*)] are 80–120 and 300–600 nmol/ml, respectively (Hedberg et al. 2007). The ability of carnivores to synthesize taurine varies greatly among species and even the different breeds of the same species. For example, unlike domestic cats, tigers, lions and other felids (e.g., the cheetah, puma, jaguar, and leopard; Chesney and Hedberg 2009; Gelatt 2014), most of dog species are able to synthesize taurine from cysteine in the liver (Hayes 1998). However, certain breeds of dogs [e.g., giant breed dogs (Newfoundland) and American Cocker Spaniels] and some individuals do not synthesize taurine due to genetic defects and must require a dietary source of taurine to maintain health and prevent disorders, such as dilated cardiomyopathy and retinal lesions (Backus et al. 2003; Fascetti et al. 2003; Kittleson et al. 1997). Anderson et al. (1979) found that 0.1% taurine in the diet supports sufficient growth in kittens and prevents tissue depletion of taurine. However, with a taurine-free diet, photoreceptor degeneration occurs in the retina due to taurine depletion, while glycine and glutamine concentrations increase in the area centralis of the retina and in the heart (Anderson et al. 1979). Concentrations of glutamine also increase in the lens of the eye, which alters the glutamine: glutamate ratio (Anderson et al. 1979). Cats and dogs use solely taurine to conjugate bile acids via *N*-acylamidation, but other species use both glycine and taurine to do so (Czuba and Vessey 1981). Bile acid conjugation plays an important role in the digestion and absorption of dietary lipids, as well as liver physiology and the intestinal microflora (Hagey et al. 2010; Wu 2018). At present, little is known about bile acid-conjugating enzymes in zoo animals, including carnivores. However, studies with 677 vertebrate species (103 fish, 130 reptiles, 271 birds, 173 mammals) have shown significant variation in bile salt composition among orders but not between families, genera, or species (Hofmann et al. 2010).

Some Felidae species (e.g., the bobcat, ocelot, Chinese desert cat, kodkod, Siberian lynx, and domestic cat) have been reported to synthesize felinine (2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid; (2*R*)-2-amino-3-[(3-hydroxy-1,1-dimethylpropyl)thio]propanoic acid]) (Hendriks et al. 1995; Miyazaki et al. 2008; Westall 1953). In addition, certain felids (e.g., the domestic cat and the lion), as well as humans with hypothyroidism and hypercholesterolemia and other select mammals (e.g., the rat, rabbit, guinea pig, and dog) are known to produce isovalthine (2-amino-5-carboxy-6-methyl-4-thiaheptanoic acid) (Kuwaki et al. 1963; Mizuhara and Oomori 1961). Furthermore, the domestic cat, other select members of the Felidae family, and humans generate isobuteine [2-amino-6-carboxy-4-thiaheptanoic acid; *S*-carboxyisopropylcysteine; *S*-(2-methyl-2-carboxyethyl)cysteine] (Kodama et al. 1980; Oomori and Mizuhara 1962).

Felinine, isovalthine, and isobuteine are unusual sulfur-containing AAs in that they contain both a sulfur atom in the main chain and a branched side chain with a methyl group. Their syntheses require glutathione and either an isoprene unit or a branched-chain α-ketoacid, as illustrated for feline in Fig. 12.2. Specifically, in the livers of those species, glutathione conjugates with isopentenyl pyrophosphate [an intermediate of cholesterol biosynthesis (Rutherfurd et al. 2002)], isovaleric acid [a metabolite of leucine (Rutherfurd-Markwick et al. 2005)], and possibly isobutyric acid (a metabolite of valine) to yield 3-methylbutanol-glutathione (3-mercaptobutanol-glutathionine; γ-glutamylfelinylglycine), *S*-(iso-propylcarboxymethyl)-glutathione, and *S*-(iso-ethylcarboxymethyl)-glutathione, respectively. These conjugation reactions are catalyzed by glutathione *S*-transferase in the cytosol of hepatocytes. The glutathione conjugates are released from the liver and transported in the blood to the kidneys, where they are metabolized via γ-glutamyl transferase (a membrane-bound

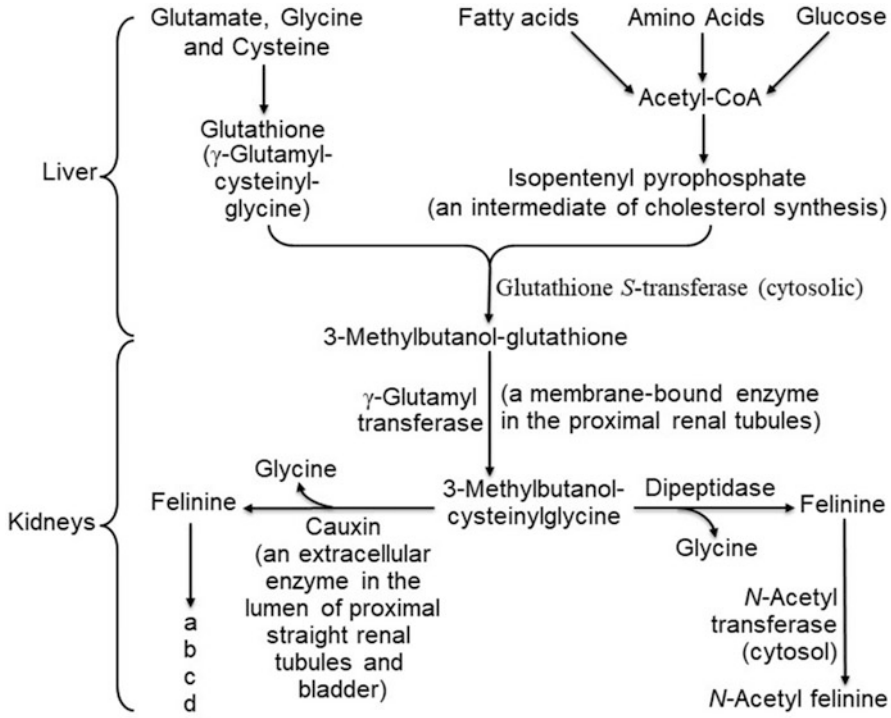

Fig. 12.2 Synthesis and metabolism of felinine in domestic cats. In the liver of domestic cats, glutathione *S*-transferase catalyzes the conjugation of glutathione with isopentenyl pyrophosphate yield 3-methylbutanol-glutathione. The latter is released from the liver and transported in the blood to the kidneys, where it is metabolized via γ-glutamyl transferase (a membrane-bound enzyme in the proximal renal tubules) to form 3-methylbutanol-cysteinylglycine. This cysteinylglycine derivatives is hydrolyzed by dipeptidases (e.g., aminopeptidase M) in the cytosol of the proximal renal tubules to generate felinine, with glycine as a co-product. Felinine is locally *N*-acetylated by *N*-acetyltransferase to *N*-acetyl-felinine. Additionally, 3-methylbutanol-cysteinylglycine is hydrolyzed by the extracellular cauxin (a carboxylesterase secreted by the proximal straight renal tubules of the kidneys) in the lumen of the renal tubules and the bladder to yield felinine, with glycine as a co-product. In the cytosol of the proximal straight renal tubules, feline is further metabolized into methylated products (a, b, c and d). a = 3-mercapto-3-methyl-1-butanol; b = 3-mercapto-3-methylbutyl formate; c = 3-methyl-3-methylthio-1-butanol; and d = 3-methyl-3-(2-methyldisulfanyl)-1-butanol. Felinine and its derivatives are excreted in the urine

enzyme in the proximal renal tubules) to form 3-methylbutanol-cysteinylglycine, *S*-(iso-propylcarboxymethyl-cysteinylglycine, and *S*-(iso-ethylcarboxymethyl-cysteinylglycine, respectively. These cysteinylglycine derivatives are hydrolyzed by dipeptidases (e.g., aminopeptidase M) in the cytosol of the proximal renal tubules to generate felinine, isovalthine, and isobuteine, respectively, with glycine as a co-product.

Some of the resultant sulfur-containing metabolites are locally *N*-acetylated by *N*-acetyltransferase to their corresponding acetyl derivatives (i.e., *N*-acetyl-felinine, *N*-acetyl-isovathine, and *N*-acetyl-isobuteine, respectively). Additionally, 3-methylbutanol-cysteinylglycine, *S*-(iso-propylcarboxymethyl-cysteinylglycine, and*S*-(iso-ethylcarboxymethyl-cysteinylglycine are hydrolyzed by the extracellular cauxin (a carboxylesterase secreted by the proximal straight renal tubules of the kidneys) in the lumen of the renal tubules and the bladder to yield felinine, isovalthine, and isobuteine, respectively, with glycine as a co-product. In the cytosol of the proximal straight renal tubules, feline is further metabolized into 3-mercapto-3-methyl-1-butanol, 3-mercapto-3-methylbutyl formate, 3-methyl-3-methylthio-1-butanol, and 3-methyl-3-(2-methyldisulfanyl)-1-butanol (Miyazaki et al. 2008). Similar modifications of isovalthine, and isobuteine may also occur. Felinine,

isovalthine, and isobuteine, as well as their derivatives are excreted in the urine.

The syntheses of felinine, isovalthine, and isobuteine are influenced by dietary intakes of methionine and cysteine (Hendriks et al. 2008; Rutherfurd-Markwick et al. 2005), and possibly dietary lipids (in the case of felinine), leucine (in the case of isovalthine), and valine (in the case of isobuteine) when the dietary provision of methionine, cysteine, glycine, and BCAAs is not limiting. Interestingly, the production of felinine by felids is gender-specific as its excretion in the urine is much higher in males than in females (Rutherfurd-Markwick et al. 2005), but the urinary excretion of isovalthine by adult cats is not gender-specific (Hendriks et al. 2004).

The biological significance of felinine, isovalthine, and isobuteine, as well as their derivatives remains largely elusive. It is possible that these sulfur-containing AA and their metabolites serve as non-toxic, non-reactive, and relatively stable end products of Met and Cys to prevent excessive formation of toxic and highly acidic substances (e.g., H_2S, SO_2, and H_2SO_4) from Met and Cys. Of particular note, Miyazaki et al. (2008) have suggested that felinine is a territorial marker for intra-species communications and is also a putative precursor of a pheromone that serves as a chemical signal to attract females. This explains, in part, an important role of dietary AAs in the physiology and behavior of zoo animals of either the same or different species.

Either inadequate nutrition (especially deficiencies in certain AAs) or excessive AAs lead to nutrition-related diseases and disorders (Oberbauer and Larsen 2020; Wu 2020a). Urolithiasis (the process of forming stones in the kidenys, bladder and/or urethra) occurs when mineral crystals precipitate from the urine and form uroliths in the urinary tract (Kleiman et al. 2010). There are different types of uroliths that may form from different nutrients and minerals in the diet. In canids, a high-protein diet may cause ammonium urate stones or cystine uroliths (Kleiman et al. 2010). Cystine has a poor solubility at physiological pH and in acidic urine, and may lead to cystine uroliths in dogs that have a defect in reabsorption of cystine and other basic AAs in the kidneys (Kleiman et al. 2010). As previously stated, felids are strict carnivores and require taurine in the diet, but canids, bears, and giant anteaters also have a dietary requirement for taurine (Kleiman et al. 2010). Dilated cardiomyopathy, bilaterally symmetrical hyper-reflective retinal lesions, poor reproduction, and progressive exercise intolerance and dyspnea have all been associated with a taurine deficiency in those animals (NRC 2006). These nutrition-related diseases highlight the importance of balanced diets with adequate AA composition in addition to the optimal overall protein content.

The giant anteater (*Mymercophaga tridactyla*) is an insectivore, a specific type of carnivore, which also commonly experiences side effects of taurine deficiency, such as dilated cardiomyopathy, in captivity (Nofs et al. 2018). Exact nutrient requirements for the giant anteater have not been established, but analyses of some diets revealed taurine levels between 0.11 and 0.18 g/kg DM (Nofs et al. 2018). As a comparison, the recommended taurine level for dry food for cats, another carnivore, is 1.0 g/kg DM (AAFCO 2012). Assuming that taurine homeostasis in giant anteaters is regulated by urinary excretion of taurine and that urinary taurine concentration varies directly with body taurine status, Nofs et al. (2018) analyzed urinary taurine concentrations in response to taurine and methionine supplementation to a commercially available insectivore diet. It was found that urinary taurine excretion increased with increasing dietary taurine intake and also increased with methionine supplementation, indicating that giant anteaters can synthesize adequate amounts of taurine from methionine (Nofs et al. 2018). Figure 12.3 illustrates how methionine is metabolized to homocysteine, which is further converted to cysteine by cystathionine γ-lyase; cysteine is then converted into taurine (Fig. 12.3). These findings suggest that giant anteaters can synthesize adequate

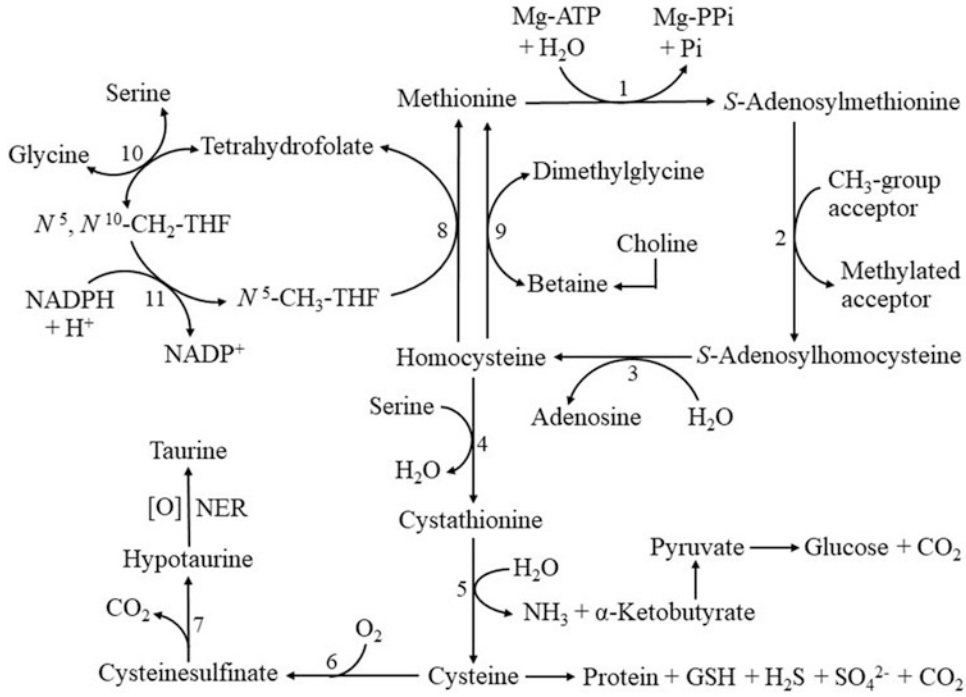

Fig. 12.3 Synthesis of taurine from sulfur-containing amino acids (methionine and cysteine) in animals. The enzymes catalyzing the indicated reactions are: (1) *S*-adenosylmethionine synthase; (2) methylase; (3) *S*-adenosylhomocysteinase; (4) cystathionine β-synthase; (5) cystathionine γ-lyase; (6) cysteine dioxygenase; (7) cysteinesulfinate decarboxylase; (8) methionine synthase; (9) betaine:homocysteine methyltransferase; (10) serine hydroxymethyltransferase; (11) N^5-N^{10}-methylene- tetrahydrofolate reductase; GSH = glutathione; α-KB = α-ketobutyrate; NER = nonenzyme catalyzed reaction; N^5, N^{10}-CH_2-THF = N^5, N^{10}-methylene tetrahydrofolate; N^5-CH_3-THF = N^5-methyl-tetrahydrofolate

amounts of taurine as long as the diet contains sufficient amounts of methionine or cysteine (Nofs et al. 2018).

For carnivorous species in zoos, whole prey items are commonly used as a dietary source, as well as enrichment to mimic species-typical behavior (Kerr et al. 2014). However, these whole prey diets tend to exceed the protein requirements established by the NRC (2006) for dogs and cats or livestock species and do not focus on specific AA requirements. Dierenfeld et al. (2011) found that all the domestic meats tested were limiting in arginine, leucine, methionine + cystine, and phenylalanine + tyrosine compared to the requirements for obligate carnivores. However, this is not true for beef (Wu et al. 2016) and some animal-source feedstuffs (Li and Wu 2020). Generally, lysine is considered as the first limiting AA when calculating ideal protein ratios, but these ratios are species-specific (Dierenfeld et al. 2011). The cecectomized rooster assay was determined to be an appropriate model for evaluating AA digestibility of animal products that may be fed as whole prey to captive exotic felids to validate that these food sources are meeting the nutritional requirements (Kerr et al. 2014). Compared to The Association of American Feed Control Officials (AAFCO 2012) recommendations for domestic cats, ground duck had a slightly lower combined concentration of methionine + cysteine than that recommended for growth, reproduction, and adult maintenance (Kerr et al. 2014). Because some methionine and cysteine in feedstuffs are oxidized under acid hydrolysis conditions at 110 °C, caution should be taken to ensure that the content of these two sulfur-containing AAs in protein is analyzed properly (Dai et al. 2014). For ground duck,

150 to 180 day-old mice, 30- to 45-day -old rabbits, and rabbits more than 65 days of age, concentrations of taurine in their blood were lower than values recommended by AAFCO (2012) and Kerr et al. (2014).

Protein quality and concentration can also have an effect on the microbiota in the gut of carnivores (Madsen et al. 2017). Even though carnivores do not rely heavily on microbial fermentation in the gut for energy, the microbiota population has an effect on gastrointestinal and whole body functions, such as digestion, inflammation, and pathogen resistance (Lubbs et al. 2009; Wasimuddin et al. 2017). Captive animal populations tend to have less diversity in their microbome compared to their free-ranging counterparts due to differences in their diet (Wasimuddin et al. 2017). According to Wasimuddin et al. (2017), captive cheetahs have a higher prevalence of potential pathogenic bacteria than free-ranging cheetahs when analyzing fecal samples with 16S rRNA gene high-throughput sequencing. Lower quality protein may not be adequately digested in the small intestine, which allows more AAs in dietary protein to enter the large intestine and increases the activity of its proteolytic bacteria (Amstberg et al. 1980; Lubbs et al. 2009). Also, more protein in the lower bowel may result in increased production of ammonia, sulfur-containing compounds, indoles, and phenols, all of which become toxic at high concentrations in the body (Lubbs et al. 2009). In domestic cats fed a high-protein diet, there was a shift from carbohydrate-fermenting bacteria to proteolytic bacteria, which may be pathogenic (i.e. *Clostridium*) (Lubbs et al. 2009). Similarly, Cheetahs in captivity experience a high prevalence of *Heliobacter* infections, leading to chronic gastritis (Wasimuddin et al. 2017), and both lions and cheetahs are known to suffer from *Clostridium sordelli* and *Clostridium perfringens* (de la Fe et al. 2006). Captive marine carnivores, such as the Australian sea lion, also experience changes in the gut microbiota, compared to wild sea lions due to less diverse protein sources (Delport et al. 2016). Reducing total protein content and balancing all proteinogenic AAs in the current commercial diets may be beneficial for improving intestinal health in carnivores.

12.3 Herbivores

Herbivores eat predominantly plant matter and have symbiotic microorganisms in the gut that help to digest plant matter by anaerobic fermentation to supply the animal with energy (Wu 2018). Herbivores can be divided into two different subgroups: pregastric fermenters and postgastric fermenters. Ruminants are pregastric fermenters and have a compartmentalized stomach containing a rumen where microbial fermentation occurs (i.e. cattle, sheep, deer, giraffe, kangaroos, and antelope; Kleiman et al. 2010). By definition, ruminants regurgitate their food to remasticate, resalivate and reswallow for further digestion (Kleimen et al. 2010). In contrast to carnivores, ruminants do not have a high dietary requirement for AAs and vitamins because the microorganisms of the rumen have the ability to synthesize protein from non-protein and non-AA nitrogen such as urea and ammonia (Kleiman et al. 2010; Wu 2013). Nonruminant pregastric fermenters also have a compartmentalized stomach for microbial fermentation, but do not regurgitate their food for further digestion (i.e. hippopotamuses, kangaroos, and langur primates; Kleiman et al. 2010). Postgastric herbivores have a large cecum and colon where microbial fermentation occurs (i.e. horses, capybaras, rabbits, rhinoceroses, elephants, and apes; Kleiman et al. 2010). The growth, development, health, and survival of herbivores (including ruminants) depend on the unique characteristics of their digestive systems (Wu 2005). All herbivores are able to synthesize taurine from cysteine in their liver, but the rates of the synthesis of taurine vary among animal species (Hou et al. 2020; Jacobsen and Smith 1968; Sturman and Hayes 1980; Wright et al. 1986).

Nutrient requirements of zoo animals are based on similar domestic species with established nutrient requirements; however, these are not always accurate comparisons. Serum concentrations of AAs in free-ranging giraffes from two game

reserves in South Africa were compared with serum concentrations of AAs in steers and sheep which showed apparent differences in concentrations of cystine, isoleucine, and valine (Schmidt et al. 2007). The concentrations of free cystine in free-ranging giraffes from Double Drift Game Reserve and Kariega Game Reserve were 0.19 mg/dL (7.9 μM) and 0.35 mg/dL (14.6 μM), respectively, compared to 4.52 mg/dL (188 μM) in sheep (Schmidt et al. 2007). The concentrations of free cystine in the serum of zoo giraffes (United States) fed an alfalfa-based diet and free-ranging giraffes (South Africa) were 0.00 mg/dL (0.0 μM) and 0.22 mg/dL (9.2 μM), respectively (Schmidt et al. 2009). The concentration of free cysteine in the serum of captive sheep is similar to the concentration of total free cysteine (cysteine + ½ cysteine; 188 μM) in the plasma of adult sheep fed an alfalfa-based diet (Kwon et al. 2003). However, the reported concentrations of cystine (the major oxidized dimer form of cysteine in animals; 0.0 to 15 μM) in the serum of adult giraffes are too low to be compatible with life and may not represent its true values, but rather might be due to problems with its analysis because the determination of this AA is a technical challenge (Wu 2013). This underscores the importance of accurate analyses of AAs in studying the protein and AA nutrition of animals.

Similar to carnivores, herbivores can also experience urolithiasis, the precipitation and formation of mineral crystals from the urine in the urinary tract (Kleiman et al. 2010). Sheep may have a high cysteine requirement for wool production, but Schmidt et al. (2007) has shown that concentrations of cysteine in the serum of giraffes are significantly reduced when compared to those for sheep, suggesting that the use of the data on dietary nutrient requirements of sheep to establish dietary nutrient requirements for giraffes may not be fully justified. Further studies are warranted to validate these intriguing findings before recommendations for changes in the diets of zoo giraffes are recommended. In blood, most (97%) cysteine is spontaneously oxidized to cystine (Wu et al. 1997). Among all physiological AAs, cysteine has the lowest solubility (0.46 mM) in water at 25 °C and neutral pH (Wu 2013). Thus, high concentrations of cystine in the diet could contribute to the prevalence of urolithiasis in captive giraffes.

Isoleucine concentrations in the serum of giraffes were 2.07 and 2.01 mg/dL from the two game reserves compared to 0.79 and 0.87 mg/dL in steers and sheep, respectively (Schmidt et al. 2007). Concentrations of valine in serum of giraffes from the two game reserves were 4.64 and 4.60 mg/dL, compared to 1.53 and 2.00 mg/dL in steers and sheep, respectively (Schmidt et al. 2007). Isoleucine and valine are branched-chain amino acids (BCAAs) along with leucine, and all the three BCAAs must be balanced to gain advantage of their physiological functions (Wu 2009). For example, BCAAs are important for protein synthesis by activating the mechanistic target of rapamycin cell-signaling pathway (Wu 2009; Zhang et al. 2019). Skeletal muscle can synthesize glutamine and alanine from BCAAs and glucose (the primary precursor of α-ketoglutartae and pyruvate). Glutamine has a variety of metabolic functions including gluconeogenesis, cell proliferation, synthesis of NAD(P), regulation of protein turnover, and synthesis of purine, pyrimidine, ornithine, citrulline, arginine, proline, and asparagine (Wu 2009). A balance of dietary BCAAs is crucial for optimal health of all animals to prevent antagonisms among the AAs. However, dietary requirements of giraffes for the BCAAs and other AAs should not be based solely on their concentrations in the serum or plasma of giraffes or other ruminant species (such as cattle and sheep), because the circulating levels of AAs are influenced by many factors (e.g., physiological, pathological, and environmental) other than diets and because there are significant differences in concentrations of AAs in serum among animal species (Wu 2018).

Caution should be exercised when feeding zoo animals a diet similar to that for their domesticated counterpart so as to prevent disruption of the gut microbiome. As previously stated, the microbial population of the gut in herbivores is essential for the digestion of fiber and production of short chain fatty acids and AAs. Gibson et al. (2019) found a significant difference in diversity of the gut microbiome in captive black rhinoceroses, compared to wild black rhinoceroses. The captive rhinos showed an increase in glycolysis and AA syntheses in the

microbial populations, suggesting an imbalance of nutrients in their diets (Gibson et al. 2019).

12.4 Omnivores

Omnivores are animals that consume both plant and animal matter (i.e. pigs, bears, foxes, raccoons, many primates, giant pandas, maned wolves, and some canids). Because the composition of AAs differ between plant- and animal-source feedstuffs (Hou et al. 2019; Li and Wu 2020), dietary intakes of many AAs (particularly methionine, cysteine, glycine, proline, and tryptophan) by these animals critically depend on their food sources. The digestive physiology of omnivores allows the consumption and digestion of meat and plant material, but the intestines of these animals except for certain species (e.g., grizzly bears, black bears, and giant pandas) have a limited capacity for the microbial fermentation of plant fibrous material in the gastrointestinal tract (Kleiman et al. 2010; Pritchard and Robbins 1990). Unlike carnivores, omnivores do not have a strict requirement for meat but rather base their diets on seasonally available feedstuffs in their habitat (Kleiman et al. 2010). Most omnivores are able to synthesize taurine from cysteine in their livers, with the rates of synthesis depending on species (Jacobsen and Smith 1968; Sturman and Hayes 1980; Wright et al. 1986). It is important to consider the ratio of plant- and animal-source feedstuffs in the natural diet of omnivores because over- or under-feeding of macro and micronutrients may result in nutrition-related diseases. Maned wolves tend to eat a higher proportion of plant material than other species of wolves that consume primarily meat (Kleiman et al. 2010). In U.S. zoos, maned wolves are fed diets primarily consisting of red meat, which has high concentrations of sulfur-containing AAs, leading to a decrease in urinary pH and the formation of cystine uroliths that can also occur in some herbivores and carnivores (Phipps and Edwards 2009; Kahn and Line 2005). However, protein-restrictive diets result in taurine deficiency and fecal inconsistency in maned wolves (Sanderson et al. 2001), suggesting that this animal species may have little or no ability to synthesize taurine. Canids that develop cystinuria also have an increased chance of developing a carnitine deficiency (Sanderson et al. 2001). Like cystine, carnitine is reabsorbed by the renal glomerulus into the blood circulation via a sodium-dependent transport system (Wu 2018). Carnitine is derived from methionine and lysine, and required for the transport of long chain fatty acids from the cytosol into mitochondria for oxidation and ATP production (Wu 2018). Because of these issues, much research is needed to formulate a specific diet for captive maned wolves for optimal health.

Bears are considered omnivores and have the digestive physiology of carnivores (e.g., having a single stomach and a short intestine), whereas giant pandas (also known as the panda bear) with the digestive system of carnivores live as herbivores consuming almost exclusively bamboo. Giant pandas do not rely primarily on microbial fermentation of plant fibrous material to meet their nutrient requirements, but are able to survive by eating a large amount of bamboo (e.g., up to 6% of body weight in DM per day by a 120-kg adult) despite their inefficient digestive system for utilizing plant fibrous material (Dierenfeld et al. 1982; Schaller et al. 1985). Bamboo contains 8.6% CP, 74.6% cell wall material (including 29.7% hemi-cellulose, 26.5% cellulose, and 7.3% lignin), and 4.8 kcal/g gross energy (all on the DM basis; Dierenfeld et al. 1982). For comparison, an adult steer (540 kg) consumes DM at 2.6% of body weight per day (Gilbreath et al. 2020). Interestingly, the passage of digesta through the gastrointestinal tract of the giant panda is very rapid (< 12 h), and the digestibility coefficients of bamboo DM (largely crude fiber), hemicellulose, and cellulose in adult giant pandas are 20%, 27%, and 8%, respectively (Dierenfeld et al. 1982). Cellular contents (AAs, protein, sugars, and starch) are the main sources of nutrients for giant pandas.

The gut microbiome of giant pandas closely resembles the gut microbiome of a carnivore with a high abundance of genes encoding for enzymes for AA degradation and a low abundance of genes for enzymes related to cellulose- and

hemicellulose-digestion (Guo et al. 2018; Xue et al. 2015). Specifically, despite its ability to metabolize dietary cellulose (Zhu et al. 2011), the gut microbiota of giant pandas is abundant in *Escherichia, Shigella* and *Streptococcus* bacteria that are normally found in carnivores for protein digestion (Xue et al. 2015) and in genes that are associated with the degradation of glutamine and glutamate (glutaminase, glutamate decarboxylase, GABA-transaminase, and succinic semialdehyde dehydrogenase; Fig. 12.4), similar to carnivores and other bears (Guo et al. 2018). Thus, we surmise that there is active nitrogen metabolism and recycling in the intestine of giant pandas for AA utilization, as reported for such omnivores as humans, pigs, rats and ruminants (Bergen and Wu 2009). This, however, may not be able to fully compensate for the low AA content of bamboo and its low digestibility (Dierenfeld et al. 1982), such that giant pandas may not have adequate protein nutrition for optimum growth, gestation and lactation. In support of the suggestion, the female giant panda ovulates only once a year in the Spring season, and implantation of her fertilized egg is delayed for 2 to 3 months until the leaves and shoots of bamboo become more abundant and contain more nutrients (e.g., AAs and calcium) to support embryonic growth and development (Schaller et al. 1985; Zhang et al. 2018). Despite the reproductive and foraging strategies of gestating giant pandas, as well as a gestation length of 96 to 158 days between insemination and parturition (Zhang et al. 2009), the average birth weight of their offspring (almost 50% singletons and 50% twins) is only 90–130 g (Schaller et al. 1985). For comparison, in domestic pigs, which usually gestate 10 to 14 live fetuses, average fetal weights on days 60, 90, and 114 (term) of gestation are 130, 596, and 1486 g, respectively (Wu et al. 2013). Improving the supply of AAs (particularly arginine and glutamine) may enhance fetal survival and growth in giant pandas, as reported for swine (Wu et al. 2010, 2011).

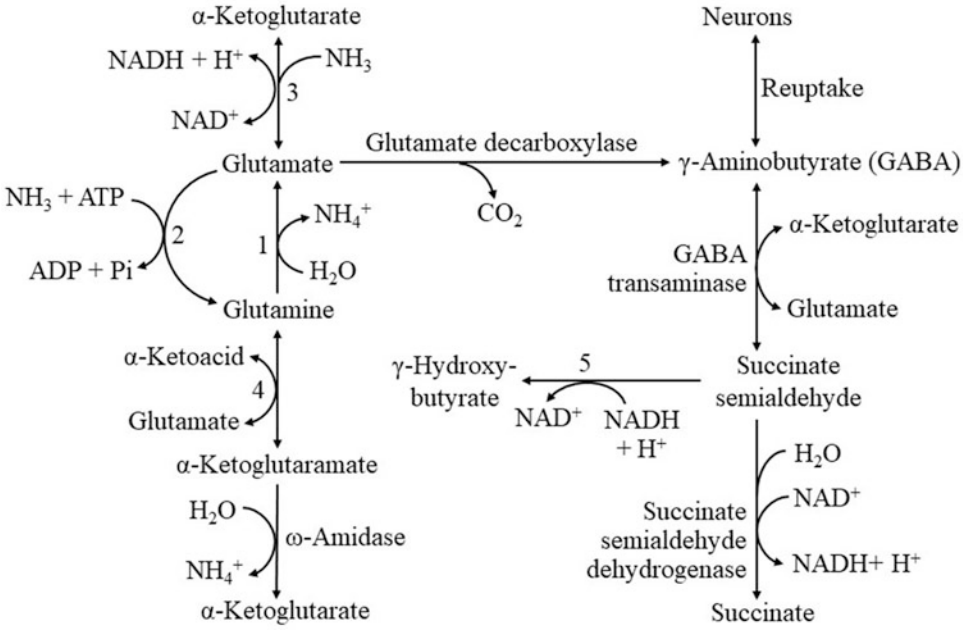

Fig. 12.4 Catabolism of glutamine and glutamate in zoo animals. The enzymes in these specific pathways of glutamine and glutamate metabolism are up-regulated in pandas, as well as other bears and carnivores. In comparison, herbivores have greater expression of enzymes associated with the synthesis of glutamine and glutamate (i.e. glutamine synthetase, glutamate synthase, and glutamate dehydrogenase). The enzymes catalyzing the reactions are: (1) phosphate activated glutaminase; (2) glutamine synthetase; (3) glutamate dehydrogenase; (4) glutamine transaminase; and (5) succinate semialdehyde reductase

The nutrient requirements for most subhuman primates are based on dietary nutrient requirements of humans. The CP of normal diets of captive apes, lemurs and marmosets provides between 9.5% and 13% of the energy intake, compared to the normal 10% to 12% in humans (King 1978). However, nutrient requirements of primates are only based on a few species of primates and specific needs may vary among species; and protein requirements seem to be different for New World primates, compared to Old World primates (Crissey and Pribyl 2000). The protein requirement of New World primates may be closer to 25% of the diet, but the NRC (1978) has established the minimum protein requirement of primates to be 16% for all stages of life (Crissey and Pribyl 2000). Flurer and Zucker (1988) observed coprophagy in marmosets fed a diet lacking in histidine and arginine, but did not observe coprophagy in marmosets fed a diet of the same protein content that contained both histidine and arginine. Histidine is an essential AA for one-carbon unit metabolism, protein biosynthesis, formation of major dipeptides in skeletal muscle and the brain such as carnosine, and conversion to histamine by decarboxylation (Wu 2013). Histidine can cross the blood-brain barrier like most AAs. Paradoxically, elevated levels of histidine and homocarnosine have been detected in the brains of rats, guinea pigs and infant monkeys that experience protein malnutrition (Taylor and Snyder 1972; Enwonwu and Worthington 1973) likely due to enhanced intramuscular protein and peptide hydrolysis. In protein-deficient monkeys, elevated levels of histidine in the brain were accompanied by decreased levels of arginine, threonine, isoleucine, leucine, and valine in their plasma, which compete with histidine to cross the blood-brain barrier (Enwonwu and Okolie 1983). Along with histidine, histamine levels in the brain were also increased in protein-deficient monkeys (Enwonwu and Okolie 1983). Histamine in the brain acts as a regulator of central acetylcholine secretion (He and Wu 2020). Protein deficiency and a specific AA deficiency may lead to impaired thermoregulation, elevated plasma levels of cortisol, reduced plasma levels of growth hormone, edema, and psychomotor dysregulation in primates (Enwonwu and Okolie 1983).

12.5 Dietary Requirements of Captive Carnivores, Herbivores and Omnivores for AAs

Animals have dietary requirements for AAs, but not protein (Wu 2016). Traditional methods to formulate diets for mammals (Bergen 2020; Oberbauer and Larsen 2020; Wu et al. 2014; Zhang et al. 2020), birds (He et al. 2020), crustaceans (Li et al. 2020b), and fish (Li et al. 2020c) have been based on the dietary CP content, which includes AAs, as well as non-protein and non-AA nitrogen. Data on dietary CP content may provide some clues into the requirements of zoo animals for dietary protein and AAs. For example, in summarizing the consensus agreement of the Giraffe Nutrition Workshop in 2005, Schmidt and Schlegel (2005) thoughtfully stated that "given the nutrient requirements of domestic ruminants and diet studies of wild giraffe, there is no nutritional reason to expect that the total dietary CP requirement of a mature giraffe is more than 12% of the complete diet (DM basis) when DM intake is at least 1.2% of the animal's body weight. Diets containing 10 to 14% CP (DM basis) will likely provide the maintenance needs of adult giraffe." The maintenance needs should include those for: (a) AAs that are irreversibly lost through catabolism, as well as excretion via the skin, urine and feces; and (b) AAs that are required for regulating immune and antioxidative responses, as well as the integrity of tissues such as the gastrointestinal tract, liver, eyes, heart, brain, and the skin. Because some non-protein and non-AA nitrogen (e.g., added melamine) may have no nutritive value and even be toxic to animals, and because AAs in feedstuffs can now be analyzed readily by advanced methods, such as high-performance liquid chromatography (Dai et al. 2014), dietary

AAs, instead of CP, should be recommended for zoo animals for their optimal growth, development, lactation, reproduction, and health. Because there are differences in digestion and metabolism of nutrients among carnivores, herbivores and omnivores, as noted previously, these animals likely have very different patterns of requirements for dietary AAs.

To date, little information is available regarding dietary requirements of zoo animals (including nonhuman primates) for AAs. Domestic animals (e.g., pigs, chickens, and sheep) may be used to assess the digestibility of AAs in proteins of commercially available avian and mammalian whole prey diet items targeted for consumption by zoo animals (e.g., Kerr et al. 2014). In addition, model animals can be used to estimate the nutrient requirements of captive animals with similar digestive physiology and metabolism (Edwards 2003). Furthermore, data from human studies (Wu 2016; Young and Borgonha 2000) can be based to recommend the requirements of nonhuman primates for dietary AAs. Diets should be optimal for the growth, development, reproduction, survival and health of all animals. These common criteria should be used for defining dietary requirements of various species of zoo animals for AAs. However, it should be borne in mind that additional criteria for recommending nutrient requirements for domestic animals (e.g., growth performance, feed efficiency, and productivity) may be different from those for zoo animals (e.g., longevity and social behavior).

Based on work with swine and poultry, as well as companion animals (Baker and Czarnecki-Maulden 1991), the "ideal protein" has been considered to optimize the provision of EAAs for zoo animals, including carnivores (Dierenfeld et al. 2011). Because this nutritional concept does not take into consideration the AAs that are synthesized in animal cells, we must think "out of the box" to recommend that the diets of carnivores, like other animal species (Wu 2014), include all proteinogenic AAs. According to the review of AA composition in common raw meats from domestic (e.g., beef, chicken, horse, pork, and turkey) and "wild" (e.g., antelope, bison, boar, guinea fowl, and rabbit) animals, Dierenfeld et al. 2011 stated that arginine, leucine, methionine plus cysteine, and phenylalanine plus tyrosine are limiting in all meats examined, regardless of source, compared to requirements established for obligate carnivores. However, it remains uncertain whether or not the previously recommended dietary requirements of the animals for the reference AA "lysine" and other EAAs are accurate, because tissue-specific metabolism of all EAAs can be affected by the dietary intakes of so-called "nutritionally nonessential AAs" that are not included in the "ideal protein" (Wu 2013). It is unlikely that animal meats would not meet the requirements of carnivores for dietary AAs. In the wild, a carnivore eats whole prey animals (including such internal organs as the liver, kidneys and heart). Thus, it is more appropriate to estimate AA requirements of carnivores on the basis of the composition of AAs in the whole body rather than meat. This does not mean that zoo carnivores should be fed the whole carcasses of prey animals due to concerns over food safety. The composition of AAs in the bodies of various species of animals (mammals, birds and fish) is similar (Wu 2013, 2018). In contrast to the previously analyzed meats (Dierenfeld et al. (2011), the animal body and animal-source feedstuffs (e.g., chicken by-product meal and poultry by-product meal) provide more arginine and leucine than lysine (Li and Wu 2020; Wu 2013; Wu et al. 2016). Chicken viscera digest and spray-dried peptone from enzyme-treated porcine mucosal tissues supply more leucine than lysine (Li and Wu 2020). As shown in Table 12.1, all alternative animal protein products contain a large amount of taurine and proteinogenic AAs [particularly arginine, glutamate, glutamine, glycine, proline, 4-hydroxyproline, serine, sulfur-containing AAs (methionine, cysteine and taurine), and tryptophan] that are crucial for intestinal integrity and health, one-carbon metabolism, anti-oxidative reactions, and immune responses in all tissues of the animals (Hou et al. 2015; Liu et al. 2020; Wang et al. 2013, 2020; Wu et al. 2019; Zhang et al. 2019). In addition, meat provides creatine that is essential for muscular and neurological development (Wu 2010).

Table 12.1 Content of total amino acids (peptide-bound plus free amino acids) in the whole body of pigs and in animal-derived feedstuffs[a]

	Pig body		CBPM		PBM (PFG)		CVD		SDPM	
Amino acid (AA)	AA content (% of DM)	% of lysine (g/g)	AA content (% of DM)	% of lysine (g/g)	AA content (% of DM)	% of lysine (g/g)	AA content (% of DM)	% of lysine (g/g)	AA content (% of DM)	% of lysine (g/g)
Ala	3.00	109	4.63	100	4.11	112	4.42	82.2	3.95	86.3
Arg	3.09	112	4.85	105	4.28	117	4.31	80.1	4.05	88.3
Asn	1.64	59.7	2.66	57.6	2.65	72.3	2.68	49.8	1.71	37.3
Asp	195	71.0	4.01	87.0	3.99	109	3.93	72.9	4.19	91.4
Cys[b]	0.60	21.9	1.09	23.6	1.08	29.4	1.31	24.2	1.11	24.2
Gln	2.34	84.9	3.96	85.9	3.52	96.3	4.00	74.2	3.14	68.5
Glu	3.86	140	5.45	118	4.91	134	6.80	126	6.50	142
Gly	5.36	195	6.06	131	7.09	194	8.85	164	5.58	122
His	0.95	34.4	1.39	30.0	1.36	37.0	0.80	14.8	1.45	31.6
Hyp	1.73	62.9	1.89	41.0	2.32	63.4	1.86	34.5	0.89	19.4
Ile	1.61	58.6	2.77	60.2	2.46	67.1	4.12	76.4	2.75	60.1
Leu	3.12	113	5.38	117	4.47	122	6.55	122	4.85	106
Lys	2.75	100	4.61	100	3.66	100	5.39	100	4.58	100
Met	0.85	31.0	1.46	31.6	1.43	39.0	1.70	31.5	1.35	29.5
Phe	1.56	56.8	2.75	59.7	2.40	65.5	3.97	73.7	2.63	57.3
Pro	3.93	143	4.39	95.1	5.24	143	5.93	110	3.56	77.8
Ser	2.02	73.5	3.11	67.5	2.71	74.0	6.92	129	3.80	82.9
Thr	1.60	58.1	2.83	61.3	2.64	72.2	2.14	39.7	3.21	70.1
Trp	0.51	18.4	0.77	16.8	0.65	17.8	1.10	20.4	0.71	15.4
Tyr	1.24	45.0	2.33	50.6	1.94	53.1	2.75	51.0	2.52	55.0
Val	1.93	69.9	3.39	73.5	3.01	82.1	5.97	111	3.43	74.9
TPAA	45.7	–	69.8	–	65.9	–	85.5	–	66.0	–
Taurine	0.14	–	0.21	–	0.40	–	0.14	–	0.18	–

[a]Adapted from Wu et al. (2013) for the 30-day-old pig and from Li and Wu (2020) for the animal-derived feedstuffs. The molecular weights of intact amino acids were used for the calculation of AA content in the pig body and the feedstuffs
[b]Cysteine + ½ cystine

CBPM chicken by-product meal, *CVD* chicken visceral digest, *DM* dry matter, *Hyp* 4-hydroxyproline, *PBM (PFG)* poultry by-product meal (pet-food grade), *SDPM* spray-dried peptone from enzymes-treated porcine mucosal tissues, *TPAA* total proteinogenic amino acids

Based on the AA content of the pig body (Wu 2013) and diets for domestic animals [e.g., sheep (a herbivore ruminant; Satterfield et al. 2013), swine (an omnivore mammal; Wu et al. 2011), and chicken (an omnivore bird; He et al. 2020; Wu 2014), we recommend the requirements of captive carnivores (young and adult; Table 12.2), herbivores (young and mature; Table 12.2), and omnivores (young, adult and lactating mammals; as well as young and mature birds; Table 12.3) for dietary true protein and AAs as percentages of the total diet. Similarly, data on the requirements of crustaceans (Li et al. 2020b) and fish (Li et al. 2020c) for dietary AAs in aquaculture can serve as useful references to formulate diets for these classes of animals in the zoo. As reported by Hou et al. (2016), the ratios of AAs to lysine in animal diets differ from those in the animal body to various extents, depending on individual AAs. This is because dietary AAs are degraded by the small intestine at different rates during the first pass and AAs in plasma are utilized by the whole body at different rates (Wu 2013). The recommendations based on AA composition in the body provides an initial framework for feeding practices and further studies. As an animal becomes older, its rate of metabolism (including basal protein metabolism) per kg body weight decreases (Wu 2018). However, this also includes reductions in the conversion of

Table 12.2 Recommended requirements of zoo carnivores and herbivores for dietary amino acids[a]

	Carnivores		Herbivores (adult)		Herbivores (young)[b]	
Amino (AA)	AA content in diet (% of DM)	% of lysine in diet (g/100 g)	AA content in diet (% of DM)	% of lysine in diet (g/100 g)	AA content in diet (% of DM)	% of lysine in diet (g/100 g)
Ala	3.00	109	0.93	131	1.30	131
Arg	3.09	112	0.84	119	1.18	119
Asn	1.64	59.7	0.73	103	1.02	103
Asp	195	71.0	0.83	117	1.16	117
Cys	0.60	21.9	0.27	37.5	0.37	37.5
Gln	2.34	84.9	1.29	181	1.80	181
Glu	3.86	140	1.12	158	1.57	158
Gly	5.36	195	0.70	98.4	0.98	98.4
His	0.95	34.4	0.31	43.8	0.43	43.8
Hyp	1.73	62.9	–	–	–	–
Ile	1.61	58.6	0.60	84.4	0.84	84.4
Leu	3.12	113	1.19	167	1.66	167
Lys	2.75	100	0.71	100	0.99	100
Met	0.85	31.0	0.23	32.8	0.33	32.8
Phe	1.56	56.8	0.70	98.4	0.98	98.4
Pro	3.93	143	1.13	159	1.58	159
Ser	2.02	73.5	0.72	102	1.01	102
Thr	1.60	58.1	0.54	76.6	0.76	76.6
Trp	0.51	18.4	0.18	25.0	0.25	25.0
Tyr	1.24	45.0	0.53	75.0	0.75	75.0
Val	1.93	69.9	0.71	100	0.99	100
TPAA	45.7	–	14.3	–	20.0	–
Taurine	0.10	–	0.00	–	0.02	–

[a]Vaues are AA content in diet. The molecular weights of intact amino acids are used for the calculation of AA content in the diet. Intakes of dry matter by zoo animals range from 1% to 6% of their body weight, depending on species, age, and physiological state

[b]Before the normal weaning age. Note: within the first 1 month after weaning, the dietary content of all amino acids is reduced by 10%. A high intake of dietary protein in post-weaning mammals increases risks for intestinal dysfunction

DM dry matter; *Hyp* 4-hydroxyproline, *TPAA* total proteinogenic amino acids

phenylalanine into tyrosine and of methionine into cysteine in older animals than in younger animals. Consequently, much attention should be paid to adequate dietary intakes of both tyrosine and cysteine by ageing animals. Although adult animals gain little protein in the body or have a reduced requirement for dietary lysine, their small intestine still requires a relatively large amount of dietary threonine to produce mucins for intestinal protection. Likewise, adults also need dietary tryptophan for the production of bioactive metabolites (e.g., serotonin, melatonin, and indoles) to maintain neurological and intestinal functions. Thus, compared with young nonruminants, the dietary ratios of cysteine, tyrosine, threonine and tryptophan to lysine for adult ruminants may be greater (e.g., +10% for cysteine/lysine and tyrosine/lysine; +12% for threonine/lysine and tryptophan/lysine; Wu 2018). However, this may not be true for ruminants, because the ability of their rumen to synthesize cysteine, tyrosine, threonine and tryptophan in adults is greater than that in the young ruminant.

Intakes of DM by zoo animals range from 1% to 6% of their body weight, depending on species, age, and physiological state. For example, within the same given species, young animals have a greater metabolic rate and, therefore, consume more feed per kg body weight, compared with adults (Wu 2018). Likewise, at the same relative developmental stage, birds have a greater metabolic rate and, therefore, consume more feed per kg body weight, compared with ruminants

Table 12.3 Recommended requirements of mammalian and avian omnivores in zoos for dietary amino acids[a]

	Mammalian omnivores						Avian omnivores			
	Mammals (adults)		Mammals (young)[b]		Mammals (lactating)		Birds (adults)		Birds (young)	
Amino acid (AA)	AA content in diet[c] (% of DM)	% of lysine in diet (g/g)	AA content in diet[d] (% of DM)	% of lysine in diet (g/g)	AA content in diet[c] (% of DM)	% of lysine in diet (g/g)	AA content in diet[c] (% of DM)	% of lysine in diet (g/g)	AA content in diet[c] (% of DM)	% of lysine in diet (g/g)
Ala	0.81	97.4	1.38	95.6	1.05	104	0.90	102	1.36	102
Arg	0.83	100	1.44	99.8	1.73	171	0.95	109	1.41	105
Asn	0.57	68.5	0.97	67.1	0.83	82.5	0.49	56.2	0.75	56.1
Asp	0.81	97.4	1.38	95.6	1.19	118	0.58	66.3	0.89	66.2
Cys	0.25	30.4	0.39	26.8	0.33	32.5	0.32	36.4	0.43	32.1
Gln	1.26	152	2.17	151	1.74	173	1.13	129	1.72	128
Glu	1.41	170	2.42	168	2.29	226	1.57	179	2.38	178
Gly	0.90	108	1.53	107	0.95	93.8	1.56	177	2.35	175
His	0.33	39.6	0.56	38.6	0.49	48.8	0.31	35.2	0.47	35.1
Ile	0.54	65.4	0.94	65.4	0.83	82.5	0.61	69.3	0.92	68.7
Leu	1.10	132	1.90	132	1.78	176	0.96	110	1.46	109
Lys	0.83	100	1.44	100	1.01	100	0.88	100	1.34	100
Met	0.25	30.4	0.39	26.8	0.32	31.3	0.37	42.2	0.54	40.1
Phe	0.61	73.0	1.04	72.1	0.97	96.3	0.53	60.3	0.81	60.2
Pro	0.96	116	1.64	114	1.57	155	1.63	185	2.46	184
Ser	0.49	59.3	0.85	58.7	0.93	92.5	0.61	69.3	0.93	69.2
Thr	0.58	70.0	0.89	62.1	0.71	70.0	0.62	70.3	0.90	67.2
Trp	0.18	21.3	0.27	18.5	0.23	22.5	0.15	17.0	0.22	16.0
Tyr	0.47	56.3	0.81	56.2	0.78	77.5	0.40	45.2	0.60	45.1
Val	0.59	71.5	1.03	71.3	0.91	90.0	0.71	80.3	1.07	79.9
TPAA	13.8	–	23.4	–	20.6	–	15.3	–	23.0	–
Taurine	0.00	–	0.05	–	0.00	–	0.00	–	0.00	–

[a]Vaues are AA content in diet. The molecular weights of intact amino acids are used for the calculation of AA content in the diet. Intakes of dry matter by zoo animals range from 1% to 5% of their body weight, depending on species, age, and physiological state

[b]Before weaning. Note: Within the first month after weaning, the dietary content of all amino acids is reduced by 10%. A high intake of dietary protein in post-weaning mammals increases risks for intestinal dysfunction

[c]The true digestibility of amino acids in dietary protein and the content of dry matter in the diet are assumed to be 88% and 90%, respectively

[d]The true digestibility of amino acids in dietary protein and the content of dry matter in the diet are assumed to be 92% and 90%, respectively

DM dry matter; *TPAA* total proteinogenic amino acids

(Wu 2018). Because embryos and fetuses are particularly sensitive to ammonia concentrations in blood (Herring et al. 2018), high intakes of dietary protein are not recommended for females before breeding or during early gestation. Diets for dams during late gestation can be the same as those for early gestation. However, as the fetus grows rapidly during the last trimester of pregnancy, the amount of the diet fed to the dams can be increased appropriately (e.g., by 20 to 25% over that during early gestation). Based on studies with swine (Wu et al. 2017, 2018), dietary supplementation with arginine (e.g., 0.4% of the diet) shortly before the implantation of blastocysts can be beneficial for reducing the concentrations of ammonia in plasma, enhancing placental angiogenesis, and improving embryonic/fetal survival in zoo animals.

Our recommended values for dietary AA requirements for zoo carnivores, herbivores and omnivores may not be optimum for all AAs and all animal species, but they are expected to serve as

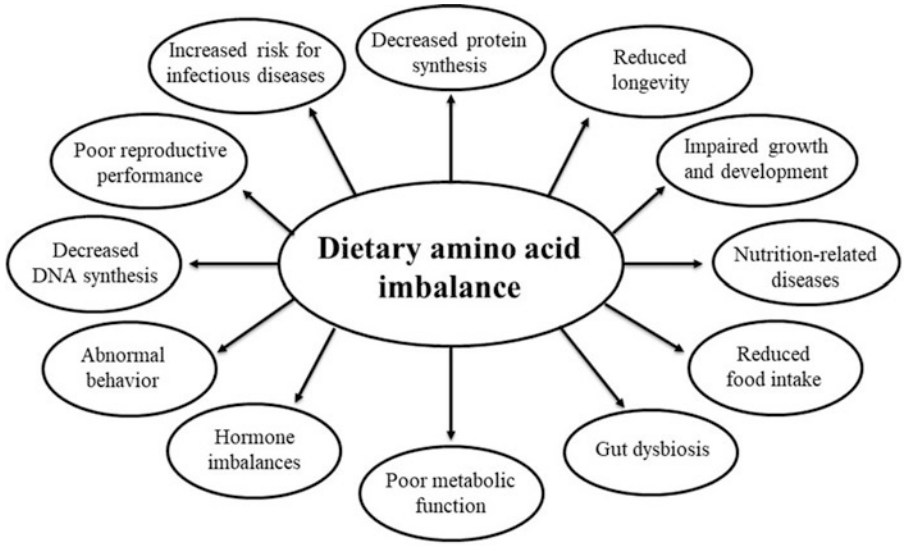

Fig. 12.5 Consequences of dietary amino acid (AA) imbalances in zoo animals. Amino acids are essential for the maintenance, growth, development, health, and survival of all animals, including those in the zoo. Inadequate AA nutrition or excessive intake of AAs can lead to nutrition-related diseases, impaired immunity, as well as the dysregulation of necessary physiological and metabolic functions in carnivores, herbivores, and omnivores. The diets of zoo animals should contain optimum balances and amounts of all proteinogenic amino acids. Taurine must be included in the diets of carnivores that do not synthesize this nonproteinogenic amino acid

helpful guidelines for feeding practices and future research, as noted previously. Because the metabolism of animals is affected by physiological, environmental and pathological factors, their optimum requirements for dietary AAs are not one set of fixed data, and may undergo dynamic changes with changing conditions. This calls for a range of the recommended requirement values, which need to be modified under practical feeding conditions. Therefore, the data in Tables 12.2 and 12.3 should be considered only as references and revised as new research findings become available.

12.6 Conclusion

In summary, domestic livestock species with established dietary nutrient requirements provide a baseline to use as a reference in formulating dietary requirements for exotic zoo animals since the processes used to determine dietary nutrient requirements are not practical for zoo animal species. However, it is important to take into account the major differences between domestic and wild species that could influence dietary nutrient requirements such as habitat, diet, behavior, and physiology. Malnutrition of protein and AAs can lead to many different nutrition-related diseases and disorders that may threaten the vitality and fecundity of zoo animal species (Fig. 12.5). Zoo animals will not thrive in captivity if their health is not optimal. Especially for endangered species, it is imperative that captive populations successfully thrive in order to conserve the Earth's biodiversity. Therefore, adequate provision of dietary AAs is crucial for successful management, sustainability and expansion of all zoo animals, including captive carnivores, herbivores and omnivores.

Acknowledgments We thank Dr. Cheryl Morris for helpful discussion on the nutrition of zoo animals. This work was supported by Texas A&M AgriLife Research (H-8200).

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1285, https://doi.org/10.1007/978-3-030-54462-1