EFFECTS OF FEED RESTRICTION ON MILK PRODUCTION AND METABOLISM IN MID-LACTATION DAIRY COWS

BY

IVAN ANSIA VAZQUEZ

THESIS

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Master's Committee:

Professor James K. Drackley, Chair
Professor Hans H. Stein
Assistant Professor Felipe C. Cardoso
Associate Professor Juan J. Loor

ABSTRACT

Availability of glucose precursors and a proper interorgan coordination during the metabolic cascade of adaptations occurring during periods of lower DMI, are the vital importance to achieve a successful transition from late gestation to lactation. The aim of the study was to determine metabolic responses to a short-term period of negative energy balance induced by feed restriction (FR) and the effect of abomasal supplementation of different amino acids (AA) or glucose. Seven multiparous Holstein cows (93 ± 15 DIM) were randomly assigned to 7 treatments in a 7 × 4 incomplete Latin square design. In 6 treatments, daily DMI was restricted to provide 60% of energy requirements during 5 d; the 7th treatment consisted of ad libitum (AL) intake. Feed was provided once daily at 0900 h. Effects of FR (AL vs RC), day, time within day, and interactions were evaluated with ANOVA using the MIXED procedure of SAS. Evaluating the effect of FR, milk yield ($P < 0.01$), milk protein concentration ($P = 0.03$) and yield ($P < 0.01$), and lactose yield ($P < 0.01$) were lower for RC, whereas milk fat ($P < 0.01$) and urea N concentrations were higher ($P < 0.01$). Treatment RC induced lower plasma insulin ($P = 0.01$) and glucose ($P = 0.04$) concentrations, with quadratic ($P < 0.01$ for both) decreasing trends reaching nadir on d 3. Concentration of NEFA was higher ($P < 0.01$) and increased quadratically ($P < 0.01$) with its maximum on d 3 during FR. Serum BHBA increased linearly ($P = 0.04$) for RC (RC x d; $P = 0.16$) with its peak at d 4. Catabolism of amino acids (AA) increased early during FR as indicated by plasma urea N increasing ($P < 0.01$) quadratically ($P < 0.01$), with its peak on d 2 and decreasing afterward. Accounting for all the amino-N circulating in form of urea or eliminated in milk as MUN, the decrease in concentration of all the AA in circulation analyzed here was not sufficient for the amount of urea synthetized. Therefore, it seems probable that body tissue protein was rapidly mobilized, to produce the energy required to support the higher ECM especially through milk fat and lactose. Plasma 3-methylhistidine increased linearly

($P < 0.01$) denoting protein tissue mobilization of contractile fibers. A group of AA (Glu, Val, Leu, Tyr, Phe, Ser, His, Thr, Asn, Ala, Pro, Met) decreased in a quadratic manner with the nadir at d 2 and 3, while Asp, Trp and Ile decreased linearly. Concentrations of other AA increased (Gln, Gly, Cys) or did not vary (Lys, Arg) during FR. Plasma AA concentrations decreased after feed delivery in both diets, coinciding with the increase of insulin, except for Glu that increased in all treatments and Gln that increased after feeding only during FR. Metabolic adaptations to low insulin during FR seemed to select catabolism of AA as the first energy source before later relying more on fatty acids. Based on responses of plasma AA and insulin to feeding, protein synthesis in tissues likely remained sensitive to insulin within day.

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To My Grandfather Manuel Vazquez Pereiro,

all this is for you and because of you.

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INTRODUCTION

Negative energy and protein balance characteristic of the transition period triggers a metabolic cascade of reactions that have the purpose of counteracting this imbalance. The hormonal reaction stimulated by the drop in dry matter intake across the transition period will induce mobilization of tissue. Adipose tissue will be mobilized and fatty acids oxidized by the liver in order to provide ketone bodies for peripheral uptake. However, excess of ketone bodies concentrations can lead to undesirables metabolic complications. In addition, tissue protein is degraded for amino acid (AA) supply for protein and glucose synthesis, or for ATP synthesis after complete oxidation. In fact, skeletal muscle and other peripheral tissue play an important role in the supply of AA and their recycling from the liver. Since most AA are received by the liver from arterial supply, AA that are not only used by the overloaded liver are important sources of energy in other organs. Even though AA are not large suppliers of glucose in most normal situations, they still are part of a considerable quantitative contribution to gluconeogenesis in critical situations such as the transition period. Cows show less severe reactions to a NEB during a more advanced phase of lactation than do early postpartum cows, however feed restriction models applied in mid-lactation cows have been validated as a model to mimic the metabolic adaptations to a lower DMI phase such as the one that characterizes the transition period. The objective of the work presented in this thesis is to understand the most demanded fates that AA have in a situation of nutrient deficiency. For that matter, the relation and synchrony of protein catabolism with other metabolic adaptations such as lipid mobilization, are the vital importance to evaluate the role that AA have on this adaptations. In addition, the identification of the AA with the largest decreases in concentration will point out also possible candidates for supplementation in situations of NEB such as the transition period.

CHAPTER 1 LITERATURE REVIEW

Introduction

The effects on milk production and metabolism during negative energy balance (NEB), such as occurs during the transition period, have been studied successfully over the last decades using mid-lactation cows in a feed-restriction condition (Akbar et al., 2013; Gross et al., 2011; Velez & Donkin, 2005). Feed-restriction models may not be able to simulate the natural decrease of dry matter intake (DMI) around calving, but they have been suitable to increase knowledge about the metabolic adaptations to nutrient deficiency at a molecular level (Bradford & Allen, 2005), without the collateral effects of the substantial hormonal regulation that occurs during the transition period (Akbar et al., 2013). One of the purposes of this experiment was to supply data for a better understanding of energy and protein metabolism by using this model.

Restricted DMI leads to an ordered cascade of metabolic changes in metabolism of long-chain fatty acids and glucose. Under this condition, the evidence indicates that there is an excess of lipogenic compounds and long-chain fatty acids, in detriment to amino acids (AA) and glucogenic compounds (Drackley, 1999). This excessive lipid mobilization from adipose tissue that occurs during feed restriction or around calving is strongly related to the increased probabilities of metabolic disorders and infectious diseases such as ketosis, displacement of abomasum, mastitis, and retained placenta among others (Cameron et al., 1998; Drackley et al., 2005; Goff & Horst, 1997). The enormous potential to jeopardize milk production, welfare, and, consequently, economic performance of cows, makes management of this imbalance among energy sources a major key to a successful transition.

Changes during Feed Restriction

Even though cows show less severe reactions to a NEB during a more advanced phase of lactation than do early postpartum cows (Carlson et al., 2006; Gross & Bruckmaier, 2015), feed restriction models applied in mid-lactation cows have been validated as a model to mimic the metabolic adaptations to a lower DMI phase such as the one that characterizes the transition period (Drackley et al., 2001; Grummer, 1993). Restricted feed models typically offer between 50 and 60% of calculated energy requirements (NRC, 2001). In the majority of studies, such restrictions caused an increase in fat mobilization measured as nonesterified fatty acids (NEFA; Ferraretto et al., 2014; Gross et al., 2013; Veenhuizen et al., 1991), a concomitant decrease in milk production from 30% to a 90 % among studies (with this big variation probably due to differences in DMI), decreased body weight (BW), and decreased body condition score (BCS) in comparison with full-fed control treatments (Ferraretto et al., 2014; Gross et al., 2011). However, other important indicators of NEB have shown a variety of responses. While Moyes et al. (2009) and Ferraretto et al. (2014) observed a significant decrease in glucose and insulin during feed restriction, other authors reported no effect on glucose (Gross et al., 2011; Gross & Bruckmaier, 2015; Laeger et al., 2012). When measured, other indicators of a high degree of fat mobilization such as β-hydroxybutyrate (BHB) and liver triglyceride (TG) content did not show a common response during short periods of restriction among studies (Gross et al., 2013). Researchers have observed a lag between the mobilization of adipose tissue TG and a substantial increase in plasma BHB (Doepel et al., 2002; Gross et al., 2011; Nielsen et al., 2003). Evidently, the infiltration of TG in the liver begins after increased concentration of NEFA in plasma (Vazquez-Añon et al., 1994). However, Kuhla et al. (2009) found an increase of 274% (DM basis) in total liver fat concentration after 60 h of almost total restriction (wheat straw was fed to provide 16%

of the energy in the control group) in comparison with the group fed for ad libitum DMI. Moyes et al. (2009) also found higher circulating concentrations of serum TG, cholesterol, and BHB during 5 d of feed restriction. Presumably, the length and severity of the restriction period, the demands of milk production, feeding frequency (restricted but daily), and blood sampling timing are causes for this wide variation of effects among studies with feed restriction.

Hepatic Metabolism of Lipids

The main organ responsible for coordinating such vital tasks is the liver, which is the central regulator of the metabolic adaptations to counteract the abrupt changes of the mixed fuels that power metabolism, and is also the first organ to receive and suffer its consequences (Drackley, 1999; Drackley et al., 2001; Grummer, 1993). It is well stablished that the liver increases its metabolic functions rapidly around calving (Drackley et al., 2001), due to an increased blood flow and an increased oxygen utilization rate (Reynolds, 2000).

Increased blood concentrations of NEFA derived from mobilization of adipose TG reserves causes an increased uptake of NEFA by the liver (Drackley, 1999) and a consequent lipid accumulation in the form of TG after the acute rise of plasma NEFA (Vazquez-Añon et al., 1994). When the pace of the esterification of fatty acids in the liver exceeds the oxidation of TG fatty acids and TG export in the form of very low density lipoproteins (VLDL), the syndrome of hepatic lipidosis, commonly called “fatty liver”, may occur (Drackley, 1999; Grummer, 1993). The bovine liver does not carry out a significant export of TG as VLDL per se; thus, the possibility to develop a fatty liver represents a risk for the lipid recycling metabolism during NEB and therefore for the wellbeing of the cow (Katoh, 2002).

Hepatic lipidosis can impair liver function due to different factors and in different ways. The increased accumulation of TG in the liver can indirectly restrain glucose synthesis due to increased concentrations of ammonia derived from a low rate of urea synthesis, thereby inhibiting the glucogenic capacity of hepatocytes (Overton et al., 1999). Decreased hormonal metabolism and endotoxin detoxification can result from TG infiltration in hepatocytes (Bobe et al., 2004). Fatty liver also has negative consequences on the production and physiological functions of the lipoproteins responsible to export lipids from the liver and return the residual lipid from peripheral tissues to the liver (Bobe et al., 2004; Katoh, 2002), and also may decrease energy precursors such as citrate and glycogen (Bobe et al., 2004; Doepel et al., 2002)

A different pathway for the metabolism of NEFA in the liver other than esterification or export is β-oxidation in the mitochondria and peroxisomes (Katoh, 2002). This route is not the primary one but is activated under feed restriction conditions or NEB (Dann & Drackley, 2005; Drackley, 1999). The acetyl-coenzyme A (Acetyl-CoA) derived from mitochondrial β-oxidation can be oxidized further if it is bonded with oxaloacetic acid to enter into the TCA cycle (Katoh, 2002). However, the intense activity of gluconeogenesis in the liver and the shortage of glucose precursors (Grummer, 1993) characteristic in a feed restriction or NEB condition results in a direct production of ketone bodies, mainly acetoacetate and BHB (Goff & Horst, 1997; Katoh, 2002; White, 2015). These ketone bodies in the blood are used as an energy source for organs such as heart, liver, and mammary gland (MG) (Gropper et al., 2009). However, among other effects BHB acts as a central anorexigenic signal inhibiting feed intake (Laeger et al., 2012). An excess concentration of ketone bodies therefore will have negative effects on cow health and production (Goff & Horst, 1997; Li et al., 2016; Piantoni et al., 2015; White, 2015).

Glucose Metabolism

The delicate and risky balance between NEFA mobilization and utilization makes it even more crucial to meet the basic requirements for glucose when supply of nutrients is insufficient when DMI does not support all requirements for pregnancy and subsequent lactation. Glucose is an essential nutrient that needs to be always maintained within a narrow concentration range in the blood, to be available for tissues like brain, kidney medulla, erythrocytes, and MG (Aschenbach et al., 2010). Overton (1998) estimated an increase in the metabolic glucose demand from 1000-1100 g/d to 2500 g/d from the fortnight before parturition until the fortnight after. Along the same line, Bell (1995) estimated that the requirements for glucose, AA, and fatty acids increase around 2.7, 2.0, and 4.5 times, respectively, in comparison with the requirements during the last phase of gestation.

Since ruminants ferment the majority of carbohydrates ingested from the feed into volatile fatty acids (VFA; also called short-chain fatty acids) in the rumen, the liver has the vital function to regulate the processes to achieve the required glucose concentration in the blood. The two main processes are glycogenolysis and gluconeogenesis. Glycogenolysis consists of the release of glucose that is stored in the liver as glycogen, however this is a limited resource and is depleted quickly in cows with high adipose TG mobilization (Veenhuizen et al., 1991). Therefore, the synthesis de novo of glucose (gluconeogenesis) is the more important mechanism to provide the necessary amounts of glucose to maintain optimal concentration in circulation (De Koster & Opsomer, 2013).

Of the total glucose available to the cow, <25% usually is provided from direct intestinal absorption from feed. The portal-drained viscera (PDV; consisting of gastrointestinal tract, pancreas, spleen, mesenteric and omental fat) plays an important role in accounting for this low

percentage. Through metabolism of glucose by its tissues, the PDV decreases the glucose available after intestinal absorption through the enterocytes by ~30% before reaching the blood (Larsen & Kristensen, 2009b). Therefore, the majority of glucose must be synthesized in the liver (Galindo et al., 2011). The principal precursors of glucose are, in order of quantitative contribution, propionate (60 to 74%), lactate (16 to 26%), alanine (3 to 5%), valerate and isobutyrate (5 to 6%), glycerol (0.5 to 3%), and other AA (8 to 11%) (De Koster & Opsomer, 2013; Galindo et al., 2015).

Depending on feed intake, propionate is the first and main precursor for gluconeogenesis. Propionate is one of the VFA produced by carbohydrate fermentation in the rumen and is absorbed through the reticulorumen wall into the portal blood. Propionate absorption and uptake by the liver will drive gluconeogenesis (Aschenbach et al., 2010; Larsen & Kristensen, 2009b). Even more, it has been demonstrated that the ability of the liver to convert propionate to glucose is modulated according to propionate supply (Armentano et al., 1991). Other studies have demonstrated that liver cells are able to increase the rate of conversion to glucose during situations of low supply of propionate, such as the transition period (Drackley et al., 2001). However, in conditions of low production of propionate and high fatty acid oxidation (e.g., feed restriction, pre- and post- partum), there will be a change in the glucose precursors available, resulting in a greater use of lactate, glycerol, and AA (especially alanine) (Erfle et al., 1971; Hammon et al., 2009; Larsen & Kristensen, 2013; Reynolds et al., 2003).

The adaptations toward alternative glucose precursors have been delineated recently at the gene expression level (Akbar et al., 2013; Loor et al., 2007; Velez & Donkin, 2005). Physiologically, ketogenesis can boost gluconeogenesis during the export of acetoacetate through the mitochondrial membrane. This transport is made by exchange for pyruvate, which is one of

the intermediates in the pathway of lactate and alanine (Ala) to enter the gluconeogenesis cycle (Zammit, 1990). The expected high infiltration and accumulation of fatty acids in the liver cells that occurs during adipose TG mobilization can increase blood ammonia concentrations due to the lower ureagenesis capacity in the liver (Zhu et al., 2000). In contrast, Overton (1998) found that the capacity to produce glucose from alanine did not decrease when blood ammonia is increased. The latter observation opens the possibility for a better energetic efficiency with substrates different than propionate during situations of lower feed intake.

Regulation of Glucose Metabolism

One of the primary hormones responsible for the regulation of gluconeogenesis and glucose use is insulin. Propionate is an insulin secretagogue, thus high concentrations will be expected in a normal feeding situation with high production of propionate (Doepel et al., 2002; Grummer, 1993). Insulin directs the gluconeogenesis pathways toward the use of propionate in detriment to other substrates. Insulin inhibits several key enzymes of gluconeogenesis from non-propionate precursors and decreases the availability of other glucose precursors, but does not inhibit propionate conversion to glucose (De Koster & Opsomer, 2013; Smith et al., 2008). The lack of inhibitory effect of insulin on gluconeogenesis from propionate led some to think about a possible evolutionary adaptation to avoid the harmful effects of accumulation of propionate (Aschenbach et al., 2010).

To coordinate usage of different fuels, liver in ruminants has the capacity to signal the specific substrate to be used for gluconeogenesis. In other species, lipogenesis and fatty acid oxidation in liver are regulated reciprocally by the lipogenic intermediate malonyl-CoA, which inhibits carnitine palmitoyltransferase (CPT-1), the enzyme responsible for controlling the uptake of long chain fatty acids into the mitochondria where β-oxidation occurs. Insulin increases

lipogenesis and so also malonyl-CoA, and thus inhibits concurrent fatty acid oxidation. Although ruminant liver has little lipogenic activity, concentrations of malonyl-CoA are regulated similarly by insulin (Brindle et al., 1985). According to Zammit (1990), methylmalonyl-CoA, an intermediate of propionate metabolism, also inhibits CPT-1 as does malonyl-CoA. Thus, with decreased propionate supply the β-oxidation of fatty acids would be increased. Because lactate and Ala do not produce methylmalonyl-CoA, ketogenesis is compatible and complimentary with gluconeogenesis from sources other than propionate (Drackley et al., 2001).

With the same premises, this "symbiotic" relationship between insulin and propionate maintain a stable fatty acid balance among organs. For instance, insulin has a direct antilipolytic effect on adipose tissue (Weber et al., 2016), avoiding the release of NEFA in normal feeding conditions; on the other hand, propionate plays a strong anti-ketogenic role by inhibiting β-oxidation (Armentano et al., 1991). The lower concentrations of insulin around calving (Reynolds et al., 2003) allow for an increased hepatic uptake of substrates other than propionate to produce glucose (Drackley et al., 2001). Therefore, these homeorhetic adaptations that occur during a period of NEB facilitate the supply of alternative glucose precursors when DMI is diminished for any reason.

However, not all organs have the same insulin-response mechanism. The MG and placenta do not require insulin stimulation for glucose uptake, unlike adipose tissues and muscle. The cow undergoes a temporary insulin-resistant phase during the transition period, when the demand for glucose increases dramatically (Bell, 1995). In this period, utilization of glucose by peripheral insulin-dependent tissues is decreased, which drives glucose towards the organs with high demand such as MG and placenta. The non-sensitive condition of the MG, therefore, is coupled with a high priority for glucose utilization (Aschenbach et al., 2010). The result allows

the cow to support the growing fetus and the onset of lactation based on the endogenous production of glucose (Bell, 1995; De Koster & Opsomer, 2013).

Inter-Organ Metabolism of Amino Acids

Amino acids uptake by the liver can end up in two completely different sets of transformations: anabolic processes as part of export (albumin, hormones, and immune proteins) or constitutive proteins; or catabolic processes like gluconeogenesis and complete oxidation (Larsen et al., 2015). The storage of AA in animals is limited; once the requirements for synthesis of protein and other nitrogenous compounds are completed, excess AA will be deaminated. The N will end up incorporated in urea that is either excreted or recycled into the rumen (Doepel et al., 2009), and the AA carbons skeletons will be available to provide energy as reviewed earlier (Bender, 2012). Therefore, there is potential supply of energy from AA due to their capacity to support gluconeogenesis as alternatives to propionate, or to their ability to be completed oxidized during catabolic processes.

Even though AA are not large suppliers of glucose in most normal situations, they still are part of a considerable quantitative contribution to gluconeogenesis in critical situations such as the transition period. Reilly and Ford (1971) showed in sheep that total glucose production was positively correlated with AA supply. Bergman (1978) defined that Ala and glutamine (Gln) together explain between 40 to 60% of the potential glucogenic supply of all AA in sheep. Overton (1998) found that the relative increase of capacity for bovine liver tissue to produce glucose at d 1 and 21 postpartum in comparison to d 21 prepartum was larger for Ala than for propionate (Drackley et al., 2001). In accordance, Reynolds et al. (2003) measured that, at d 11 postpartum, the contribution of propionate to glucose production reached its nadir whereas Ala was at its maximal contribution. The same conclusion was reached by Larsen and Kristensen

(2009[a]), who found that Ala and Gln, together with glycine (Gly), were the non-essential AA (NEAA) with the greatest liver extraction in the early postpartum period.

The two main organs receiving increased nutrient supply around parturition are the developing fetus before calving and the MG after calving, which results from the decreased glucose oxidation in skeletal muscle and decreased uptake by adipose tissue thanks to the aforementioned temporal insulin-resistant phase during the transition period (De Koster & Opsomer, 2013). Consequently, AA are an important part of these requirements, but not only as the basic units to synthesize protein. Bell (1995) pointed out that between 30 to 40% of the substrate oxidized by the fetus is attributed to AA, but only ~30% of the AA-N uptake is deposited as tissue protein. This implies that the requirements of the fetus in terms of metabolizable protein are significantly greater than the actual requirement for growth. The remarkably high requirement for protein in the late gestation by the fetus (220 g/d) contrasts with the almost irrelevant requirement for fat deposition (12 g/d) (Bell et al., 1992). In the same way, bovine placenta also has a considerable catabolism of AA during late lactation (Reynolds et al., 1986). Bell et al. (2000) estimated that the dry cow requires around 742 g/d of AA supply during late pregnancy. The significant catabolic fate of AA during the last phase of pregnancy is the result of the oncoming metabolic adaptations toward the new lactation. Steel and Leng (1973) observed an increasing endogenous production of glucose according to the stage of gestation and fetal number in feed-restricted sheep. More recently, Doepel (2002) found that the plasma concentration of 3-methylhistidine (3-MH), which is used as an indicator of muscle protein catabolism (Blum et al., 1985), was higher and increased earlier prepartum for cows fed a low crude protein (CP) diet in comparison with those fed a high CP diet.

The onset of lactation imposes an even greater gap between the MG requirements and the supply of nutrients through the feed. The high demand for glucose is required to produce lactose, which is the main osmotic solute in the milk and so determines directly the amount of milk produced (Bell, 1995; Rigout et al., 2002). Between 50 to 85% of the whole-body consumption of glucose occurs in the MG (De Koster & Opsomer, 2013). Nonetheless, accounting for all the dietary precursors of hepatic gluconeogenesis, there is still a shortage of the estimated glucose required to supply the lactose for milk production. Muscle protein serves as the main AA pool from which cows pull AA to boost gluconeogenesis or protein synthesis during the transition period to close that gap (Bell, 1995; Ji, 2013; Larsen & Kristensen, 2013; Van der Drift et al., 2012). The imbalance of required AA and supplied AA is even more substantial during the first and second week postpartum (Bauman & Elliot, 1983). Regardless of the amount of dietary CP during prepartum, Doepel (2002) determined that, according to the evaluation of the muscle mobilized, the greater demand for AA was in the first week postpartum. Reflecting the key role of AA mobilization from muscle, Brockman and Bergman (1975) determined that muscle protein supported the increasing rate of glucose production from Ala. Appuhamy et al. (2011) concluded that the increased milk protein production in early postpartum cows also was supported by muscle protein mobilization. Bell et al. (2000) estimated that in order to meet the requirements for glucose and AA for milk production during the 10 d postpartum, a high producing cow would need to mobilize around 1000 g of tissue protein per day. These results are in agreement with those of McNeill et al. (1997), who reported a greater loss of carcass protein in ewes fed a diet below their protein requirements during the last month of pregnancy.

Synthesis of lactose by MG cells is not only function of whole-body glucose availability, but also depends on the uptake and use by the MG itself. There is no strong positive relationship

between lactose yield and whole-body glucose rate of appearance (WB-Ra; represents sum of synthesis de novo, absorption of glucose, and glycogenolysis) in accordance with the extensive research about glucogenic precursors (Lemosquet et al., 2009). Galindo et al. (2015) found an increase of energy corrected milk (ECM) and lactose yield with a higher WB-Ra of glucose during casein infusion. Similar results were reported by Lemosquet et al. (2009), who found an increase of milk and lactose yields during abomasal casein infusions in comparison with ruminal infusion of propionate; however, the whole-body WB-Ra of glucose was increased with both treatments compared with controls. Since the mammary uptake of glucose was the same for both treatments, the calculated half-udder balance of carbons suggests that more anabolic pathways were activated with casein infusion to support increased milk components and yield. Given that the appearance of glucose is a function of direct absorption, synthesis, and release from reserves, these results (Lemosquet et al., 2009) must be interpreted carefully. Under different conditions, Doepel et al. (2009) reported that from dry period to lactation (and therefore higher portal absorption), the amount of lactose produced by the mammary gland could not be supported by the net release of glucose from the splanchnic tissues, and they suggested that other metabolic processes must have been activated.

Absorption of AA by MG cells depends on the amount of AA that cross the gland (blood AA concentration × blood flow) per unit of time, and the process by which AA are transported through the cell membrane of the mammary cells (Arriola Apelo et al., 2014; Mepham, 1982). Amino acids can end up being part of milk protein, retained as structural protein, metabolized to other components (e.g., CO_2, NEAA, urea) or simply pass unaltered to the milk (Mepham, 1982). In general terms, the classification of AA made by Mepham (1982) based on the difference between the individual AA uptake by the MG and the output in milk remains valid.

Group 1 (His, Met, Phe + Tyr, Thr, and Trp) consists of the AA whose ratio uptake/output is close to 1. Group 2 (BCAA plus Lys) includes AA with higher uptake than secretion. Finally, Group 3 (NEAA except Tyr) are the AA with higher output in the milk than the mammary uptake (Raggio et al., 2006).

Dietary supply of AA is one of the main tools to correct and improve the imbalance of glucose precursors during the transition period. Amino acid supply includes the microbial protein and the undegraded feed protein that passed from the rumen. A small part of the protein ingested reaches the small intestine intact, and free AA do not survive long in the rumen. In order to minimize the degradation of AA in the rumen and also to avoid the uncertain profile of AA released after rumen fermentation, feeding rumen protected amino acids (RPAA) is the well-established commercial approach (Atasoglu & Wallace, 2003). Abomasal infusion of the desired free AA has been a common technique used in research conditions for many years (Papas et al., 1974; Schwab et al., 1975).

Previously in this review, the important withdrawal by the PDV of the glucose absorbed intestinally was mentioned. Such a noticeable reduction also occurs among the glucogenic precursors (Aschenbach et al., 2010) such as AA, which is what we are concerned about in this study. Arriola Apelo et al. (2014) summarized that the use of AA by the PDV includes three primary fates: cell turnover, production of export proteins, and catabolism to provide energy. Berthiaume et al. (2001) measured net fluxes of AA across the gastrointestinal tract (GIT). Their observations suggested that the GIT uses around 30% of the total AA absorbed. For instance, Glu, Asp, and Gln had net portal fluxes close to zero, and even negative in the case of Gln, which indicates the huge importance of those AA to the GIT. Lobley et al. (2007) also showed that Leu and Met had a significant disappearance in the PDV (23 and 11%, respectively) due to their

oxidation to provide energy. This remarkable use of AA by the PDV suggests that an indirect positive effect of the supply of AA is to spare glucose rather than contribute to the direct effect of increasing the liver supply of glucose. In fact, some studies have found an increase in the WB-Ra of glucose or an increased hepatic release of glucose with an increased supply of AA, even when the net hepatic removal of AA by the liver remained invariable (Doepel et al., 2009). Furthermore, Galindo et al. (2015) found that abomasal supply of casein increased the WB-Ra of glucose without altering either the hepatic release or the arterial utilization of glucose. From the same study, Larsen et al. (2015) calculated that the BCAA were the most catabolized AA in the PDV among the essential AA (EAA), and that Asp, Glu, and Tyr were the most catabolized among the NEAA during the casein infusion.

Accounting for the effect of the liver in AA removal, net removal of EAA by splanchnic tissues (i.e., PDV plus liver) can be the same or even greater than the removal by the MG for production of milk protein (Hanigan et al., 2001). Most of this removal is explained by the considerably larger AA influx received by the splanchnic tissues, which in turn comes from the AA released from the MG. Therefore, Arriola Apelo et al. (2014) asserted that the effect of splanchnic tissues is mainly dependent on the EAA released by the MG. Thus, the different AA affinities and blood flows of these two tissues will determine the concentration of AA in circulation (Arriola Apelo et al., 2014). Given that most of the EAA that the liver receives come from arterial supply and that liver removal is not a direct function of portal absorption (Doepel et al., 2009), providing AA that are scarcely removed by the liver (e.g., BCAA and Lys) could improve AA utilization in MG and splanchnic tissues and thereby reduce AA catabolism (Arriola Apelo et al., 2014).

Other recent studies have demonstrated that the transition period does not impose a radical change in the net liver uptake of AA, but instead the increasing hepatic affinity postpartum for lactate would quantitatively support the larger amount of glucose release (Larsen & Kristensen, 2013), while AA would be derived mostly for milk protein synthesis (Doepel et al., 2009). Even with increased net fluxes of AA through splanchnic tissues, the net uptake of AA by the liver remains almost constant from pre- to postpartum (Doepel et al., 2009). The tremendously important roles of muscle mobilization and the alternative pathways activated in the liver and MG account for the required AA supply for the increasing production of milk and milk components (Dalbach et al., 2011). Larsen and Kristensen (2009[a]) also suggested that the remainder of EAA required to account for the production of milk protein during the first days of lactation may be provided by an inter-organ transfer of AA where the liver is not involved.

Another mechanism originating from an increased AA supply that results in a greater availability of glucose is the higher starch digestion in the small intestine as a result of an increased secretion of α-amylase from the pancreas (Richards et al., 2003). Swanson et al. (2004) found an increase of trypsin and chymotrypsin secretion as well as α-amylase during abomasal casein infusion, although none of the hormones and blood metabolites analyzed helped to understand how this effect is produced. Findings by the same authors (Swanson et al., 2003) during in vitro experiments indicated that cholecystokinin (CKK), which is one of the hormones responsible for stimulating secretion of digestive enzymes in pancreatic juice in response to luminal nutrient flow, only increased the concentration of trypsin and α-amylase when the pancreatic tissue had been extracted from calves that received casein infusion or when tissue was cultured with AA (i.e., recommended media concentration of AA for in vitro tissue incubation plus Gln). Behavior of CCK has been addressed by many researchers (Furuse et al., 1992;

Konturek et al., 2003; Zabielski, 2003), but a clear definition of the link between CCK and small intestinal starch digestion is still missing. More recently, Brake et al. (2014) compared different duodenal protein supplies and found that casein and EAA increased pancreatic α-amylase production, while Glu and NEAA increased small intestine starch digestion.

Besides the direct nutritional effect of AA as an energy source, the supply of AA might increase the synthesis of apolipoprotein B, which is the main component of the compounds necessary for export of excess fatty acids in the liver as TG and cholesterol esters in the form of VLDL (Bell et al., 2000). Bauchart (1998) demonstrated that increased intestinal supply of Lys and Met can increase hepatic concentration of apolipoprotein B100 in postpartum cows; as a consequence they also found less liver TG and less ketone bodies in plasma.

Regardless of how AA are used (milk protein synthesis, catabolic processes to provide energy, synthesis of tissue for turnover or growing fetus) or where the uptake of AA occurs (PDV, liver, MG, muscle tissue), it has been clarified after this review that AA metabolism plays an important function in the homeostatic evolution from the last phase of pregnancy to post-calving and consequent lactation. A simpler way to evaluate the net utilization of AA during that period is with the analysis of the concentration of free AA in arterial or venous blood. The pool of AA in the body is much bigger than the pool in plasma, but any substantial alteration in the supply or utilization of AA should be reflected in the plasma AA concentration (Meijer et al., 1995). These concentrations are a reflection of the AA (and other metabolites as well) available for use, no matter where they come from (mobilization, infusion, or feed digestion). Maeda et al. (2012) monitored the jugular plasma concentrations of AA during the transition period (-30 d to +60 d) in healthy cows and reported that except for Gly and Ser, which increased continuously from the prepartum period until +60 d after calving, the rest of the AA decreased their

concentration as cows were getting close to calving, with the day of calving having the lowest value. Thereafter, all AA except Glu and Gln recovered quickly and even exceeded their prepartum concentrations between 15 and 30 d after calving. However, Glu and Gln remained lower than prepartum values at 60 d after calving. Verbere et al. (1972) monitored daily the concentration of AA for 1 wk around calving and found that all AA decreased their concentration before parturition with the lowest value on the day of calving. At day +3 all AA had recovered their initial value with the exception of Gln. Meijer et al. (1995) measured the AA concentration in plasma and muscle from 2 wk before to 15 wk after calving. Except for Gly and Ser that increased from prepartum to early lactation, the remainder of the AA showed similar patterns to those described in the other studies, decreasing until the day of calving and thereafter recovering. However, no recovery was found for Gln, Met, and Phe. Muscle concentrations of AA increased as parturition neared, but concentration of Gln decreased. All EAA (except Leu and Val) and all NEAA (except Ala, Gln, Glu, and Gly) recovered their pre-calving values by 15 wk after calving. Similarly, Doepel (2002) reported a decrease at calving of most of the AA, suggesting their use for gluconeogenesis and milk protein. That suggestion paired with the observed increase of glucose at calving (Sun et al., 2016), probably due to the stimulation of gluconeogenesis and glycogenolysis by the increased glucocorticoid concentration at calving (Vazquez-Añon et al., 1994). Once again, Gln was the only NEAA that decreased from day +1 to +21. Results from Dalbach et al. (2011) determined that Glu and Gln were the only AA with no increase in plasma arterial concentration from calving until 29 DIM.

A few studies have analyzed the effect of feed restriction in mid-lactation cows on protein metabolism at the AA level. In order to study the effect of different metabolites as hunger or satiety signals, Laeger et al. (2012) analyzed their concentration in the blood and cerebrospinal

fluid. Regarding AA, after 4 d of feed restriction Leu and Lys increased in plasma probably due to their mobilization from protein tissue breakdown, and concentration of 3-MH also increased as a reflection of tissue protein mobilization (Blum et al., 1985). Arginine increased in plasma, likely because of its role in the urea cycle (Bender, 2012) to recycle the N from the catabolism of AA. In contrast, Trp decreased likely because of its role as a precursor for milk protein or as serotonin and melatonin precursor. On the other hand, the author (Bender, 2012) proposed that Ser, Tyr, and Thr may have acted as anorexigenic signals since their concentrations in the cerebrospinal fluid decreased.

Summary and Objectives

According to this brief but broad review, the role that the 20 AA play during the adaptation processes that take place during the transition to lactation is not exactly known but it is clear that role is important. Protein and energy metabolism are closely related in many aspects. Free AA are required as substrates (for catabolism) or are produced (anabolism) on a continuous basis during the homeostatic processes of the transition. Whether synthesizing protein, being deaminated to produce energy via ATP, producing export proteins, or being glucose precursors, the demand for AA by the organs responsible for the success of the transition is constant. This demand is not equal for all AA, and even differs for AA that belong to the same classification group (e.g., EAA vs. NEAA). The metabolic changes triggered by feed restriction will be assessed by comparing positive (ad libitum diet) and negative (feed-restricted diet) controls.

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CHAPTER 2. EFFECTS OF FEED RESTRICTION ON MILK PRODUCTION AND METABOLISM IN MID-LACTATION DAIRY COWS

INTRODUCTION

Multiple factors around parturition from natural changes in homeorhetic hormone concentrations (Laeger et al., 2013) to farm feeding management (Janovick et al., 2011) will cause an inherent decrease of DMI (Bell, 1995; Drackley, 1999). The lower DMI will trigger a synchronized cascade of metabolic adaptations destined to maintain optimal concentrations of glucose in circulation and energy supply at the cellular level (Aschenbach et al., 2010; De Koster & Opsomer, 2013). Mobilization of stored lipids is a major mechanism to provide energy (Bell, 1979; Drackley et al., 2001). However, excessive use of storage lipid can lead to metabolic malfunctions due to hepatic accumulation and excessive ketone bodies formation (Bobe et al., 2004; Drackley et al., 2005; Zammit, 1990). Therefore, availability of other precursors of glucose and energy, such as amino acids (AA), plays a vital role during periods of negative energy balance (Arriola Apelo et al., 2014; Bequette & Nelson, 2006; Larsen et al., 2015; Overton et al., 1999). In fact, body tissue protein mobilization also is an important mechanism switched on around parturition, as a source of AA (Lobley, 1998; Nishizawa, 1989; Van der Drift et al., 2012).

Distribution and efficient use of required AA during a period of lower DMI requires inter-organ coordination between liver and peripheral tissues (Arriola Apelo et al., 2014; Larsen & Kristensen, 2009). Uptake by the mammary gland has a relevant importance because of the relative amount consumed in comparison with the splanchnic tissues (Hanigan et al., 2001), and also because the mammary gland is able to alter extraction efficiency according to its requirements (Mackle et al., 2000; Mepham, 1982). Accordingly, the negative protein balance during the transition period is reflected in lower concentrations of the majority of essential and

non-essential AA in circulation (Doepel et al., 2002; Larsen et al., 2015; Maeda et al., 2012; Zhou Z. et al., 2016), which recover "pre-transition" values sooner or later during lactation. Nevertheless, analysis of the AA individually during the transition period can sometimes lead to misleading conclusions due to the effect of different feeding or grouping techniques applied (Dann et al., 2006; Doepel et al., 2002). In addition, an excessive time between sampling might prevent detection of quick changes in metabolite concentrations (Laeger et al., 2012; Zhu et al., 2000).

Feed-restriction models do not simulate exactly the natural negative energy balance occurring across the transition period, but have been a valid system to appreciate the metabolic adaptations to nutrient deficiency at a molecular level (Block et al., 2003; Bradford & Allen, 2005; Chelikani et al., 2004; Gross & Bruckmaier, 2015; Nielsen et al., 2003) without the collateral effect of hormonal influences characteristic of parturition and onset of lactation (Akbar et al., 2013). However, research investigating the effects of feed restriction on protein metabolism is not recent (Baird et al., 1972; Lomax & Baird, 1983), or it was focused on roles other than energy purposes (Ferraretto et al., 2014; Laeger et al., 2012; Rius et al., 2010).

The objective of the research reported in this chapter was to compare and analyze the effects of feed restriction on the relationships between protein and energy metabolism across time. The hypothesis was that the timing, synchrony, and magnitude of variation in blood metabolite concentrations will indicate the preference and requirements of specific AA for maintenance of milk production and basal metabolism when DMI is deficient.

MATERIALS AND METHODS

All procedures involving animals were approved by the Institutional Animal care and Use Committee at the University at Urbana-Champaign (IACUC protocol #15167)

Experimental Design

The experiment was conducted as a 7 x 4 Youdin Square (incomplete Latin Square) design with experimental periods of 10 d length, where 7 cows received 1 different treatment during each of the consecutive 4 periods. During d 1 to d 5 of each period, cows in 6 out of the 7 treatments had the amount of feed offered restricted to provide only 60% of their NE_L (Mcal/d) requirements at the start of each period. Additionally, feed-restricted cows during the first 5 d received the infusion treatments and frequent sampling of blood and milk was performed. During the following 5 d (d 6 to d 10) cows were fed for ad libitum DMI as a recovery and wash-out period. A detailed trial timeline can be observed in Figure 1.

Animals and Diets

Seven multiparous Holstein cows past peak lactation (93 ± 15.5 d in milk) were used to evaluate the effects of different AA and glucose abomasal infusions on energy and protein metabolism derived from a 5-d feed restriction period. All cows were housed in tie stalls and were milked in a parallel parlor at the Dairy Research Unit of the University of Illinois during the duration of the trial.

All cows had their ruminal cannula fitted previous to this experiment, and thus no cannulation surgeries were performed for this experiment. Cows were adapted to the tie stalls and to feed for 7 d before beginning the respective treatments.

The diet, fed as a total mixed ration (TMR), was formulated according with the NRC (2001) requirements for a lactation diet. The diet composition and nutrient content is detailed in Table 1. The TMR was mixed daily and offered at 0900 h. The restricted amount to offer during the restriction phase was calculated as the 60% of the specific NE_L requirements of each cow at the beginning of each treatment period; body weight (BW), milk production (kg /day), number of lactations, and days in milk were the parameters involved in this calculation. The restricted feed amount also provided 60 % of the EAA required approximately (Table 2). Dry matter content of the TMR was calculated every day before feed delivery in attempt to offer accurately the same amount of TMR DM during the experiment. Dry matter content was measured using a Koster tester (Koster Moisture Tester; Koster Crop Tester, Inc., Strongsville, OH). The Koster tester provided a fast and reliable method with small requirement of time and effort (Oetzel et al., 1993) . Additionally, the TMR was sampled every second day and analyzed by Dairy One Cooperative Inc., Ithaca, NY; forages and concentrate components were sampled every week. Near Infrared Reflectance (NIR) spectroscopy was the method elected to analyze the feed samples because of the speed of getting the results, which allowed us to adjust the feed amount offered according to the restriction requirements.

During the last 5 d of each period cows were fed ad libitum and refusals were weighed to calculate the DMI.

Blood Sampling

Indwelling catheters were inserted into a jugular vein of each cow on the afternoon of the day before d 1 of each period. The procedure area was prepared using a #40 clipper blade to remove hair in a 7 cm × 14 cm area centered over the jugular groove. The area was cleaned with povidone iodine scrub and alcohol. Wearing sterile gloves (Triflex, Cardinal Health, McGaw

Park, IL), the jugular vein was partially occluded with digital pressure to identify a location for catheter placement. After that, pressure on the vein was released and 3 to 5 mL of lidocaine solution were injected subcutaneously over the region of the jugular vein. The area was cleaned again with povidone iodine scrub and alcohol. While tenting the skin, a small (0.5 cm) stab incision through the skin was made with a #15 stainless steel disposable scalpel (Integra Miltex, Integra LifeSciences Corporation©, Lyon, France). The jugular vein was partially occluded with digital pressure. A 16-gauge 8.25 cm. I.V. catheter (BD Angiocath, Becton, Dickinson and Co., Franklin Lakes, NJ) were inserted through the stab incision and towards the jugular vein at approximately 60 degrees to the vein. When blood entered the catheter, the hub of the catheter was moved almost parallel to the vein and the catheter was advanced into the vein without moving the stylet. A T-Port extension set (B. Braun Medical Inc. Bethlehem, PA) was attached to the hub of the catheter and extension set and the catheter was flushed with 3 to 5 mL of heparizined saline (10 mL Heparin + 1000 mL NaCl 0.9% solution, B. Braun Medical Inc.). The hub of the catheter was secured to the skin with a simple interrupted suture of a non-absorbable suture material (Braunamid, B. Braun Medical Inc.). Using the same suture in a horizontal mattress pattern, the skin was folded dorsal and ventral to the catheter over the catheter to protect the catheter site. The extension set was secured to the neck with one or two simple interrupted sutures. Catheters were filled with sterile saline, capped, and secured with surgical wrap around the neck.

Abomasal Infusions

At the beginning of the adaptation period, the infusion line was placed in each cow. Abomasal infusion was accomplished by passing an infusion tubing through the rumino-omasal orifice into the abomasum by way of the rumen cannula. An infusion tube (Tygon tubing), fitted

at the end with a 60-mL plastic bottle with the bottom removed and with an approximately 8 cm rubber flange, was inserted through the rumen cannula and passed into the abomasum via the rumino-omasal orifice. To do so, the flange was compressed within the hand and the tubing guided through the orifice by hand. Once inside the abomasum, the flange was allowed to open, which prevented the tubing from being dislodged from the abomasum. The tubing remained in place for the duration of the experiment (40 d). The infusion apparatus was connected to an external infusion line via a hole in the rumen cannula plug. The line was connected to the external line by way of a quick-disconnect fitting. The location of the abomasum and the infusion line placement was performed following the directions by Litherland et al. (2005).

The correct placement of the infusion line was confirmed each day of the treatment period shortly before the infusion time. Furthermore, the infusion lines were flushed with distilled water before the infusion to eliminate possible clogs caused by digesta that got into the line from the end inserted in the abomasum.

Treatment solutions were infused using a rotary peristaltic pump (Sentinel™ Enteral Feeding Pump, Alcor Scientific Inc., Smithfield, RI). The infusions were initiated at the same time that the restricted amount of feed was offered; the doses (225 mL/h) were programmed to last 4 h to simulate the effect of each treatment if they would be included in the feed. In this chapter, only the effect of feed restriction was evaluated. Therefore, only the treatments where water was infused are described.

The treatments were as follows:

1. AD LIBITUM **(AL):** This treatment was used as a positive control.

During this treatment cows were fed the same ration as the cows in the restricted

treatments but for ad libitum DMI. The TMR offered and refusals were individually weighted to calculate and ensure a daily orts of around 10 to 15% of the amount of feed delivered on an as-fed basis. Cows assigned to this treatment did not receive any infused treatment. However, to simulate the same conditions among all treatments, cows were infused with 0.9 mL of distilled water added to the infusate volume during the same 4 h as the other treatments.

2. RESTRICTED CONTROL **(RC)**: This treatment was used as a negative control. During this treatment cows were fed the calculated restricted amount of feed according with the calculation previously explained. Cows were infused with only distilled water in the same way as cows in the AL treatment.

Measurements

Milk Samples

Cows were milked twice daily at 0600 and 1700 in a parallel parlor. Milk was sampled at each milking and milk production was recorded during length of the experiment. One aliquot of each daily milking sample was preserved (800 Broad Spectrum Microtabs II; D&F Control Systems, Inc., San Ramon, CA), refrigerated, and then analyzed (Dairy Lab Services, Dubuque, IA) for contents of fat, protein, milk urea nitrogen (MUN), lactose, and total solids. Another aliquot was immediately frozen at -20°C.

Body Weight

Body weight was measured on d 1 of the restriction before feeding and also after the 5-d restriction on d 6 before the first ad libitum offer of TMR.

Blood Sampling

Blood samples (40 mL) were withdrawn via the catheter on d 1 to 5 at 0800, 0900, 1200, 1300, 1400, and 2300. After sampling was completed on d 5 in each period, the catheters were removed. From d 6 to d 10, to have an indication of metabolic state between the intensive sampling periods, one blood collection was made at 0900. Samples were transferred into evacuated tubes (BD Vacutainer, BD and Co., Franklin Lakes, NJ) containing clot activator for serum and K_2EDTA for plasma. After blood collection, tubes for plasma were placed on ice and tubes for serum were kept at 21°C until centrifugation (~15 min). Serum and plasma were obtained by centrifugation at 2,000 × g for 15 min at 4°C. Aliquots of serum and plasma were frozen (−20°C) until further analysis. Concentrations of NEFA, BHB, total protein, albumin, total alkaline phosphatase, aspartate aminotransferase, gamma-glutamyl transferase, total bilirubin, total cholesterol, glutamate dehydrogenase, and triglycerides were determined at the University of Illinois College of Veterinary Medicine diagnostic laboratory by automated analysis methods. Globulin concentration was calculated by the difference between total protein and albumin. Plasma glucose concentration was analyzed by the University of Missouri Experiment Station Laboratory. Concentrations of plasma AA and N components were analyzed by Ajinomoto Co., Inc. (Tokyo, Japan). Insulin, leptin, serum amyloid A and haptoglobin were analyzed using commercial ELISA kits from Mercodia (catalog no. 10-1201-01), MyBioSource (catalog no. MBS703026) and Tridelta Development Ltd. (catalog no. TP 802 and TP801) respectively. Variables included in acute-phase response and liver function sections plus leptin, were only analyzed in one sample (0900) per day.

Statistical Analysis

Comparisons were made using the MIXED procedure of SAS version 9.4 (SAS Institute, 2012) according to the following model:

$$Y_{ijklm} = \mu + Cow_i + Treatment_j + Period_k + Day_l + Hour_m + (Treatment \times Day) + (Treatment \times Time) + \varepsilon_{ijkl}$$

Effects of treatment, period, day, and hour were included as fixed effects in the class statemen while cow was included as a random effect. t. The repeated fixed effects of day nested within period was used in the REPEATED statement with cow nested in treatment the subject. For each variable with equal time spacing between samples, the subject was tested for 3 different covariance structures: autoregressive order 1, compound symmetry, and spatial power. For variables with unequal spacing, spatial power law, Gaussian and SPH were tested. The covariance structure that resulted in the smallest Bayesian information criterion was chosen (Littell, 2006). The degrees of freedom were estimated with the Kenward-Roger specification in the model statement. Other measurements including initial (d1) BW, initial milk production (kg/d), number of lactations, and days in milk (DIM) were tested as covariates including them in the model statement, and then removed according with their significance explaining the variance in the model and with their influence on the Bayesian criteria. Furthermore, a set of covariates was created and included to account for the carry-over effect between consecutive treatments on each variable (Doelman et al., 2015).

In order to gain power during the feed restriction effects analysis, an additional model was performed to estimate the effect of all feed-restricted treatments grouped together (RES) and compared with AL, where the data points affected directly by the infusion itself were removed.

This comparison will be also referred as the effect of “diet”. The MIXED procedure was applied according with the following model:

$$Y_{ijkl} = \mu + Cow_i + Diet_j + Period_k + Day_l + (Diet \times Day) + \varepsilon_{ijkl}$$

The PROC UNIVARIATE procedure was applied to each variable to check for normality and for the presence of outliers. Graph plots and P-values from the Shapiro-Wilk and Kolmogorov-Smirnov tests were used to evaluate both homogeneity and normality of residuals. When appropriate, variables were transformed to accomplish the above-mentioned criteria. Non-transformed LSMEANS and standard errors (SE) were reported. The CONTRAST statement was used to compare AL and RC, or AL and RES. When interactions between treatment and day were significant, the SLICE statement was applied in order to identify the difference among treatments and days. The linear and quadratic trends were evaluated using the ESTIMATE statement from PROC MIXED, to classify the evolution along time according with feed condition (RES or AL), or with the infusion treatments on each variable. The smaller P-value from either linear or quadratic trend was reported. Correlations between concentrations of AA in plasma, and between milk components were identified using the CORR procedure.

RESULTS AND DISCUSSION

Production Variables

Milk and blood variables reacted markedly to the 5-d feed restriction model in mid-lactation cows. Success of this model had been validated in similar conditions by others (Baird et al., 1972; Lomax & Baird, 1983; Moyes et al., 2009; Toerien & Cant, 2007; Velez & Donkin, 2005). To establish the basis of the metabolic adaptations that occur during the feed restriction period, the comparison of variation in concentrations of plasma metabolites, milk production, and milk components between AL and RC are described in this section.

According with the previously mentioned calculations to determine the amount of feed to be delivered during treatments under feed restriction, the average DMI for the RC treatment (12.4 kg /d) was 54.7% lower ($P < 0.0001$) than in the AL (27.5 kg/d) treatment (Table 3, Fig. 2). The DMI during feed restriction was constrained to be constant, while during ad libitum feeding, the intake increased following a quadratic trend along the 5 experimental days with its peak on d 3 (Fig. 2). This reduced DMI is within the range (65% to 40%) observed in other studies where the purpose was to compare the metabolic responses during restriction with the previous fed state, or with other treatments during ad libitum conditions. This reduction has been proven to be effective in causing the desired metabolic cascade of adaptations (Akbar et al., 2013; Carlson et al., 2006; Moyes et al., 2009). The short period of feed restriction likely indicates that the significantly lower BW after feed restriction (-69 kg) at the end of the period (Fig. 3) was probably mostly due to the reduced DMI (Laeger et al., 2012) but also to a lesser degree the lipid storage mobilization (Drackley et al., 2001).

Dry matter intake is the main factor that drives milk production (Dado & Allen, 1994; Drackley et al., 2006) and vice versa (Cardoso et al., 2013; Drackley et al., 2005; Janovick et al., 2011). Therefore, a decrease in milk production in our study would be expected just by the reduced DMI in treatments under feed restriction. The average milk production for RC (30.5 kg/d) was -19% lower (P = 0.002) than daily milk production for AL (37.7 kg /d). Similar decreases have been reported previously in similar conditions (Contreras et al., 2016; Ferraretto et al., 2014; Velez & Donkin, 2005). Across time, milk yield for RC decreased continuously (P = 0.008, Fig. 4) in a linear trend (P = 0.001), while for AL milk had a weak increasing tendency (P = 0.063) matching the same pattern as DMI (Fig. 2).

Due to the energetic and protein imbalance, the high availability of fatty acids from fat mobilization, the lower duodenal AA flux, and the countervailing hormonal adjustments also will alter milk composition (Arriola Apelo et al., 2014; Contreras et al., 2016; Doepel et al., 2004). As a result, daily averages of milk protein percentage and yield were lower (P = 0.034 and P = 0.001, respectively) during RC (2.95%, 0.9 kg/d) in comparison with AL (3.09%, 1.15 kg/d). Both variables manifested a linear (P < 0.0001, Fig. 4 and 5) decreasing effect across time (P < 0.0001 and P = 0.001 respectively). During AL treatment, milk protein percentage and yield were not different across time (P = 0.16 and P = 0.43, respectively). Similar effects also were reported by Gross et al. (2011) and Carlson et al. (2006). In contrast, milk fat percentage was higher (P = 0.005) during RC (4.30%) than during AL (2.53%), following a weak linear tendency (P = 0.1, Fig. 8) but without a significant change across the 5-d period (treatment by day, P = 0.34). However, considering the group formed by all restricted treatments (RES), milk fat did increase (RES by day, P < 0.0001). No differences (P = 0.99) were detected in milk fat yields between AL and RC, with no variation across periods (P = 0.28, Fig. 8). This finding is in

agreement with the results of other experiments using short-term feed restriction periods (Chelikani et al., 2004; Nielsen et al., 2003). Other authors also did report differences in fat yields (Contreras et al., 2016). Despite being numerically lower during RC, the high standard deviations found in our study did not allow for more significant differences (Fig. 8).

Milk production is sustained largely by the mammary production of lactose, which is the biggest osmotic solute present in milk (Bell, 1995) and therefore determines the milk volume. Lactose is secreted into the alveolar lumen, and the concomitant transport of water drives the consequent milk production (Rigout et al., 2002). Glucose is the principal precursor of lactose, and is able to promote its synthesis in mammary cells and also induce cell viability and proliferation (Ye et al., 2016). Milk lactose percentage decreased linearly ($P < 0.0001$, Fig. 9) during RES, but remained steady ($P = 0.87$) in AL during the 5 d. Nevertheless, there were no significant effects of day or treatment with day (Table 3). Neither were there differences ($P = 0.2$) between daily average lactose percentage between AL and RC (4.66% and 4.56%, respectively) since only at d 5 of the period did RC have lower lactose concentration than AL (Fig. 9). However, milk lactose yields per day were greater ($P = 0.001$) for AL treatment (1.75 kg/d) than during RC (1.38 kg/d), in agreement with the result observed by Velez and Donkin (2005). Lactose yield per day decreased following a linear trend (Fig. 10). Milk lactose content has been reported to decrease during feed restriction or fasting in many studies (Carlson et al., 2006; Chelikani et al., 2004; Contreras et al., 2016; Moyes et al., 2009), while others did not find that effect (Gross et al., 2011; Nielsen et al., 2003) even though plasma glucose was decreased. Since synthesis of lactose also depends on glucose uptake by the mammary gland, other anabolic mechanisms are probably activated to promote lactose synthesis during glucose shortage (Lemosquet et al., 2009). As a consequence of feed restriction, the percentage of milk solids

decreased during RC (Fig. 11), but total solids concentrations were not different between AL and RC (P = 0.68). However, the yield of total solids decreased progressively across time (P = 0.013) in a linear trend (Fig. 12), resulting in a lower (P = 0.007) average yield for RC.

Milk production (kg/d) was negatively correlated with fat percentage (-0.31), protein (-0.35) percentage, and DIM (-0.46), while the correlation was positive with lactose (0.27) content (Table 9). Therefore, even though the homeostatic and homeorhetic responses to NEB have a smaller magnitude in mid-lactation (Gross & Bruckmaier, 2015), the adaptive mechanisms of synthesis of milk components and milk production still applied in the same way as in the onset of lactation.

These adaptations in milk components to counteract the decreasing milk production led to only a tendency (P = 0.078) for lower energy-corrected milk (ECM) production during RC (Fig. 13). A similar tendency was found (P = 0.065) considering all treatments under feed restriction (Table 3). This compensatory effect for a reduced energy intake also was reported in other experiments under similar conditions as well as during early post-partum (Gross & Bruckmaier, 2015; Nielsen et al., 2003). This effect also explains why even though feed efficiency (milk yield/DMI) decreased (RC by day, P = 0.007) linearly (P = 0.001) during RC, no differences were detected between treatments (Fig. 15) or between AL and RES (Table 3). In contrast, when FE is corrected by using ECM (Fig. 14), there was an opposite reaction in which there was no interaction (Trt by day, P = 0.1), but there was a strong effect of treatment (AL vs RC, P = 0.022). The latter result was also observed but in a reverse direction when energy balance (EB) was calculated (Fig. 16). Therefore, even though energy intake was reduced, cows successfully adapted to try to provide the same energy output through milk components, as reflected by the lack of difference in TS percentage between AL and RC. Deserving special mention is the milk

components with higher energy content like milk lactose, whose concentration in milk did not vary significantly, and milk fat, whose concentration increased during feed restriction (Table 3).

An imbalance between the requirements and the intake of nutrients will cause an adjustment of N metabolism (Nousiainen et al., 2004). Excessive amounts of ammonia will be produced from ruminal degradation in case of an excess protein intake, or also from AA catabolism in tissues to provide energy during NEB (Morris, 2002; Reynolds, 2006). This surplus of ammonia will be detoxified by conversion into urea in the liver (Bender, 2012c). Since urea reaches rapidly a balance in all body fluids, milk urea N (MUN) constitute a good indicator of AA catabolism (Nousiainen et al., 2004). The average daily concentration of MUN was higher (P = 0.007) during RC (15.26 mg/dL) than during AL (10.48 mg/dL). While MUN did not vary (P = 0.92, Fig. 17) during the 5 experimental days in the AL treatment, it did increase (P = 0.037) during RC, following a quadratic pattern (P = 0.004) with a peak at d 2. Similar findings were reported during short-term feed restriction periods in other studies (Carlson et al., 2006; Velez & Donkin, 2005). According with this data, MUN reflects that catabolism of AA occurred during RC. The response was fairly fast since at d 2 cows were still in the beginning of the experimental period. This could mean either that catabolism of AA is the first option as source of glucose and energy in NEB or that capacity to detoxify the NH_3 derived from catabolism in the ureagenic pathway decreased in favor to other export routes such as the glutamine pathway (Bender, 2012c; Noro & Wittwer, 2012; Zhu et al., 2000).

Energy-Related Metabolites and Hormones

As a consequence of the reduced feed intake, the daily average concentration of NEFA during RC treatment was 19% higher (P = 0.001, Table 3) than the daily average in AL due to fat mobilization (Drackley, 1999; Katoh, 2002). The pattern across time showed that NEFA

increased ($P < 0.0001$) during RC treatment following a quadratic trend ($P < 0.0001$, Fig. 18). After a linear increase of 170% to reach its peak at d 3 (0.412 mEq/L), NEFA concentration decreased slowly thereafter until d 5. In contrast, NEFA did not show variation across time ($P = 0.14$) for AL, resulting in a significant difference in d 2 and subsequent days (Fig. 18). This rapid increase in NEFA was also found in other experiments during short periods of feed restriction. For instance, Nielsen et al. (2003) reported a linear increase of 121% for NEFA during 3 d of feed restriction of 65% in DMI, and more recently Contreras et al. (2016) reported a 75% higher NEFA concentration in a group of cows with intake 47% lower in comparison with the ad libitum fed group.

The contrasting effects of lipolysis of triglycerides plus reesterification of NEFA within adipocytes are the reasons for the increase of NEFA in blood during periods of fat mobilization, and both processes are regulated by the antilipolytic effect of insulin (De Koster & Opsomer, 2013; Weber et al., 2016). Since propionate is an insulin secretagogue (Grummer, 1993), the reduced DMI will lead to a reduced flux of volatile fatty acids to the liver ending with the consequent decrease of insulin (Harmon, 1992). Even though there were no a remarkable effect of treatments on insulin concentration overall ($P = 0.09$), the RC treatment did result in lower ($P = 0.013$) daily mean concentration of insulin than AL (0.44 vs 0.64 μg/L). Similarly, Contreras et al. (2016) reported just a tendency for lower insulin concentration in feed restricted cows after 4 d of restriction. Even more, Velez and Donkin (2005) did not find any effect of feed restriction on insulin concentration after 5 d of limited feeding. In contrast, Ferraretto et al. (2014) and Carlson et al. (2006) found a considerable decrease of insulin concentration (<50%) after 4 d with a feed restriction of 50% of DM. The differences among studies can be due to differences between lactation stage of animals (Gross et al., 2011), blood samples timing (Piantoni et al.,

2015), composition of the offered diet (Harmon, 1992), and differences between the animals themselves (Ferraretto et al., 2014; Gross & Bruckmaier, 2015). Nevertheless, in our study insulin decreased ($P < 0.0001$) in a quadratic manner ($P < 0.0001$, Fig. 20) across days with the lowest value at d 3 and thereafter remaining invariable until d 5.

The observed insulin depression matches with the increase of NEFA concentration. Both converged with d 3 being their inflexion point. This synchrony mimics the usual behavior of both metabolites around calving (Drackley et al., 2001; Grummer, 1993; Vazquez-Añon et al., 1994). Moreover, these two metabolites act accordingly in the same opposite direction within day (Allen, 2014) as observed in this study. While NEFA concentration was lower in the samples from 3, 4, and 5 h after feeding (Fig. 19), insulin concentration was higher than the values pre-feeding (Fig. 21). This insulin-NEFA reaction to feeding had the same effect across all treatments. However, the magnitude of the differences in concentrations of NEFA relative to changes of insulin within day were 2 times higher during feed restriction than during AL treatment, reflecting the difference the interactions of diet by time (Table 8). While variations within day of insulin only tended to differ between diets ($P = 0.07$, Table 3), variations of NEFA were totally different depending on the diet ($P = 0.003$). Similar results were reported by Piantoni et al. (2015) who found no variations of NEFA 4 h after feeding in cows in late lactation, but a marked effect in cows during their post-partum period (12.6 ± 3.8 DIM). This different reaction between insulin and NEFA reveals that lipolytic and antilipolytic mechanisms are heavily influenced by the hourly feeding behavior (Allen et al., 2005), and that even the insulin sensitivity of tissues has more importance than the insulin concentration during NEB conditions other than the post-partum period (Janovick et al., 2011; Piantoni et al., 2015).

Leptin is another protein hormone involved during processes unleashed by low DMI. Leptin is considered a satiety hormone, thus its main purpose is to send signals to decrease feed intake (Ahima & Flier, 2000) and also interacts with other hormones implicated in regulation of feed intake such as growth hormone (GH) and insulin (Allen et al., 2005; Block et al., 2003). Leptin is synthetized by adipocytes, and therefore the status of adipose tissue regulates the expression of leptin and its concentration in blood is correlated with the amount of total body fat stores (Ahima & Flier, 2000; Block et al., 2001). In contrast leptin is not related to differences in hepatic fat infiltration (Hammon et al., 2009). Consequently, other than just a restricted feeding situation, a phase of NEB also would alter leptin concentration due to adipocyte fat mobilization. Furthermore, it has been demonstrated that leptin has anti-inflammatory effects and also boosts the immune system (Johnson & Finck, 2001). Many studies proved a marked decreasing plasma concentration of leptin during the onset of lactation, a period that is characterized by lower DMI and NEB caused by the increasing milk production (Block et al., 2001; Hammon et al., 2009; Reist et al., 2003). Besides the clear relation with NEB, Liefers et al. (2003) also found a significant increase in detection of first estrus postpartum in cows with higher plasma leptin concentration. Alterations of leptin were also provoked by short periods of feed restriction (Block et al., 2003; Chelikani et al., 2004) or by altering the energy balance in periods of milking or no milking (Block et al., 2001). In our study, RC had a slightly lower numeric concentration of leptin than AL (4.99 vs 4.87 ng/ml) but with no statistical difference ($P = 0.32$). Neither treatment showed a significant trend over time either (Table 5, Fig. 22). Similarly, Reist et al. (2002) did not find a correlation between leptin and other energy-related blood metabolites in the first 100 wk postpartum. However, it is valuable to mention that during our study, there was a tendency for leptin in AL to be higher than the group formed by all treatments with feed

restriction (RES) in the experiment (P = 0.061) and also that the RES group showed a linear decrease (P < 0.0001) over the 5 d.

Increasing plasma concentrations of glucagon is another endocrine response to maintain the desired mix of fuels in order to resist changes in glucose concentration in circulation (Aschenbach et al., 2010). Glucagon activates the release of glucose from liver glycogen, promotes gluconeogenesis from propionate (Donkin & Armentano, 1995), and also regulates malonyl-CoA, which plays a key role in regulating fatty acid oxidation in liver cells (Brindle et al., 1985). The beginning of the lactation is characterized by lower DMI, where the pancreas balances the concentrations of insulin and glucagon in circulation to achieve that goal. Hammon et al. (2009) reported that glucagon and the glucagon/insulin ratio increased after parturition until 2 wk postpartum in cows with high and low liver content of fat. The lack of information regarding glucagon concentration in mid-lactation cows under feed restriction, complicates the discussion of our results. Early lactation cows in a restriction condition were studied by Toerien and Cant (2007), who found that even though there were no significance differences, glucagon concentration tended to increase during the 18 h after beginning of the restriction, and then 6 h later decreased to similar values as the initial point. The authors pointed out that this later decrease 24 h after restriction was synchronized with the biggest drop in milk production, meaning that the mechanism required to save glucose may no longer be necessary after that. Our results showed no important differences between AL and RC treatments (P = 0.67). However, due to the extremely high standard errors obtained, only comparing AL to the RES group leads to clearer but still cautious conclusions. There was an interaction of diet with day (P = 0.001). During feed restriction, glucagon concentration remained constant on d 1, 2 and 3; thereafter, it decreased and remained lower during d 4 and 5 (Fig. 23, Table 4). In accordance with Toerien

and Cant (2007), it seems that glucagon concentration remained high to compensate for the decrease of glucose and later reach a constant concentration in tune with glucose during the last 3 d of the period. The same pattern was pointed out by Heitmann et al. (1987) in sheep and steers, where the decrease of insulin was coupled with constant values of glucagon during fasting. This would lead to a higher ratio of glucagon/insulin and therefore trigger the concomitant energetic homeostatic processes. As hypothesized by de Boer et al. (1985), glucagon synthesis could be inhibited by ketone body and NEFA concentrations, or it could be also limited by availability of substrate what would explain the decline toward the end of the period when rumen content is very limited and BHBA and NEFA concentration are high.

The principal aim of all these adaptations is to maintain an optimal balance of the energy fuels for the cells in a situation of lower glucose concentration due to a reduced feed intake (Drackley, 1999). In this study, glucose concentration per day in RC was lower ($P = 0.031$) than AL (59.3 vs 64.4 mg/dL). Glucose concentration decreased (RC by day, $P < 0.0001$) linearly from d 1 to d 3 (-12%) and later remained invariable until the end of the feed restriction period (Fig. 25). This slight decrease led to a non-significant difference between AL and all treatments under feed restriction ($P = 0.16$). This could be a reflection of the homeostatic processes triggered by metabolism to keep an optimal and constant flux of glucose in the blood (Bell, 1995). These adaptive mechanisms seemed to produce different results in plasma glucose concentration in some of the studies reviewed. For instance, Carlson et al. (2006) found no difference after 4 d of restriction and they attributed this to the measured depletion of liver glycogen that would be used to release glucose into the blood stream. On the other hand, Ferraretto et al. (2014) did find a significant decrease after 4 d of restriction in similar conditions. Probably, the lower energy density (NE_L=1.65 Mcal/kg) in Ferraretto's diet, the fact that cows on

their study were in late lactation with 204 ± 29 DIM (versus 132 ± 36 DIM), and with a bigger drop in milk production (-10 kg/d) than during Carlson's trial (-4.9 kg/d) were some of the reasons why glucose behaved differently in the 2 studies. Glucose concentration decreased after feeding for all treatments with no important differences between treatments (P = 0.89), similar to Piantoni et al. (2015) and Ferraretto et al. (2014). During the postprandial phase glucose is driven toward insulin sensitive cells due to the increase of insulin stimulated by the VFA from the feed digestion (Allen et al., 2005; Ferraretto et al., 2014), decreasing the glucose concentration in plasma. However, similarly to the variation of NEFA in reaction to changes in insulin concentrations, there were a different reaction of glucose in RC and RES in comparison with AL. While insulin response to feeding within day tended to differ depending on the diet (diet by hour, P = 0.071), glucose variation within day was practically the same regardless of the diet restriction (diet by hour, P = 0.75). This different reaction is even more remarkable during the night sampling (14 h after feeding) where large differences in concentrations of insulin (Fig. 21) between AL and RC match with similar concentrations of glucose between treatments (Fig. 26). Therefore, it seems that there was a difference in the insulin sensitivity of tissue more than a reaction to the concentration change, since the decrease of both metabolites across the period have similar significance (Table 3). This finding is in agreement with Piantoni et al. (2015), who reported no proportional relation between changes in insulin with changes in NEFA and glucose across groups of cows with different energy balance (late lactation and early postpartum), or as observed in humans and mice where feed or caloric restriction increases insulin sensitivity (Barnosky et al., 2014; Cheng et al., 2017).

Once the increasing flux of NEFA through the blood reaches the liver, they might be oxidized to provide the energy required (Drackley, 1999) during feed restriction. To avoid the

dangerous consequences of an excessive accumulation of fatty acids within the liver (Vazquez-Añon et al., 1994), the hydrolysis of stored triglycerides (TG) and their export in form of very low density lipoproteins (VLDL) and cholesterol must be very efficient (Grummer, 1993). During a period of feed restriction, the concentration of TG in plasma released from the liver will rise (Gross et al., 2015) in a rate proportional to the concentration of NEFA in plasma (Grummer, 1993). In our study, restricted diets increased (P = 0.02) TG concentration in all treatments. Cows under RC treatment had a daily average concentration of TG of 10.5 mg/dL, being 30 % higher (P = 0.015) than cows in the AL treatment (8.0 mg/dL). Plasma TG increased (RES by day, P = 0.033) in a quadratic pattern (P = 0.001, Fig. 27) with a maximum at d 3 and decreasing progressively thereafter, following a pattern similar to NEFA (Fig. 18). Increases in TG also were reported in some studies in which cows were subjected to feed restriction (Carlson et al., 2006; Gross & Bruckmaier, 2015; Moyes et al., 2009) but not in another (Capuco et al., 2001). As reported by Ohgi et al. (2005), liver TG infiltration takes place after NEFA concentration reaches its maximum concentration (Grummer, 1993; Vazquez-Añon et al., 1994). In our study, the plasma concentration of TG decreased after concentration of NEFA reached its peak in blood, suggesting that hepatic infiltration of TG might have started around d 3 since TG concentration in blood also decreased that day in the RES group (Table 4).

The NEFA recently re-esterified into TG must be exported from the liver. Ruminants have a low capacity for TG export from liver in comparison with non-ruminant species (Pullen et al., 1990). The VLDL are the lipoproteins in charge of the export of TG and cholesterol. In this study, there were no differences (P = 0.47) in serum concentrations of cholesterol between AL (190.9 mg/dL) and RC (195.3 mg/dL). This finding is in agreement with Capuco et al. (2001), who found no difference after 6 d of feed restriction at 80% of the AL group intake. However, it

is noteworthy that RC showed a weak quadratic increasing (P = 0.19, Fig. 28) trend of cholesterol. Moreover, considering all the restricted treatments (RES), feed restriction induced a tendency to higher cholesterol (P = 0.11), manifesting a linear increasing trend (Table 5). The short period of time under restriction, the severity of the restriction, or differences in other factors that affect the capacity of cholesterol export may be reasons for lack of a greater difference between AL and restricted intake. For instance, Gross et al. (2015) reported an increase of cholesterol after 3 wk of restriction to 50% of ad libitum DMI, and DiMarco et al. (1981) reported a 10% higher serum cholesterol after 9 d of fasting. In accordance with this export from the liver, Kuhla et al. (2009) measured that liver of mid- lactation cows after 60 h of feeding a straw diet (18 ±1.1 MJ NE_L vs 110.2 ±11.2 MJ NE_L in AL group) had 73% less liver cholesterol concentration (DM basis) than the AL group. In summary, this variation matches with the natural increase of blood cholesterol that occurs after calving (Gross & Bruckmaier, 2015; Ohgi et al., 2005) during a healthy transition period (Bobe et al., 2004).

During periods of NEB and intense mobilization of adipose TG, high concentrations of NEFA lead to increased β-oxidation of fatty acids within hepatic mitochondria. The lack of glucogenic precursors to bind the acetyl-CoA product of β-oxidation for entry into the TCA cycle for complete oxidation to CO2 (Zammit, 1990), leads to an increase in formation of ketone bodies instead (Katoh, 2002). Hence, higher concentrations of ketone bodies, primarily as β-hydroxybutyrate (BHB), have been widely reported during the postpartum period (Bell, 1995; Drackley, 1999; Grummer, 1993) or even before parturition (Grum et al., 1996), as well as during short periods of feed restriction during mid-lactation (Gross et al., 2011; Moyes et al., 2009; Nielsen et al., 2003). However, cows in late stages of lactation have a less intense reaction to increase ketone bodies formation (Carlson et al., 2006; Gross & Bruckmaier, 2015; Gross et

al., 2013). This modest response was observed in our study, where there were no differences (P = 0.46) between RC (0.59 mmol/L) and AL (0.61 mmol/L) for overall BHB concentrations. However, the 2 treatments differed in their evolution across time (Table 5, Fig. 29). While AL did not have any important deviation from d 1 on subsequent days (P = 0.8), as expected, RC displayed a weak tendency across days (P = 0.16) to follow a linear increasing trend (P = 0.042) that reached its maximum at d 4, after the peak of NEFA concentration occurred (Fig. 18). This "delayed" reaction is in agreement with other studies (Gross et al., 2011; Gross & Bruckmaier, 2015; Van Den Top et al., 1996), and follows the biologic process previously mentioned wherein TG infiltration (Vazquez-Añon et al., 1994) and ketone bodies formation (Goff & Horst, 1997) likely would start when NEFA uptake exceeded the capacity for TG export and complete oxidation. Similarly, considering all the treatments under RES, the interaction of diet with day became stronger (P < 0.0001) with the BHB peak at d 4 (Table 4). The short period under restriction that resulted in a significant increase at d 4, the different initial values, and the relatively large standard errors likely did not allow for bigger differences in BHB concentrations between the two treatments.

Ketones bodies are also produced from the butyrate produced during rumen fermentation (Brockman, 2005). During the postprandial phase, butyrate is highly metabolized into BHB (Emmanuel, 1980) by the epithelium lining the rumen walls. This process is in accordance with our data, where independent of treatment or fed vs fasting condition, BHB increased 88% by 3 h post-feeding (Fig. 30). The net portal flux of BHB does not vary substantially, regardless of the amount of butyrate produced in the rumen (Menahan et al., 1966; Nozière et al., 2000). In agreement, we observed in our study that there were no differences in BHB concentration between RC and AL on d 1, 2, or 3 during the post-absorptive phase even though the amount of

feed ingested was different. Our data also agrees with Piantoni et al. (2015), who reported a positive correlation between DMI and the increase in BHB after feeding only in late lactation cows and not in post-partum cows. However, the amount of butyrate produced in the rumen strongly affects the concentration of BHB in arterial plasma (Herrick et al., 2016; Nozière et al., 2000). Therefore, the higher concentration of BHB during the RC treatment could be a mix between BHB that was produced by the liver due to the greater availability of NEFA in circulation (Katz & Bergman, 1969) because of lower insulin concentration in the pre-feeding time points, and also as a result of greater uptake of BHB by mesenteric-drained viscera (Nozière et al., 2000). Nevertheless, the samples taken before feeding displayed the greatest increase across the period (Table 6). In addition, during the fed state BHB is the main ketone body synthesized by the liver and rumen epithelium, whereas in the fasted state the BHBA to acetoacetate ratio decreases and acetoacetate can account for between 20 to 30% of total ketone body production in the liver (Heitmann et al., 1987; Katz & Bergman, 1969; Toerien & Cant, 2007) during a time when BHB production by rumen epithelium would be decreased due to lack of feed intake. Therefore, since we only measured BHB, the magnitude of hepatic ketone body production in RC or RES likely was underestimated.

Acute-Phase Response

Free fatty acids or NEFA are transported in blood by binding with albumin (Bell, 1979). Thus, in a situation of intense fat mobilization, albumin concentration potentially could be reduced by deficiency of albumin production of the liver, resulting in greater NEFA uptake into tissues (Moyes et al., 2009; Seifi et al., 2007). On the other hand, serum albumin belongs to the negative acute-phase proteins, whose production can be diminished by the effect of cytokines (Bertoni et al., 2008). Cytokines are released around calving due to diseases, stressors, or even

nutritional imbalances (Drackley et al., 2005). Cytokines, among many other effects and implications (Bradford et al., 2015), stimulate the acute phase response, which is defined by increasing positive acute-phase response proteins and decreasing the synthesis of negative acute-phase protein such as albumin (Bertoni et al., 2008). Because of both reasons combined, lower albumin concentrations have been related to higher fat infiltration in the liver (Reid et al., 1983; West, 1990). This makes albumin a good indicator of transition period performance (Trevisi et al., 2010; Zhou et al., 2016). Cows under feed restriction in the RC treatment had lower albumin concentration ($P = 0.053$) than AL. Albumin was slightly altered across time by the effect of the restriction (RC by day, $P = 0.16$), with the biggest difference on d 3 and 4 since albumin concentration declined following a quadratic trend ($P = 0.032$, Fig. 31). This temporal pattern suggests the use of albumin as NEFA transporter during periods of fat mobilization. Moyes et al. (2009) also found a decrease in albumin concentration 2 days after the beginning of the restriction with a recovery to initial concentrations 2 d later.

As mentioned in the previous section, albumin is a classic negative acute phase protein, whose synthesis is decreased in response to pro-inflammatory signals. Globulin, in contrast, is a positive acute-phase respondent and its synthesis will be stimulated by cytokines (Drackley et al., 2005). Therefore, it constitutes a good indicator of inflammatory status (Bertoni et al., 2008) and impaired hepatic lipid metabolism (Crookenden et al., 2017). No effects of diet or treatment were detected in globulin concentration (Table 3, Fig. 32)

Albumin and globulin account for the total concentration of so-called total protein (TP) in blood. Bertoni et al. (2008) observed that TP concentration in serum increased from d 7 to d 28 postpartum following an inverse pattern from NEFA, similar that reported for albumin (Seifi et al., 2007), and with no relationship between concentrations of TP and fat content in liver cells

(Reid et al., 1983). During our study, the concentration of TP did not differ between AL and RC (P = 0.92) and there were no detectable patterns over time for those treatments (Table 5, Fig. 33), similar to the response for globulin concentration. Therefore, under the conditions of our trial, it seems that only albumin was affected by feed restriction.

Haptoglobin (Hp) and serum amyloid A (SAA) also belong to the group of positive acute-phase proteins whose synthesis is stimulated by pro-inflammatory cytokines and glucocorticoids (Drackley et al., 2005; Katoh, 2002). Haptoglobin is an acute-phase glycoprotein that has been used as an indicator of liver health status due to its quick response (Bertoni et al., 2008) to an inflammatory event (Godson et al., 1996). It also plays an important role in lipid metabolism (Katoh, 2002; Katoh et al., 2002) because it is synthesized also by adipocytes (Friedrichs et al., 1995). As observed with globulin, under our conditions Hp did not seem to be activated by feed restriction since no differences were detected between diets (P = 0.25), days (P = 0.52), or interactions with day (Trt by day, P = 0.14). We observed a significant quadratic increase of Hp during AL that might be explained as due to possible inflammation in the catheter implantation site or to other unnoticed infection (Fig. 34). However, because of the large standard error of this result and the lack of correlation with other infection-related metabolites, the real reason behind this increase in Hp is not clear. On the other hand, SAA in plasma is associated with HDL because it helps transport of free cholesterol to damaged tissue for its repair (Liang & Sipe, 1995) and has other properties related with the immune system (Katoh, 2002). Similar to the response observed in Hp concentrations, there were no differences between diets (P = 0.44), among days (P = 0.47), or any interaction (Trt by day, P = 0.79) (Fig. 35).

Liver Function Indicators

Serum concentration of total bilirubin (TB) is an indicator of liver function during the homeostatic processes occurring during NEB (Trevisi et al., 2010; Zhou et al., 2016). High concentrations of TB in circulation can derive from an increase of its synthesis or, more likely, from a decrease of TB clearance (Reid et al., 1983). During periods of high fat mobilization, NEFA competes with TB for binding with albumin (Naylor et al., 1980) in the serum to be transported to tissues such as liver (Listowsky et al., 1978). Therefore, in a feed restriction condition as is our experiment, we would expect higher concentrations of TB in serum due to the impaired clearance function of the liver (Reid et al., 1983) and also because of the decrease in albumin observed (Fig. 31). The TB concentration in the RC group was 58% higher ($P = 0.013$) than AL. Total bilirubin increased across time ($P = 0.04$, Fig. 36) during feed restriction, showing a weak quadratic tendency ($P = 0.12$) that reached its peak at d 3 and smoothly decreased thereafter until d 5, which is a similar pattern to NEFA (Fig. 18). Even though Ohgi et al. (2005) only detected a slight increase of TB in the first week postpartum, the authors found higher TB concentration in serum from cows with higher fat liver content. This positive correlation between TB in serum and liver fat concentration also was proven by other studies (Bertoni et al., 2008; Reid et al., 1983; West, 1990). Hence, TB has a fast response to fat mobilization, which makes it also a good indicator of liver function during short periods of deprived feeding.

Total alkaline phosphatase (APT) has been used as another signal for impaired liver function (Aiello, 2016). Alkaline phosphatase is formed by a group of isoenzymes that are synthetized in the liver and bones, which have a long half-life (Sato et al., 2005). However, the range of values for activities found in serum is wide due to the number of non-nutritional and

physiological factors involved: blood group, age, sex, seasonal fluctuations, and many others. (McComb et al., 1979). Regarding the factor we are concerned about, even the difference between lactation and pregnancy is not clear. Gahne (1967) found lower APT activity after calving, while Sato et al. (2005) reported higher activities during the entire lactation in comparison with the dry period. In contrast, Dann et al. (2006) did not observe any effect of prepartum dietary energy on APT. More recently Bertoni et al. (2008) found no difference during 28 d post-partum between cows with different levels of liver activity index, which is based on the response of acute-phase proteins. Nevertheless, APT activity (36.5 U/L) during RC tended weakly to be lower ($P = 0.18$) than during AL (39.9 U/L). However, the difference gains significance if we consider the whole group of RES, and APT activity was lower during diet restriction ($P = 0.042$). While APT for AL stayed invariable ($P = 0.64$), for RES it decreased (RES by day, $P < 0.0001$) across period (Table 4) linearly ($P = 0.002$, Table 5) as RC did (Fig. 37). These results are in agreement with Li et al. (2016) who observed a numerically lower serum APT activity in ketotic cows (clinical and subclinical) than in healthy postpartum cows, and showing activities in the same range as ours. Again, the short period of time or the high variation, as in Li's study, probably did not allow more significant differences but at least the tendency seems to a lower activity of APT during periods of NEB. Since increased activities of APT would be expected as a sign of liver damage (Aiello, 2016), it seems that the lower activities probably derive from the malnutrition (McComb et al., 1979; Miller Wj Fau - Martin et al., 1969) rather than from hepatic impairment in the conditions of our study.

Aspartate aminotransferase (AST) is another enzyme that is valuable to evaluate liver function. AST catalyzes the transfer of the amino group from 2-oxo-glutarate to aspartate resulting in creation of glutamate and oxaloacetate (Bender, 2012d). Higher serum activities of

AST means that there is a leakage into the blood due to damage in the liver cell membrane (Reid et al., 1983). However, AST is not liver specific so high activities of AST could also mean damage in other tissues (Reid et al., 1983). In consequence, increasing activities of AST have been positively associated with high fat accumulation in the liver and metabolic disorders (Bobe et al., 2004; Dann et al., 2005; Ohgi et al., 2005), as well as with high blood concentrations of NEFA and BHBA (Li et al., 2016). In fact, it has been reported in numerous studies that AST increases sharply after calving and decreases progressively until reaching the lowest values during the dry period (Bertoni et al., 2008; Ohgi et al., 2005; Van Den Top et al., 1996; Weber et al., 2015; Zvonko Stojević & Maja Zdelar-Tuk, 2005). Dann et al. (2006) tested different combinations of diets during far off and close-up prepartum, and detected that cows fed with a diet with 150% of the energy requirements (NRC, 2001) during the far off period had higher AST activities than cows fed with a diet meeting 100 or 80% of the requirements. However, in the conditions of our study, we did not find any difference (P = 0.72) between cows fed ad libitum or restricted diets, and neither any significant change across time in either condition (Table 5, Fig. 38). As a matter of fact, AST activity decreased during all treatments (AL included; Table 4), but only the RES grouping showed a quadratic decreasing trend (P = 0.009) with its peak on day 3.

Gamma glutamyl transferase (GGT) is an enzyme that participates in glutathione breakdown, forming cysteine and in this way keeping a correct oxidative stress balance (Yokoyama, 2007). Therefore, its activity could be increased due to greater oxidative stress such as during the transition period (Drackley et al., 2005). The GGT also acts as mediator catalyzing the transfer of a gamma-glutamyl group to different molecules such as water, AA, and peptides (Schulman et al., 1975). Nevertheless, in many studies there were no differences in GGT serum

activities between healthy cows and cows with different levels of ketosis or liver function (Bertoni et al., 2008; Dann et al., 2005; Gröhn et al., 1983). In contrast, Ohgi et al. (2005) detected a higher activity postpartum in cows with higher liver fat. However, in this last study the values of cows with higher liver fat [18 to 27 U/L] were even lower than the group with the best liver function [27.5 to 29.3 U/L] in Bertoni et al. (2008), making it even more complicated to draw conclusions. In addition, GGT activities were not different before and after calving among different far-off diets or even between ad libitum and restricted rations during the close-up period (Dann et al., 2006). In our study, there were no differences ($P = 0.98$) of average activities of GGT between AL and RC, with no significant patterns of evolution over time in either treatment (Table 5, Fig. 39). However, considering the group of RES, restricted intake tended ($P = 0.099$) to increase quadratically ($P = 0.001$) the GGT activity. Nevertheless, GGT total activities in AL and RC (28.4 and 29.5 U/L) were above the reference range (6 to 17.4 U/L; Aiello, 2016).

Glutamate dehydrogenase (GLDH) is a liver-specific enzyme that, together with glutaminase, represents the biggest mechanism for elimination of NH_3 in the form of urea, via the glutamate pathway (Bender, 2012b). Activity of GLDH converts ammonium and 2-oxo-glutarate into glutamate (Bender, 2012b). To prevent any NH_3 passing into circulation, GLDH is located in the mitochondrial matrix of the hepatic cells bordering the central vein that drains the liver (Bender, 2012c; Reynolds, 1992). Even though activity of GLDH is not altered by moderate infiltration of fat in the liver (Reid et al., 1983), GLDH is considered an indicator of liver health (Bobe et al., 2004; Wemheuer, 1987) since it would mean that the damaged liver cells are leaking the enzyme into the blood (Aiello, 2016). For instance, several studies have reported an increase in GLDH serum activity during the early postpartum period (Hoedemaker et al., 2004;

Reist et al., 2003; Weber et al., 2015).Therefore, in almost all situations GLDH is positively correlated with NEB (Hoedemaker et al., 2004; Reist et al., 2002), with impaired liver functioning, or with metabolic disorders (Stengärde et al., 2010). During our trial, there were no differences ($P = 0.21$) in GLDH serum activities between AL and RC groups and neither was there significant variation across period (Trt by day, $P = 0.94$). Only an effect of day ($P < 0.0001$) was observed, which means that GLDH decreased during all the experiment, similar to the effect observed for AST (Fig. 38). However, RC manifested a linear ($P = 0.042$) decreasing evolution, as did the whole group of RES (Table 5), while AL did not change (Fig. 40). The big difference between both groups already on d 1 possibly prevented finding significant differences due to that trend. Our results are in accordance with Kuhla et al. (2009), who found a downregulation of glutamate dehydrogenase 1 (GLUD1; EC 1.4.1.3) after 60 h of feed restriction (only chopped straw provided to avoid rumen collapse). The reduction of circulating ammonia by this and other mechanisms, together with the reduction of TCA intermediates that would reduce the presence of 2-oxo-glutarate available for synthesis of glutamate (Noro & Wittwer, 2012), may explain why GLDH decreased during feed restriction. It is important to mention that in our study, the total activities in AL and RC (51 and 59 U/L, respectively) are in the upper edge of the ranges observed in other studies.

Protein Metabolism Variables

Moving forward to analyze the effect of feed restriction, we next analyze the consequences on protein metabolism and it adaptations to counteract dietary energy and protein deficiencies. Urea is one of the N compounds derived from catabolism of AA, together with purines, uric acid, and ammonium salts (Bender, 2012c). Enzymes participating in the urea cycle are activated when there is an excess of protein intake in order to eliminate the overload of

ammonium from the AA catabolism. In addition, those enzymes are also activated by glucagon and glucocorticoid hormones in fasting situations (Bender, 2012c; Schoneveld et al., 2007) to eliminate ammonium produced by the AA catabolized for gluconeogenesis, as reported by Kuhla et al. (2009) after feed restriction during 2.5 d or by Loor et al. (2007) after 10 to 14 d of feed restriction. Plasma concentrations of urea have been positively correlated with better liver function (Bobe et al., 2004; Reist et al., 2002; West, 1990) because TG infiltration in hepatic cells impaired ureagenesis (Strang et al., 1997) and ammonia reduced the capacity to produce glucose from propionate (Drackley, 1999; Overton et al., 1999). Moreover, Bertoni et al. (2008) also found lower plasma urea concentration in cows with lower acute phase response after calving. Therefore, in healthy cows we expect to observe an increase of plasma urea concentration after calving in comparison with during the dry period (Fiore et al., 2015; Weber et al., 2015) due to the inherent energy shortage of this stage.

In our study, daily plasma concentration of urea for RC (530 µmol/dL) tended to be higher ($P = 0.077$) than its concentration for AL (426 µmol/dl). Urea increased ($P < 0.0001$) in a quadratic trend ($P < 0.0001$), rapidly reaching its peak at d 2 with a 33% increase, and decreasing progressively thereafter (Fig. 41). The same effect was observed during all RES treatments (RES by day, $P < 0.0001$). The fast increase and recovery probably did not allow for higher significant differences of total concentrations of urea in our study and in other feed restriction studies (Carlson et al., 2006; Laeger et al., 2012) or even during the onset of lactation (Osorio et al., 2014; Zhu et al., 2000). However, plasma ammonia concentrations probably are a better indicator of liver function than urea in cows during periods of NEB as suggested by Zhu et al. (2000). Nonetheless, ammonia was not different in RC than in AL in our study ($P = 0.29$), and no relevant evolution across time was detected in any of treatments (Trt by day, $P = 0.24$, Fig.

43). Ammonia concentration in plasma decreased during the post-feeding phase with no important differences between treatments (Fig. 44) and was higher at night, exhibiting an inverse progression within day compared with urea, which increased during the post-feeding hours (Fig. 42). This coupled behavior within day demonstrates the ammonia detoxification function of urea, which increases quickly after the peak of ruminal ammonia due to feed fermentation in the rumen (Bender, 2012c; Gustafsson & Palmquist, 1993). Therefore, it seems that the higher concentration of urea during feed restriction periods serves to keep a constant optimal level of ammonia in blood. This mechanism seems to not be activated during post-calving in agreement with the higher ammonia concentrations in blood observed by Zhu et al. (2000) and with Hartwell et al. (2001), who reported no activation of the genes responsible for ureagenesis after calving.

Glutamate

Glutamate (Glu), together with Gln, are the two main AA responsible for the transamination among AA and also for the detoxification of nitrogen in form of ammonia (Bender, 2012b). Glutamate synthetized in the liver can be later be deaminated again by GLDH or transaminated into Aspartate (Asp), participating in the urea and TCA cycles (Watford, 2000). Asp, oxaloacetate, α-ketoglutarate, and Glu are interconnected due to their participation in ureagenesis, the TCA cycle, and gluconeogenesis in the liver (Noro & Wittwer, 2012). One mole of NH_4 and 1 mol of the amino group from Asp are needed to produce 1 mol of urea (Noro & Wittwer, 2012). Glutamate, besides providing α-ketoglutarate, which is another intermediate of the TCA cycle (Bender, 2012a), also provides the α-amino N required to synthetize Asp from oxaloacetate (Lobley et al., 2000; Reynolds, 1992). The double withdrawal of oxaloacetate from the urea and TCA cycle is compensated by its regeneration from the malate produced by

fumarate released in the urea cycle (Noro & Wittwer, 2012; Reynolds, 1992). In normal conditions, more oxaloacetate is synthetized than is required for both mechanisms. Therefore, after decarboxylation, it can be used as phosphoenolpyruvate (PEP) to produce glucose or to re-enter the TCA cycle as acetyl-CoA once dephosphorylated to pyruvate (Bender, 2012b). However, when an imbalance in the availability of any of the intermediates in the urea cycle occurs, the NH_4 cannot be removed and it accumulates in tissues (Visek, 1984).

In this study, plasma concentration of Glu tended to be lower (P = 0.095) during RC than during AL. While Glu concentration remained invariable (P = 0.21) during AL, in RC treatment there was a decreasing effect across time (P = 0.005) that manifested a quadratic trend (P = 0.015, Fig. 45). Within day, plasma Glu concentration had a significant variation (RC by hour, P = 0.002) and it was higher in the time points after feeding (Fig. 46) than in samples taken pre-feeding, and was lower again at night (2300 h). In contrast, Glu remained invariable during the fed condition (AL by hour, P = 0.17, Fig. 46). Because insulin also was higher after feeding (Fig. 21) and insulin drives the circulating AA towards peripheral tissues for anabolic processes (De Koster & Opsomer, 2013; Lobley et al., 2000; Noro & Wittwer, 2012), it seems that Glu, as concluded by Egan. (1968), is derived primarily for gluconeogenesis rather than for protein synthesis during milk synthesis and other anabolic processes.

Proline

Proline (Pro) is a semi-indispensable AA since it can be synthesized from Arginine (Arg) via Glu (Basch et al., 1997; Bequette, 2006). Because synthesis in the liver is not sufficient to meet the requirements for milk protein, Arg extracted by the udder is used as precursor of Pro (Bruckental et al., 1991). Since Pro is the AA with greatest abundance in the body, due to its function in collagen synthesis (Bender, 2012b; Stein et al., 1999), its demand may overcome

supply. In a nutrient stress situation, Pro can be metabolized to be used as metabolic fuel since proline oxidase will be activated, yielding Glu and then α-ketoglutarate, which is a TCA intermediate (Bender, 2012b; Gropper et al., 2009). Even collagen can be catabolized in order to supply more Pro (Phang et al., 2008). Hydroxyproline (OH-PRO) is an amino-acidic compound that participates in collagen synthesis and also is a marker of bone turnover (Peterson et al., 2005). The OH-PRO is formed from the residue of Pro when it is metabolized only during protein synthesis, and also can be oxidized by proline oxidase yielding Glu and pyruvate.

In our study, plasma concentration of Pro decreased (-26%) during RC (RC by day, $P < 0.0001$) in a quadratic trend ($P = 0.005$, Fig. 47), whereas it did not vary (AL by day, $P = 0.13$) during AL treatment. However, this decrease did not allow us to find a significant difference between average daily Pro concentration ($P = 0.24$). On the other hand, OH-PRO was neither affected by feed intake ($P = 0.22$) nor by day ($P = 0.10$) in any of the treatments, and there were no interaction ($P = 0.66$) with day. However, Larsen et al. (2014) found a quick but sharp increase of OH-PRO at 4 DIM in comparison with prepartum values, which decreased thereafter. Therefore, it seems that there were no variation in the synthesis and catabolism of OH-PRO even though its precursor Pro did decrease during feed restriction. Accordingly, Pro concentration around calving also shows a decrease during prepartum with a nadir on the day of calving, and then an increase to recover prepartum values quickly in early lactation (Larsen & Kristensen, 2009; Maeda et al., 2012; Meijer et al., 1995; Verbeke et al., 1972; Zhou Z. et al., 2016). In contrast, Laeger et al. (2012) did not find any variation after feed restriction. Within day, Pro decreased after feed delivery and then started to recover 14 hours after (2300) (Fig. 48) during all treatments (Table 6).

Asparagine

Asparagine (Asn) is a dispensable AA than is synthesized from Asp and therefore can also be degraded back to Asp and NH_3 (Wu, 2013). The NH_3 can be taken up by α-ketoglutarate or pyruvate to form Glu or Ala respectively, and the carbon skeleton goes to oxaloacetate (Bender, 2012g; Gropper et al., 2009). Even though its chemical structure is very similar to Gln, Asn is not degraded by the enterocytes (Wu, 2013). Although statistically non-significant, Laeger et al. (2012) found a decrease in plasma Asn concentration after 4 d of feed restriction. Similarly, we detected no significant lower total concentration during RC ($P = 0.47$) in comparison with AL treatment. However, Asn concentration did decrease (-37%) across days ($P < 0.0001$) in a quadratic trend ($P = 0.001$, Fig. 51), reaching its nadir at d 3. Meanwhile, there were no interaction between AL treatment and day ($P = 0.32$), with Asn remaining stable during the 5 experimental days (Table 4). Asparagine also shows a decreasing plasma concentration around calving and a posterior recovery during early lactation (Doepel et al., 2002; Maeda et al., 2012; Meijer et al., 1995; Verbeke et al., 1972). Concentration of Asn also decreased after feed delivery without differences between treatments (Table 6, Fig. 52)

Histidine

Histidine (His) is an indispensable glucogenic AA since during its catabolism it yields α-ketoglutarate as intermediate for the TCA cycle (Gropper et al., 2009). On the other hand, His also is a precursor of the central neurotransmitter histamine (Bender, 2012f). Histamine plays a role in the gastrointestinal tract as secretagogue of gastric secretions, and in the immunologic system (Gropper et al., 2009). Histidine also is required for synthesis of carnosine (in reaction with β-alanine), some of whose functions are still unknown but it is supposed to act within cells as buffer and antioxidant, and also helping during muscle contraction (Artioli et al., 2010;

Derave et al., 2010). Since it is present also in food, it can be a source of His and β-Alanine during digestion (Gropper et al., 2009). On the top of that, His seems to have a tremendous influence on milk protein synthesis and may be a limiting AA in some lactation rations (Bequette et al., 2000; Huhtanen et al., 2002).

During our trial we detected a weak tendency ($P = 0.17$) for a lower concentration of plasma His during RC treatment. Histidine concentration decreased ($P < 0.0001$) from d 1 to d 3 (-26%) and then remained at the same level until d 5, therefore manifesting a quadratic trend ($P < 0.0001$, Fig. 53). As observed for the rest of AA, His concentration during AL remained constant during the 5 d, however, His seemed to be affected by AL (AL by day, $P = 0.035$) since there was a significant decrease from d 4 to d 5 (Fig. 53). This singular alteration was due to only one cow that had a remarkable drop (-47%) of His concentration from d 4 to d 5 without a logical explanation. Histidine concentration decreased slowly after feeding and increased again at the night sampling (Fig. 54, Table 6).

Results from the literature are diverse. Histidine concentration did not decrease after feed restriction or even after starvation in mid-lactation cows (Baird et al., 1972; Laeger et al., 2012). However during the transition period and early lactation His seems to undergo a high uptake by the mammary gland (Larsen et al., 2015) or a deficiency of His since arterial levels did not return to pre-calving levels in most studies (Doepel et al., 2002; Maeda et al., 2012; Meijer et al., 1995; Zhou Z. et al., 2016).

Another relevant function of His in body protein is that it is methylated when the muscle contracting proteins (actin and myosin) are broken down during protein mobilization (Gropper et al., 2009; Plaizier et al., 2000), after activation of the ubiquitin-proteasome pathway (Hasselgren & Fischer, 1997) by the decrease of insulin (Chen et al., 2011) and higher presence of cytokines

and glucocorticoids during fasting (Dardevet et al., 1995; Hasselgren, 1999; Hasselgren & Fischer, 1997). This new form is called 3-methylhistidine (3-MH) and it cannot be recycled for protein synthesis. Therefore, it has to be excreted, and as such it is used as an index of protein mobilization (Blum et al., 1985; Doepel et al., 2002). The 3-MH increased in both treatments across days (Table 4); however, during RC the concentration increased continuously and it was greater than during AL (Fig. 55). During RC, 3-MH began to increase on d 2 and reached its peak on d 5. Nevertheless, 3-MH has to be considered as an indicator of tissue protein turnover rather than simply mobilization. The ratio 3-MH:creatinine would give a better insight of this mechanism, because creatinine has a positive strong correlation with body muscle tissue mass (Costa e Silva et al., 2014). Creatinine concentrations were not measured in this study, but we would predict that during AL, creatinine in circulation would remain constant or increase, indicating a higher synthesis than degradation. On the other hand, during RC creatinine would probably have decreased, resulting in a greater ratio of 3-MH:creatinine as expected during NEB (Doepel et al., 2002), indicating higher protein degradation than synthesis. Moreover, 3-MH is not a proportional indicator of amount of muscle mobilized because its incomplete recovery in urine suggest a pool of non-protein bound 3-MH in muscle (Harris & Milne, 1980; Lobley, 1998), as occurs in pigs (H.N.A. van den Hemel-Grooten et al., 1996) . On the top of that, muscle degradation occurs first in non-contractile myofibers, and then if any in contractile protein (MacLean et al., 1994). Therefore, amount and rate of degraded muscle cannot be accurately assessed using only 3-MH.

Another methylated form of His is called 1-methylhistidine (1-MH). Together with β-alanine they form anserine, which is a methylated form of carnosine that also is present in skeletal muscle (Houweling et al., 2012). Like 3-MH, during carnosine catabolism, 1-MH is also

eliminated in urine since it cannot be recycled in the body (Bender, 2012f). No difference in concentration of 1-MH was found between RC and AL (P = 0.57), and no significant interactions either (P = 0.13). However, AL again showed a linear increasing trend (P = 0.033), while RC increased until d 3 and decreased thereafter, manifesting a quadratic trend (P = 0.019, Fig. 57). Similarly, Laeger et al. (2012) found increases in 1- and 3-MH after feed restriction. While, as mentioned previously, 3-MH and 1-MH must be excreted through the urine, β-alanine can be catabolized to yield acetyl-CoA in tissues (Wu, 2013). However, no significant differences were found during feed restriction in RC (Table 3, Fig. 59) and no interactions of any treatments with day (Table 7) either. Consequently, no big differences were detected in carnosine concentrations between AL and RC (P = 0.99) and neither were there interactions with day (Table 3). Nevertheless, during RC, carnosine (-9%) and β-alanine (-15%) showed similar weak decreasing quadratic trends (Fig. 61 and 59 respectively) that matched the quadratic increase of 1-MH (+8%); in contrast there were no changes during the AL treatment. The same opposite pattern across time between carnosine and 1-MH was reported by Laeger et al. (2012). These results suggest that carnosine likely was effectively degraded during restriction, releasing β-alanine that also was degraded and 1-MH that was excreted.

Alanine

Alanine (Ala) is a dispensable AA that can be a precursor for pyruvate and yield the N-group for Glu synthesis (Gropper et al., 2009). Therefore Ala is considered a glucogenic AA. It is part of the important inter-organ connection called the Glucose-Alanine cycle, in which pyruvate receives the N-group from BCAA transamination in muscle to form Ala, and this Ala later yields the N-group for α-ketoglutarate and pyruvate in the liver (Gropper et al., 2009). This mechanism gains importance during NEB because it makes available Glu and pyruvate for

glucose synthesis and energy (Wu, 2013). For this reason is has been considered as one of the principal gluconeogenic precursors during the transition period (Bergman, 1978; Galindo et al., 2015; Overton, 1998). In this study, Ala decreased (-18%) ($P < 0.0001$) from d 1 to d 5 in a quadratic trend ($P < 0.0001$, Fig. 63), with d 3 presenting the biggest difference between treatments. Even though Ala concentration in AL treatments showed a linear decreasing trend ($P = 0.05$), there was no interaction between AL and day ($P = 0.34$). In addition, since in RC the Ala concentration increased a small amount by the end of the period, possibly due to release from muscle, Ala concentration was not different on d 4 and 5 between treatments. A similar decrease (-13%) was reported by Laeger et al. (2012) after feed restriction, while Baird et al. (1972) found a bigger decrease (-37%) after 6 d of complete starvation. Alanine also showed a decreased concentration from pre-calving toward calving day, and a corresponding increase during lactation (Larsen & Kristensen, 2009; Maeda et al., 2012; Meijer et al., 1995)

Branched-Chain Amino Acids

Contrary to other essential AA, the branched-chain AA (BCAA) are metabolized to common intermediates (Bender, 2012d; Bequette, 2003). Leucine is ketogenic (i.e., forms acetyl-CoA and acetoacetate), Ile is both ketogenic and glucogenic (forms acetyl-CoA and propionyl-CoA) and Val is just glucogenic (forms succinyl-CoA) (Bender, 2012d; Wu, 2013).

During shortage of dietary energy as in the fasting state, the pyruvate that comes from glycolysis of muscle glycogen receives the amino group from BCAA, forming Ala that will be later metabolized in the liver for gluconeogenesis or Gln to eliminate the amino group (Bender, 2012d). Unlike most AA, transamination of the BCAA occurs mostly in the skeletal muscle instead of in the liver (D'Mello, 2003). The keto-acids yielded from this transamination are then either transported to the liver to be back transformed to the AA (reacting with alanine), or can

also be oxidized in the muscle as a fuel source (Bender, 2012d; D'Mello, 2003). During fasting, the multi-unit complex of enzymes that form the branched-chain keto-acid dehydrogenase increases its activity, allowing for a greater capacity for BCAA oxidation in muscle (Bender, 2012d). Furthermore, during fasting the oxidation of branched-chain keto-acids is increased due to the lower expression of branched-chain keto-acid kinase (Harris et al., 2001; Joshi et al., 2006). The BCAA are also more oxidizable in the mammary gland than other essential AA, at least in sows (Kim & Easter, 2003). On the other hand, BCAA also helps to develop a proper immune response since those are required for synthesis of acute-phase proteins, immunoglobulins, and cytokines, among other proteins (Calder, 2006; Zhou Z. et al., 2016).

In our study, no differences were observed between RC and AL for the daily mean concentrations of any of the BCAA (Table 3). However, during RC, all BCAA had a decreasing (quadratic for Val and Leu, linear for Ile; Fig. 65, 67, and 69) interaction with day during RC (Table 7). In general, BCAA plasma concentrations decreased linearly until d 3 and thereafter remained steady until the end of the experimental period. Meanwhile in AL, plasma concentrations of BCAA had no variation across days (Table 7) except for Valine ($P = 0.049$), even though it did not manifest any significant dynamic trend during the 5 days (Fig. 65, Table 5).

These results are in accordance with other studies that found a significant decrease of BCAA during the onset of lactation when energy requirements are not met (Dalbach et al., 2011; Larsen et al., 2015; Maeda et al., 2012; Zhou Z. et al., 2016). Moreover, Larsen et al. (2015) found a higher catabolism of BCAA in the portal drained viscera (PDV) and mammary gland when greater supply of AA is provided during the beginning of lactation. Nevertheless, our results contrast with other studies with feed restriction performed in mid-lactation cows where

BCAA concentration increased after the feed restriction period (Baird et al., 1972; Laeger et al., 2012), especially Leu. This effect could be due to greater muscle and peripheral tissue mobilization. Furthermore, in both studies there were only two time points compared (pre-starvation and post-starvation), therefore we cannot conclude whether there was an earlier decline in the BCAA plasma concentration.

The BCAA, especially Leu, also act as signals of the availability of AA, together or separated with insulin after feeding (Bender, 2012d; Bequette, 2003). Therefore, the BCAA also stimulate the synthesis of protein (Bender, 2012d; Escobar et al., 2006).

In our study, the 3 BCAA decreased after feeding but only Leu and Ile had a significant decrease of plasma concentration (Table 6, Fig. 68 and 70), with no difference between treatments (Table 3). Valine had no significant variation within day (Table 6, Fig. 66). These results are in accordance with the anabolic effect that insulin exerts over the AA after feeding, which drives them toward tissue uptake (Lobley, 1992) to synthesize protein.

Aromatic Amino Acids

Phenylalanine (Phe), Tyrosine (Tyr) and Tryptophan (Trp) belong to this special classification because their structure is characterized by side chains containing aromatic rings (Wu, 2013). Only Phe and Trp are dietary indispensable because Tyr can be synthesized easily from Phe (Bender, 2012c). Both Phe and Tyr are both glucogenic and ketogenic since each can be metabolized to produce fumarate, which is a glucose precursor, and acetoacetate (Gropper et al., 2009). Phenylalanine hydroxylase, which is the enzyme that catalyzes the conversion from Phe to Tyr, is increased by glucagon and inhibited by insulin (Gropper et al., 2009). Degradation of Tyr is regulated by tyrosine transaminase, whose activity is induced by cortisol and other

glucocorticoid hormones, and also by its own concentration; Tyr then is converted to fumarate and acetoacetate (Bender, 2012c; Gropper et al., 2009). Therefore, in a feed restriction condition as the one established in our study, it is expected that a higher degradation of Phe will occur due to the observed decrease in insulin concentration (Table 5, Fig. 20). In addition, the concomitant increase of Tyr concentration and the possible higher plasma cortisol concentration (not measured in this study) will drive greater Tyr degradation to increase gluconeogenesis from these AA (Bender, 2012c) and reduce protein synthesis (Doepel et al., 2016). In our study, the role of Phe as precursor of Tyr was detected through their high correlation (r = 0.61, Table 10), which was the highest correlation for Phe with any compound.

During this study, total concentrations of Phe (P = 0.032) and Tyr (P = 0.001) were lower during RC treatment than AL (Table 3). Plasma concentration varied across periods (Table 7) for both AA during restriction (Fig. 71 and 73). Both Phe and Tyr decreased quadratically (Table 5) reaching a nadir on d 3. In contrast, during AL treatment Phe and Tyr plasma concentrations did not vary across days (Table 7). Within day, both AA decreased after delivering feed (Fig. 72 and 73, Table 6) without differences between treatments. This decrease during the postprandial phase corresponds to the increase of insulin concentration after feeding that drives the circulating AA into insulin sensitive tissue for protein synthesis (De Koster & Opsomer, 2013; Lobley et al., 2000). Therefore, the lower daily concentration of insulin and higher values of glucagon and perhaps glucocorticoid hormones occurring during feed restriction lead to greater degradation of Phe and Tyr (Bender, 2012c; Gropper et al., 2009). Laeger et al. (2012) did not find any effect on Phe concentration after 4 d on feed restriction (50% of the previous ad libitum intake), but did find a tendency for a lower Tyr plasma concentration and also a lower Tyr concentration in cerebrospinal fluid (CSF). This finding suggests a potential central anorexic effect of Tyr

(Laeger et al., 2012). Tyrosine is a precursor of melanin in the skin, thyroid hormones in the thyroid gland, neurotransmitters (catecholamines) and hormone precursor in neurons and adrenal medulla, respectively (Bender, 2012c; Gropper et al., 2009). Among the catecholamines synthesized from Tyr, adrenaline, noradrenaline, and dopamine have a recognized anorexic effect (Laeger et al., 2012). Thyroid hormones and catecholamines also activate mechanisms to counteract the negative energy balance. However, during feed restriction synthesis of thyroid hormones seems to decrease (Capuco et al., 2001), while catecholamines in circulation increase in order to decrease milk production and increase lipogenesis (Salin et al., 2017). Therefore, this pathway seems to be highly dependent on Phe and Trp. Similar results have been found during the onset of lactation when energy balance is negative due to the lower intake and the increasing demand of nutrients for milk production. Plasma Phe and Tyr decreased at parturition and then recovered, as lactation advanced (Larsen et al., 2015; Larsen & Kristensen, 2009; Maeda et al., 2012; Meijer et al., 1995).

Tryptophan is also glucogenic and ketogenic since it can be metabolized to pyruvate and acetyl-CoA (Gropper et al., 2009; Wu, 2013). The first enzyme that initiates Trp degradation (tryptophan dioxygenase) is activated by glucagon and cortisol (Gropper et al., 2009) and is down-regulated by insulin (Nakamura et al., 1980). Subsequent reactions through the kynurenine pathway, which accounts for 95% of Trp degradation (Wu, 2013), end up forming Ala and acetyl-CoA. In addition, Trp plays an important role as precursor for the neurotransmitter serotonin and the hormone melatonin (Bender, 2012c). Tryptophan catabolism also produces nicotinamide adenine dinucleotide (NAD^+), which is a coenzyme form of the B-vitamin niacin (Bender, 2012c; Gropper et al., 2009) and is vital for the pathway of glycolysis due to its oxidative capacity (Gropper et al., 2009). Nevertheless, oxidation of Trp through the kynurenine

pathway is regulated by its own concentration, since the presence of Trp in liver activates tryptophan dioxygenase (Bender, 2012c). Therefore, lower Trp concentration will decrease degradation to spare Trp for the serotonin pathway. Serotonin plays a crucial role in regulating mammary gland metabolism, milk synthesis, and calcium homeostasis during the transition period (Hernández-Castellano et al., 2017). This saving mechanism could be one of the reasons why in our study we did not find a significant difference in Trp concentration between RC and AL ($P = 0.37$). However, as observed for Phe and Tyr, plasma concentration of Trp decreased (-18%; $P < 0.0001$) during RC until d 3 (Fig. 73), resulting in a quadratic trend; whereas Trp concentration was stable across days in AL ($P = 0.53$). Nevertheless, is important to mention that the decrease was linear ($P <.0001$) for the RES group. Like the other aromatic AA, Trp concentration decreased after feed delivery and increased again later during night sampling (Table 6), with no difference between treatments (Fig. 74).

Laeger et al. (2012) reported a decrease (-33%) in plasma concentration of Trp after feed restriction. Increased catabolism during the transition period and the onset of lactation also has been reported, analogous to the effects on Phe and Tyr, decreasing during prepartum until the calving day and later recovering as lactation begins (Maeda et al., 2012; Zhou Z. et al., 2016). Even more, the liver increases its removal of Trp during early postpartum period (Doepel et al., 2009; Larsen et al., 2015).

Threonine, Glycine, and Serine

There are 3 different pathways for Threonine (Thr) metabolism. One is driven by threonine deaminase, which is the most important during fasting and ends up synthesizing succinyl-CoA that is a precursor in the TCA cycle (Bender, 2012a; Gropper et al., 2009; Wu, 2013) or that can be used as precursor of Ile (Bender, 2012a). Also, Thr can be a precursor of

pyruvate when the concentrations of the AA are high (Gropper et al., 2009). Finally, Thr can be metabolized in the mitochondria to produce Gly and acetyl-CoA (Bender, 2012a; Gropper et al., 2009). Therefore, Thr is a strictly glucogenic AA.

In our trial, average daily Thr concentration was lower (-11%) for RC but was not statistically different from AL ($P = 0.28$). However, during RC the plasma Thr concentration was affected by the effect of day during restriction ($P < 0.0001$), showing a quadratic ($P < 0.0001$) trend, with the biggest difference from AL treatment on d 2 and 3 (Fig. 77). In contrast, Thr concentration during AL treatment remained steady during the 5 d ($P = 0.20$). Laeger et al. (2012) also reported a similar decrease (-12%) in plasma and a larger decrease in the CSF (-24%), which the authors suggested to mean that Thr acts as a potential signal for feed intake. Like the other indispensable AA discussed so far, Thr also decreases as calving is getting close and then increases as lactation advance (Larsen et al., 2015; Maeda et al., 2012; Zhou Z. et al., 2016). According with the literature, addition of extra Thr does not seem to have a positive effect, but neither does its deletion cause a negative effect (Doepel et al., 2016). This latter result suggests that even on a low protein intake, the cow is able to meet its Thr requirements.

Glycine (Gly) is a semi-indispensable AA, since even though it is possible to synthesize it from other metabolic components (such as the AA Thr and Ser, glyoxylate, and choline), under certain conditions Gly synthesis cannot meet the requirements (Bender, 2012e). The conversion between Gly and Ser is bi-directional, so Gly is considered a glucogenic AA since through Ser it can provide pyruvate as an intermediate in the TCA cycle (Bender, 2012e; Gropper et al., 2009). Moreover, Gly is a precursor of purines, porphyrins, glutathione, creatine, and the bile salt glycocholate (Bender, 2012e; Gropper et al., 2009). During our study, Gly concentrations had a strong tendency ($P = 0.056$) to be higher (+24%) during RC than in the AL treatment. Even

though Gly was higher since d 1 (Fig. 79), the difference with AL treatment was bigger on d 4 and 5 because Gly concentration increased ($P < 0.0001$) across time for RC but remained steady for AL (AL by day, $P = 0.33$). This considerably higher concentration or even no variation in Gly concentration during NEB has been reported widely in the literature. During an induced NEB through feed restriction, Laeger et al. (2012) did not find any variation of Gly concentration, whereas Baird et al. (1972) reported a non-significant decrease (-27%). It is important to remark that the latter authors applied a complete feed restriction (starvation) while Laeger et al. (2012) just used a 50% restriction compared with the measured ad libitum intake. Regarding the transition period, several authors reported an increase from prepartum to calving day and an onward decrease as lactation began (A. Pechova et al., 2000; Doepel et al., 2002; Larsen et al., 2015; Maeda et al., 2012; Meijer et al., 1995; Zhou Z. et al., 2016) or no variation across the transition period (Larsen & Kristensen, 2009; Verbeke et al., 1972).

At least in the current study, it seems that Gly was not importantly degraded for any purpose during feed restriction. Instead, it seems that it was synthetized from other components, probably from Thr during its pathway toward the synthesis of acetyl-CoA (Gropper et al., 2009) or also from choline (Wang et al., 2013). During the latter pathway, choline must be first be oxidized to betaine in the liver. Then, after donating its methyl group and reacting with folic acid, sarcosine is generated, which is a direct precursor of Gly (Gropper et al., 2009; Wu, 2013). Even though total sarcosine concentration was not different between treatments ($P = 0.96$), there were an interaction with day only during RC ($P = 0.002$), resulting in an increasing linear trend during RC (Fig. 81). Therefore, it is probable that this pathway of Gly synthesis was activated during feed restriction (Soloway & Stetten, 1953). Higher plasma Gly concentrations also may

be due to the increase of tissue mobilization since, together with Gln, both are the AA with greatest content in connective tissue in muscle (Verbeke et al., 1972; Zhou Z. et al., 2016).

Serine is a dispensable AA that is strictly glucogenic. Transamination of Ser can yield a precursor of Gly (glyoxylate) or a substrate for gluconeogenesis (3-phosphoglycerate) (Bender, 2012e; Wu, 2013). Deamination of Ser by serine deaminase produces pyruvate, which is also a required precursor for the TCA cycle. This enzyme is activated by glucagon and other glucocorticoid hormones and deactivated by insulin (Bender, 2012e). Serine can also participate in Met metabolism by reacting with homocysteine to generate cystathionine (Wu, 2013), and also during the synthesis of phospholipids producing ethanolamine and choline (Gropper et al., 2009). Even though there were no differences between treatments in our study ($P = 0.73$), the plasma concentration of Ser during RC treatment interacted with day ($P < 0.0001$), while during AL it remained constant ($P = 0.07$). The Ser concentration during RC manifested a clear quadratic trend ($P = 0.001$), decreasing sharply until d 3 (-22%), and then remaining practically constant until the end of the period (Fig. 83). Therefore, it seems that Ser could be also a precursor of Gly during this experiment. Even though Ser and Gly had different patterns across the period, Gly had the strongest correlation with Ser ($r = 0.55$, Table 10), suggesting a ready conversion between them regardless of time.

Baird et al. (1972) also found a lower plasma Ser concentration after 6 d of starvation, and Laeger et al. (2012) reported a decrease of Ser concentration only in CSF, suggesting a potential central anorexic effect like Thr and Tyr (Laeger et al., 2012). However, in several transition studies, Ser concentration was found to remain constant (Doepel et al., 2002; Larsen & Kristensen, 2009; Verbeke et al., 1972; Zhou Z. et al., 2016) around calving, or showing the same pattern as Gly, increasing during prepartum until calving day and then decreasing during

the onset of lactation (Maeda et al., 2012; Meijer et al., 1995). Probably, the wide range in the concentrations of some of the Ser precursors such as choline, glucose, and Glu (Sun et al., 2016; Wang et al., 2013) due to dietary and other conditions in experiments with transition cows lead to this different behavior of Ser in comparison with studies like ours with feed restriction. Both AA (Ser and Gly) decreased (Table 6) within day after feed delivery (0900) and recovered by night 14 h later (2300 h) (Fig. 80 and 84) denoting the anabolic process promoted by insulin in both treatments (Lobley, 1992).

Phosphoserine (P-Ser) is an intermediate in the phosphorylated pathway of Ser synthesis. In this pathway, Ser is synthesized from glucose and Glu (Bender, 2012e; Wu, 2013). However, P-Ser was not different between RC and AL ($P = 0.78$), and was not affected by day ($P = 0.9$). It is important to remark that P-Ser decreased to a bigger or smaller extent during all treatments (Table 7). Also, during AL P-Ser was strongly altered by the day effect ($P = 0.004$), decreasing in a linear trend (Fig. 85). This could be in relation to the slight decrease (AL by day, $P = 0.07$) of SER (-9%) concentration observed during AL treatment (Fig. 83).

Ethanolamine (EA) is a component of phospholipid that results from decarboxylation of Ser followed by methylation becomes choline (Gropper et al., 2009). Phosphorylation of EA yields phosphoethanolamine (PEA), which is a derivative of ethanolamine used in phospholipid synthesis. While during AL treatment none of these compounds was altered, during RC, EA decreased (RC by day, $P = 0.03$, Fig. 87) across the experimental period and PEA tended to increase (RC by day, $P = 0.06$, Fig. 89). The PEA is a constituent part of sphingolipids, which also play a relevant role in signal transmission and cell recognition (Bartke & Hannun, 2009). However, the more likely scenario would be that, during feed restriction, Ser degradation contributed to form EA and PEA during synthesis and degradation of choline (Maldonado et al.,

2014). Increasing concentrations of PEA can be due to decreased methylation since Met concentrations also decreased during restriction. This finding is in agreement with the increasing concentrations of sarcosine, which is an intermediate in the choline-to-glycine pathway (Cernei et al., 2013).

α-Amino butyric acid (α-ABA) is a non-protein AA that is synthesized from the transamination of α-ketobutyrate; therefore, it can be derived from the metabolism of Thr, Ser, Gly, and also Met. Since α-ketobutyrate has another glucogenic pathway, the transamination toward α-ABA it is considered an indicator of liver malfunction connected with hyper-catabolism of AA and hyper-aminoacidemia (Chiarla et al., 2011). In our study, α-ABA increased ($P = 0.001$) during the whole period in a linear (Fig. 91) trend during RC, similar to the response reported by Laeger et al. (2012). However, α-ABA also increased during AL treatment (AL by day, $P = 0.021$) in a quadratic ($P = 0.007$) manner, reaching the peak on d 4 and decreasing afterwards. The 3-MH concentration seems to be correlated with the concentrations of α-ABA during sepsis in humans (Chiarla et al., 2011). As observed in our study, both metabolites showed comparable linear increase during AL, matching the behavior during feed restriction. Therefore, probably the higher concentration of AA in the AL treatment promoted a higher protein synthesis and hence the higher AA catabolism of the excess.

Sulfur (S)-Containing Amino Acids

Methionine (Met) and Cysteine (Cyst) are characterized by their side chain containing a Sulfur (S) atom (Wu, 2013). Catabolism of Met ends up producing succinyl-CoA, so therefore Met is a glucogenic AA; during that catabolic process Met also yields Cys (Gropper et al., 2009). However, its most important role is that Met is the major donor of methyl groups through S-adenosyl methionine (SAM) for one carbon transfer reactions (Bender, 2012a; Zhou et al., 2016).

The methyl donor SAM is needed for synthesis of carnitine, creatine, epinephrine, melatonin, choline, anserine, 3-MH and polyamines (Bender, 2012a; Wu, 2013). On top of that SAM also is required for methylation of DNA as an epigenetic reaction, therefore influencing gene expression (Gropper et al., 2009). Due to the relation of Met with choline and carnitine, this AA plays a relevant role in fatty acid metabolism during NEB. Recent studies have indicated the important effects of choline and carnitine in the transition period. Choline promotes the uptake of NEFA by hepatocytes and is a necessary component of VLDL synthesis (as phosphatidylcholine), and carnitine is a required component for fatty acid β-oxidation in mitochondria (Goselink et al., 2013; Sun et al., 2016). These functions have been linked with a better performance in the postpartum transition during Met supplementation (Osorio et al., 2014; Zhou et al., 2016). Accordingly, during our study there seemed to be Met degradation during the RC treatment since Met plasma concentration decreased (-19%) along the 5 d ($P = 0.001$) in a linear trend (RC by day, $P = 0.004$, Fig. 93), with the biggest drop between d 1 and d 2 (-16%). In contrast, during AL concentrations of Met did not change (AL by day, $P = 0.60$) across the period. Nevertheless, probably due to the different initial values, there were no differences ($P = 0.77$) in Met concentrations between treatments. Laeger et al. (2012) reported also a non-significant change of Met concentration after feed restriction for 4 d. Regarding Met plasma concentration around calving, while some studies had reported a quick recovery from prepartum values after the nadir at calving day or no variation (Doepel et al., 2002; Larsen et al., 2015; Larsen & Kristensen, 2009; Maeda et al., 2012; Verbeke et al., 1972), other studies found a lower value during early lactation than in prepartum (Meijer et al., 1995; Zhou Z. et al., 2016). The fact that Met is an indispensable AA and is considered one of the most potentially limiting AA in Western diets (NRC, 2001) could be one of the reasons why such a difference exists among studies. Moreover,

because the first product of Met catabolism is SAM, which stimulates Met degradation itself (Gropper et al., 2009) and is related with lipid metabolism as reviewed previously, the wide range of conditions affecting the transition period could have led to these diverse responses in peripartal Met concentrations.

Cysteine is a dispensable AA that is synthetized from Met (Wu, 2013). Cysteine can be used as precursor for synthesis of glutathione, taurine, and pyruvate, and therefore it is a glucogenic AA (Gropper et al., 2009). Since it is a required step for glutathione synthesis, Cys (and hence Met too) has an important role regulating the intracellular antioxidant status (Luo & Levine, 2009; J. S. Osorio et al., 2014; Sun et al., 2016). Nevertheless, during our study total plasma concentrations of Cys had a weak tendency to be higher during RC than during AL (P = 0.15), especially on d 4 and 5 where Cys concentration was significantly higher (Fig. 95) for RC than for AL treatment. Laeger et al. (2012) also reported a numerical increase of Cys concentration after feed restriction. As observed for Met, there are different responses detected around calving. In general, it has been reported that Cys in most cases recovered to pre-calving concentrations quickly after calving, or even that Cys did not change significantly across the transition period (Larsen et al., 2015; Larsen & Kristensen, 2009; Meijer et al., 1995; Verbeke et al., 1972). However, Zhou Z. et al. (2016) detected that Cys did not recover the pre-calving values and remained low during the early post-partum. The role of Cys as precursor of glutathione could mark the difference between studies. The demand for glutathione as antioxidant around calving could be the major factor that determine the degradation of Cys and thus of Met. As in our case, it seems that 5 d of feed restriction did not require an excessive redox function so the demand for glutathione did not require catabolism of Cys. Moyes et al. (2009) also concluded that similar feed restriction conditions applied to mid lactation cows were

not sufficient to induce a significant oxidative stress. In fact, Cys concentration increased probably due to the higher metabolization of Met via glucogenic pathway to yield succinyl-CoA.

The role that Cys plays as the only AA able to inhibit the activation of mTORC1, and therefore inhibiting protein synthesis and promoting tissue degradation, could be crucial during NEB periods and accordingly should be a good indicator of tissue mobilization (Dyachok et al., 2016). Cystathionine (Cysta) is one of the intermediates within this pathway that is a result of the reaction of Ser with Homocysteine (Bender, 2012a; Wu, 2013). No differences were detected between AL and RC ($P = 0.93$). There was no significant interaction between RC and day ($P = 0.23$), however when considering all treatments under restriction (RES), diet had a significant interaction with day (RES by day, $P < 0.0001$) and manifested a quadratic ($P = 0.001$) decreasing trend (Table 5) during feed restriction (Fig. 97). This interrelated decrease of Met, Ser, and Cysta indicates that they likely were utilized during the Cys synthesis pathway. Cysteine also is a precursor of Taurine (Tau), which is mainly located in muscle and in the CNS (Gropper et al., 2009). Taurine is not involved in protein synthesis, but has important roles as antioxidant, tissue osmolyte, bile salt component, and inhibitory neurotransmitter (Bender, 2012a; Gropper et al., 2009; Kalhan & Marczewski, 2012). Concentration of Tau in plasma during RC treatment was not different ($P = 0.97$) than during AL, and no interaction with day was detected in either treatment (Table 7). Nevertheless, it is important to mention that Tau increased during the other treatments forming the RES group (Table 4 and 3), except in GLN. Even more, Tau only increased across the period in the treatment where Cys also increased and therefore showing a strong correlation in our experiment.

Lysine

Lysine is an in-dispensable AA that allows for synthesis of only ketogenic precursors (acetyl-CoA and acetoacetate), and so it is considered a strictly ketogenic AA (Wu, 2013). The most common pathway for Lys catabolism is the saccharopine pathway, which uses α-amino adipic acid (α-AAA) as intermediate for synthesis of acetoacetate. This same intermediate also is involved in Lys synthesis from oxoadipic acid in bacteria, yeast, and some fungi (Bender, 2012f). Once Lys is incorporated into muscle protein, it is a precursor for carnitine that is required for oxidation of fatty acids (Akbar et al., 2013; Gropper et al., 2009), and also is a precursor for de novo synthesis of Arg and Pro (Bequette, 2002). It seems that in our study, there was no excessive degradation of Lys since its daily concentration was higher during RC than AL ($P = 0.05$). Neither treatment had a significant interaction with day (Table 7), although the biggest differences were detected on d 4 and 5 (Fig. 101). Even though uptake of Lys by the liver does not change substantially in response to Lys supply (Lapierre et al., 2009), during calving increased hepatic uptake of Lys occurs (Larsen & Kristensen, 2009); uptake changes to a net release around 15 d after calving.

Nevertheless, the organ where most Lys uptake occurs is the mammary gland and it is taken up beyond the requirements for milk protein synthesis (Bequette et al., 1998; Raggio et al., 2006). Oxidation of the excess is supposed to provide the N-group for synthesis of other NEAA such as Arg and Pro (Bequette et al., 1998; Bequette et al., 2002; Lapierre et al., 2003; Roets et al., 1979). Lysine is considered in most Western diets as a limiting AA for milk protein synthesis (Clark, 1975; NRC, 2001; Schwab et al., 1976) and a positive performance response after Lys supplementation has been generally observed (Arriola Apelo et al., 2014; Robinson et al., 2010). Nevertheless, it seems that during the experimental period of this trial, there were an increased Lys concentration in circulation probably due to the higher tissue protein breakdown as observed

by Laeger et al. (2012) and Baird et al. (1972), who found an increases of Lys in plasma after feed restriction or starvation, respectively. Furthermore, the decrease of milk protein production (Fig. 5) suggests that Lys was not limiting for this purpose. Similarly, Swanepoel et al. (2010) also reported a lack of effect on milk components during early lactation. Other studies assessing AA concentration around calving showed that Lys decreased from the prepartum period until calving and increased afterwards (Doepel et al., 2002; Larsen et al., 2015; Larsen & Kristensen, 2009; Maeda et al., 2012; Verbeke et al., 1972). Mammary gland has the capacity to alter AA uptake to meet its requirements (Mackle et al., 2000). Specifically, Lys seems to have a regulated mechanism that allows the same mammary catabolism even when the mammary artery supply is diminished (Guo et al., 2017), probably by decreasing the uptake for milk protein synthesis since the Lys content in milk is rarely altered (Lapierre et al., 2009; Tucker et al., 2017), and driving it toward synthesis of other NEAA alike Arg, which was also higher during RC ($P = 0.008$).

Those findings agree with our results because Lys concentration in plasma was higher during RC, despite a relevant catabolism of Lys indicated by the tendency ($P = 0.12$) for greater total concentration ofα-AAA than during AL, which suggests a regulatory role of the excess of Lys in circulation by hepatic and/or extrahepatic oxidation (Tucker et al., 2017). The plasma concentration of α-AAA had an interaction with day ($P = 0.015$), which resulted in the biggest differences on d 4 and 5 (Fig. 103) in, accordance with the days of higher Lys concentration (Fig. 101). Therefore, it is probable that similar to what occurs when there is an excess of dietary protein (Broderick, 2003), the overload of Lys concentration in plasma would be degraded to produce energy through its carbon skeleton (ketone bodies and acetyl-CoA in this case) and then eliminating the 2 N via saccharopine (Wu, 2013), yielding 2 Glu for further metabolism. It is important to mention that, at least in vitro, the liver of ruminants (cattle and sheep) is able to

synthesize Lys from saccharopine (Fellows, 1973), and the activity of the enzyme required (saccharopine oxidoreductase) was higher than in non-ruminants (Fellows & Lewis, 1973). Even though concentrations of saccharopine are very low in circulation, its precursor α-AAA is available (Fellows & Lewis, 1973). Lysine concentration decreased after feeding (Fig. 102), with no differences between treatments (Table 6).

Urea cycle

The urea cycle takes place in the periportal hepatocytes and partially in the enterocytes and other extra-hepatic tissues (Bender, 2012c). Intermediates of the urea cycle are very tightly regulated (Morris, 2002). Some of them were measured in our study and will be discussed later in this section. The enzymes responsible for the urea cycle are regulated by glucagon, insulin, and glucocorticoids among other hormones (Morris, 2002). For instance, the uptake of the ammonium required for synthesis of carbamoyl phosphate is regulated by the mitochondrial carbamoyl phosphate synthetase (CPS-I), which is the main regulator of the urea cycle and is activated in response to glucagon and glucocorticoids (Bender, 2012c; Schoneveld et al., 2007). The urea cycle needs balanced entrance of carbamoyl-P and Asp (Lobley et al., 2000). To avoid the prejudicial consequences of an imbalance on any intermediate of the cycle, ruminant metabolism has its own mechanisms of compensation (Lobley et al., 2000). For example, 32% of the aspartate-N came from recycled ammonia in fasted sheep (Milano et al., 1996). It was also suggested that catabolism of AA can be down regulated in case of an excessive blood concentration of ammonia (Lobley et al., 2000).

Enzymes involved in the urea cycle are not expressed in all cells (Morris, 2002); therefore, its completion requires an interorgan coordination (Reynolds, 1992) between liver and extrahepatic tissues. A big fraction of the dietary Arg is transformed to Orn in the small intestine,

which is later converted in the liver to citrulline (Cit). Thereafter, Cit is transported to the kidneys in order to synthetize Arg (Bender, 2012b; Reynolds, 1992). Without this interorgan cooperation, most of the dietary Arg would be degraded by the liver to produce urea and Orn due to the high activity of arginase in the liver, leaving very little Arg available for other organs (Bender, 2012b; Curis et al., 2005). Moreover, since Arg regulates positively ureagenesis (Meijer et al., 1990), this coordinated adaptation prevents an unnecessary production of urea (Bender, 2012b; Curis et al., 2005).

Urea cycle equilibrium during a short-term feed restriction also was reflected in our study. The daily concentration Arg during the RC treatment was higher ($P = 0.008$) than that during AL during the entire 5-d experimental period, but this increase did not fit in any recognized trend across time, similar to AL (Fig. 105). This suggests that an equilibrium in Arg concentration was achieved, even with increasing and variable amounts of urea synthesized (Fig. 41, Table 3). According to our findings, higher ($P = 0.07$) and increasing (RC by day, $P < 0.0001$) Cit concentrations (Fig. 107) during RC were derived from Orn, which decreased (RC by day, $P = 0.007$, Fig. 109) in an opposite trend (linear; $P = 0.018$), capturing ammonia through the merger with carbamoyl-P. Citrulline would later condense with Asp to form Argininosuccinate (not analyzed in this experiment), which is an immediate precursor of Arg (Husson et al., 2003). Therefore, it seems that during our short-term period of feed restriction, urea synthesis completed its cycle and achieved an equilibrium state of ammonia and Arg at the expense of Asp, which showed a decreasing (RC by day, $P = 0.0001$) linear trend ($P < 0.0001$, Fig. 111) during the entire period.

As mentioned earlier, some of the enzymes catalyzing the urea cycle are regulated by insulin, glucagon, and glucocorticoids. For instance, glucagon and GH upregulate the production

of urea through the synthesis of argininosuccinate, which activates argininosuccinate synthetase (ASS) (Curis et al., 2005). The uptake of ammonia into carbamoyl-P activates f N-acetylglutamate (N-AG) synthetase, while insulin downregulates both enzymes. These known processes are in accordance with our results where the higher plasma concentration of urea coincided with the higher concentration of glucagon and the decreasing concentration of insulin during the first 3 d of feed restriction. The same mechanism seems to operate with the variations observed within day. Blood samples drawn after feeding showed lower concentrations of all metabolites implicated in the urea cycle than in pre-feeding samples (Table 6, Fig. 106 108, and 109), except Asp and urea itself (Fig. 112 and 42). From these results, we can extrapolate that during the absorptive phase the higher urea concentrations were due to the production from rumen fermentation (Atasoglu & Wallace, 2003).

The liver in mammals has a specific heterogeneity of morphology that ensures that little or no ammonia reaches circulation (Noro & Wittwer, 2012). The ammonia that escapes from GLDH located in the periportal cells is later metabolized in the perivenous cells where is incorporated to Glu and then converted to Gln by glutamine synthetase (Bender, 2012c; Noro & Wittwer, 2012). Glutamine has multiple metabolic functions in the body. It is the main energy substrate for dividing cells in the intestinal epithelium and in lymphocytes (Chwals, 2004), and also acts as neurotransmitter within the brain (Taylor & Curthoys, 2004). Muscle cells play a role as Gln reserves and are able to have up to 30 times higher Gln concentration than plasma (Meijer et al., 1993). During acute metabolic acidosis, muscle rapidly releases glutamine after the metabolic pH drops (Schröck et al., 1980) and uptake from the digestive tract decreases (Taylor & Curthoys, 2004). This interorgan coordination results in a significant increase of plasma Gln concentration (Bender, 2012b), which allows the kidneys to increase their uptake of Gln (Taylor

& Curthoys, 2004). The activity of glutaminase in the renal tubes responsible for the uptake of glutamine also is activated by metabolic acidosis (Curthoys & Gstraunthaler, 2001). The resulting Glu produced in the kidneys is catabolized by GLDH into ammonia to be expelled in the urine, and at the same time bicarbonate is formed to enter into circulation and compensate for the ionic imbalance (Bender, 2012b; Taylor & Curthoys, 2004).

Cows during RC treatment in our study had a faster and compulsive ingestion of the limited amount of feed, which resulted in them ingesting all the feed offered (~12.4 kg DM) in a very short period of time (~2 to 3 hours). This probably led them to encounter an acute drop of the ruminal pH, developing a consequent temporary acidosis that would result in the above-mentioned increase of Gln concentration in plasma. This seems to be the reason behind the different behavior of plasma Gln concentrations after feeding observed between AL and RC treatments in our study (Fig. 114). Even though no significant interaction of time with diet was established (P = 0.27), it is remarkable that in all restricted treatments (RES) Gln increased after feeding while it decreased during AL (Table 6). As a result, the biggest differences between diets occurred in the time points after feeding and especially at night (Fig. 114). Daily concentration of Gln was higher in RC (P = 0.03) and tended to be higher in RES (P = 0.089) than in AL, probably due to the higher requirements to detoxify ammonia concentration from AA catabolism (Meijer et al., 1990). Glutamine also is also tightly related with the urea cycle (Bender, 2012b). During fasting, intestinal cells take up plasma Gln to synthesize Cit besides Arg (Curthoys & Watford, 1995). The synchronized and opposite patterns of Cit (Fig. 107) and Gln (Fig. 113) across the period found in our trial seems to justify that urea formation was the preferred pathway at the beginning of the dietary restriction, probably due to the higher detoxification capacity of ammonia at lower energetic cost (Bender, 2012d; Noro & Wittwer, 2012; Reynolds,

2006), or just because of the higher ammonia resulting from AA oxidation (Haussinger, 1990). Urea synthesis might have been reduced later on in the period to save bicarbonate ions and maintain an adequate cation-anion balance (Meijer et al., 1993); however, since during fasting ruminal pH increases (Galyean et al., 1981), this option does not seem probable. During RC treatment, the peak Cit concentration and the nadir concentration of Gln coincide on d 2. This result suggests that there was an increased uptake of Gln to produce Cit, driving the expulsion of ammonia through urea. Around 75% of the ammonia is converted to urea while Gln accounts for the other 25% under normal physiological conditions (Haussinger, 1990). However, as days pass it seems that urea synthesis decreases and Gln increases, suggesting a switch in the preference for a low-capacity high-affinity method to detoxify ammonia as Gln (Lobley et al., 1995). Interestingly, the negative correlation between NH_3 and Gln was greater than the correlation between NH_3 and urea in our study (Table 10). This relationship likely reflects the tremendous importance of Gln in N metabolism.

Accounting for the loss of amino-N due to the decrease of all the individual AA analyzed, and considering the increase of urea, Gln, and the other AA that increased during fasting (Lys, Cyst, Arg, Cit, Tau, α-ABA, sarcosine), there seems to be a large shortfall of amino-N sources to trigger the increase in urea observed during feed restriction. The increase from d 1 to d 5 of amino-N (~30 μg/dL) found in blood was very similar to the increase registered in milk (~37 μg/dL). However, the assumed amount of amino-N provided by cleavage of the AA in circulation only represents around 15% of the urea increase. Therefore, since urea synthesis does not require extra uptake of AA (Reynolds, 2006) as was suggested in the past (Lobley et al., 1995), tissue protein mobilization could be providing the AA required for energy generation and glucose synthesis, along with the subsequent excretion of the amino groups through urea

synthesis. According with the literature, this rapid reaction suggests that plasma proteins, liver, and gastrointestinal tract were the principal sources of AA at the beginning of the period, while skeletal muscle was increasing its contribution during the last days as indicated by the higher concentration of 3-MH (Swick & Benevenga, 1977). It must be realized that these estimates are not quantitative because fluxes of compounds and differential half-lives in circulation were not measured.

CONCLUSIONS

Feed restriction effectively caused lower BW and a greater degree of NEB. Consequently, milk yield decreased with higher milk fat percentage, lower milk protein percentage, and no difference in milk lactose content. Therefore, no differences were detected in ECM between AL and RC treatments. The lower concentration of insulin during RC quickly allowed lipid mobilization as reflected by higher NEFA and TG concentrations, and increased AA oxidation as reflected by the higher plasma urea N. The higher urea concentration was paired with lower concentrations of all essential AA except Lys, and nonessential AA except Cys, Gly, Arg, and Gln. Both Phe and Tyr deserve special mention because they were the only AA with significantly lower concentration, with Glu and Asp tending to be lower. These specific cases demonstrate their involvement as regulators and precursors in important mechanisms activated during homeostatic processes. However, in attempting to account for all the amino-N circulating in form of urea or eliminated in milk as MUN, the decrease in concentration of all the AA analyzed here was not sufficient for the amount of urea synthetized. Therefore, it seems probable that body tissue protein was rapidly mobilized at the beginning of the feed-restriction period, to produce the energy required to support the higher ECM especially through milk fat and lactose.

Despite the synchronized evolution of decreasing insulin, glucose, and AA concentrations paired with increasing NEFA and urea across the 5-d feed restriction period, the feeding effect within each day immediately provoked an analogous reactivity regardless of the diet. The spike of insulin after feeding caused a decrease of glucose and AA concentrations, which indicates an uptake for glycolysis or storage and protein synthesis, respectively, in insulin-sensitive tissues. Furthermore, NEFA concentration also dropped significantly to values close to those during AL after feeding. On the other hand, urea in circulation increased after feeding, which suggests higher urea cycle activity capturing ammonia absorption from rumen fermentation in accordance with the lower NH_3 concentration after feeding. This series of reactions suggests that the feeding effect in RC rapidly restored metabolite concentrations to their regular behavior during normal fed conditions, possibly due to higher sensitivity to insulin during periods of negative energy and protein balance.

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FIGURES

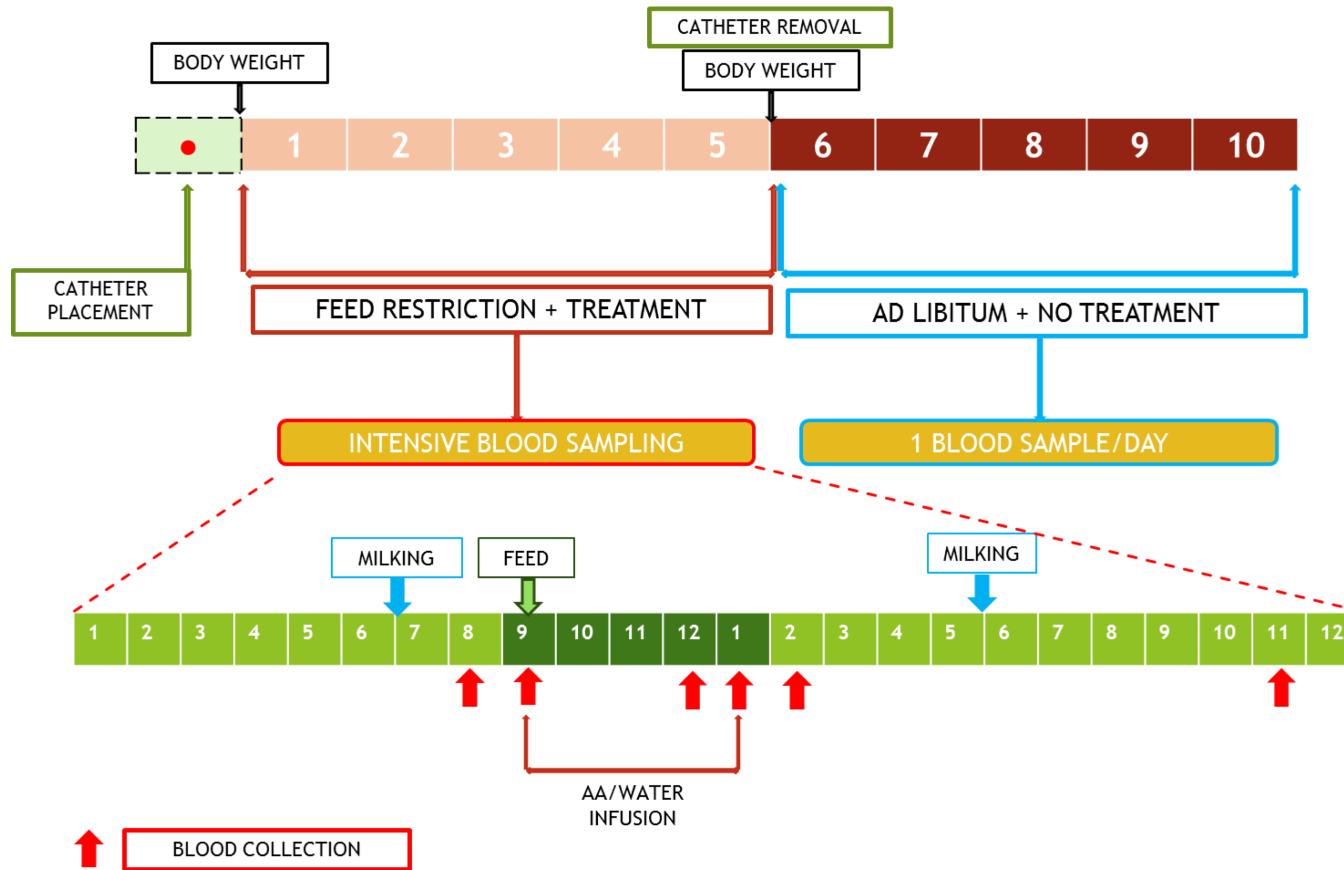

Figure 1 Schematic design of the experiment timeline and daily sampling and treatments application schedule.

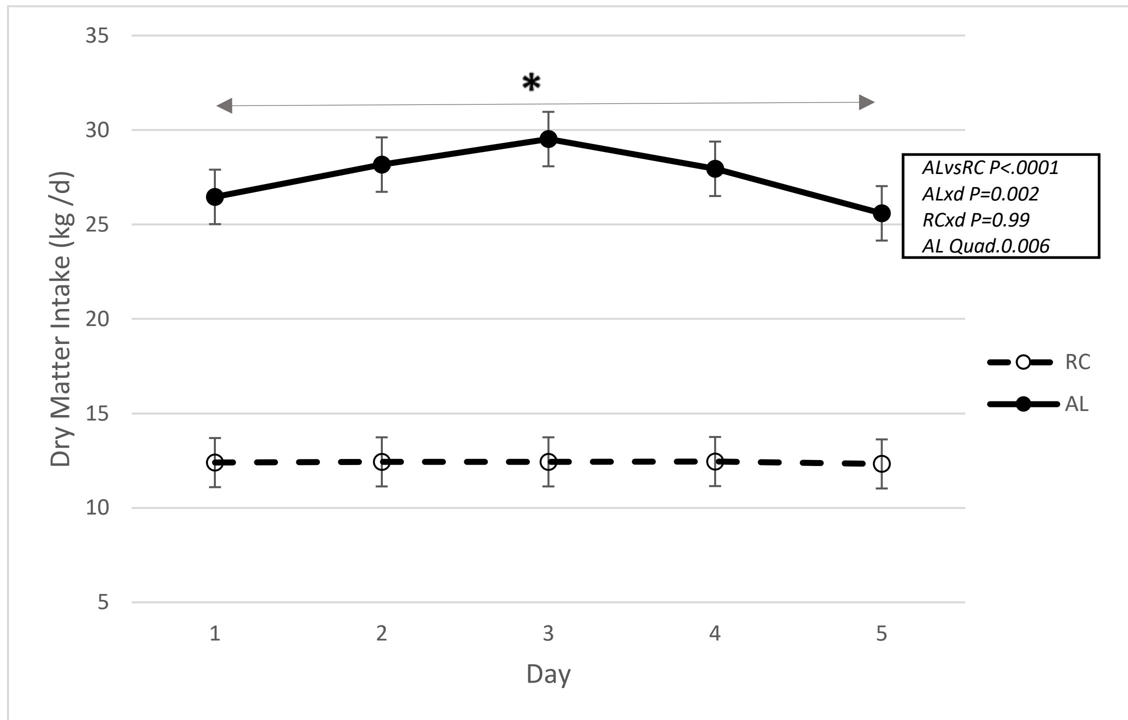

Figure 2 **Daily average of Dry Matter Intake (kg /day) in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

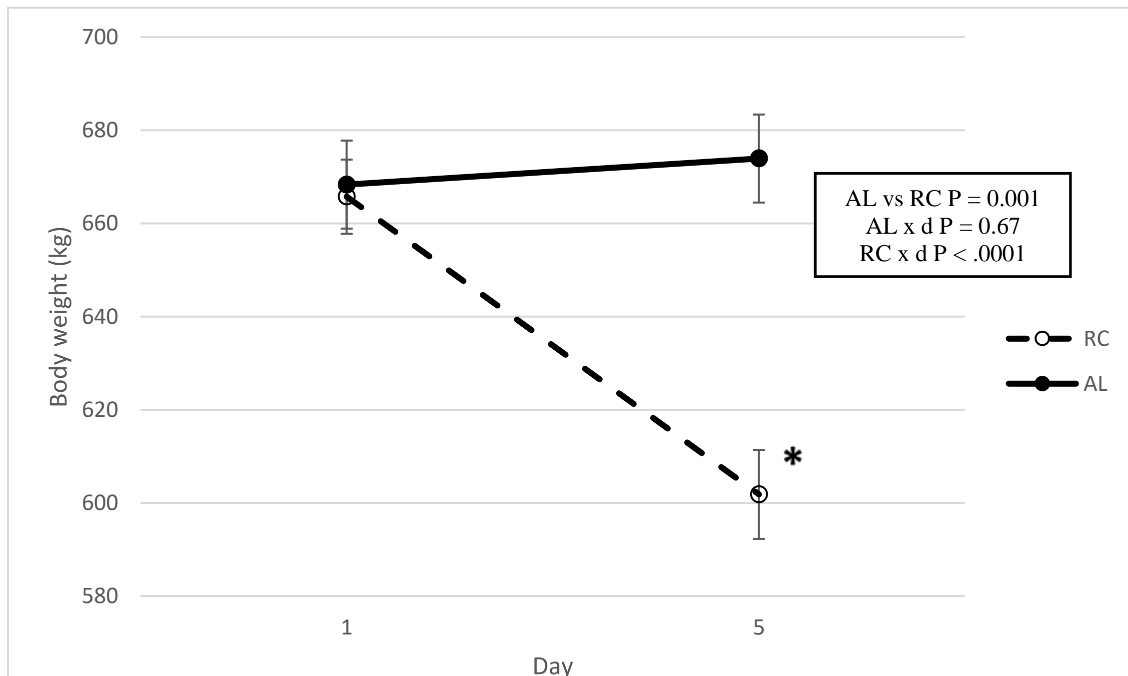

Figure 3 **Daily average of Body weight (kg) in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

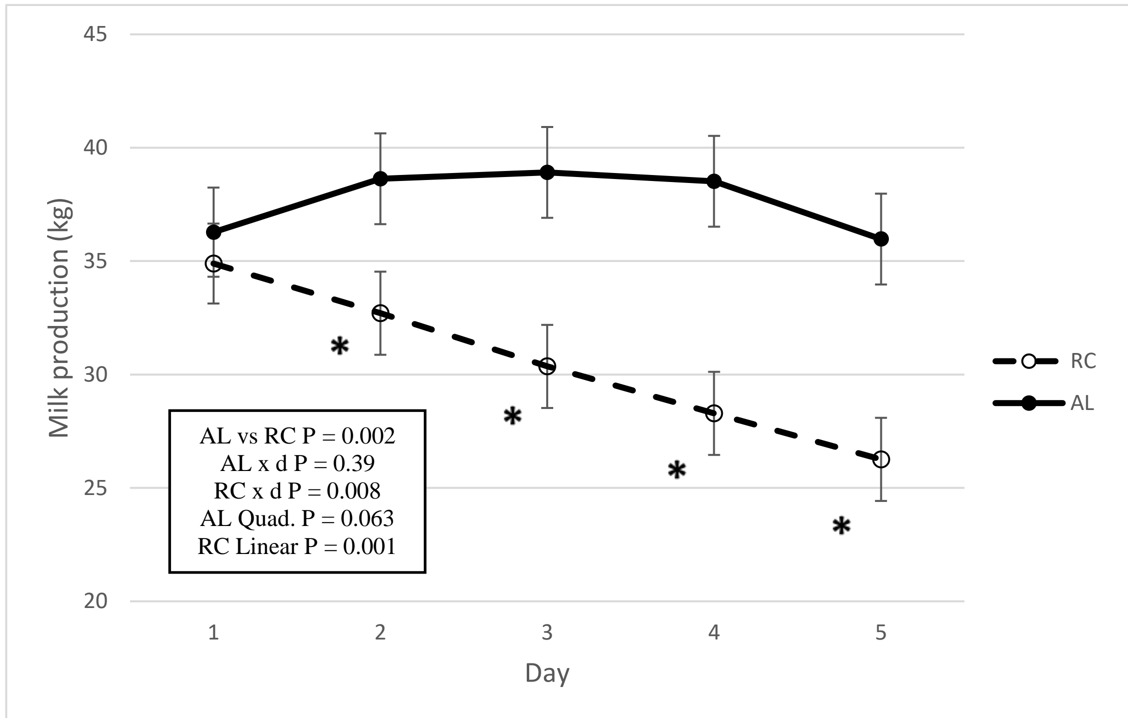

Figure 4 **Daily average milk production (kg/day) of RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

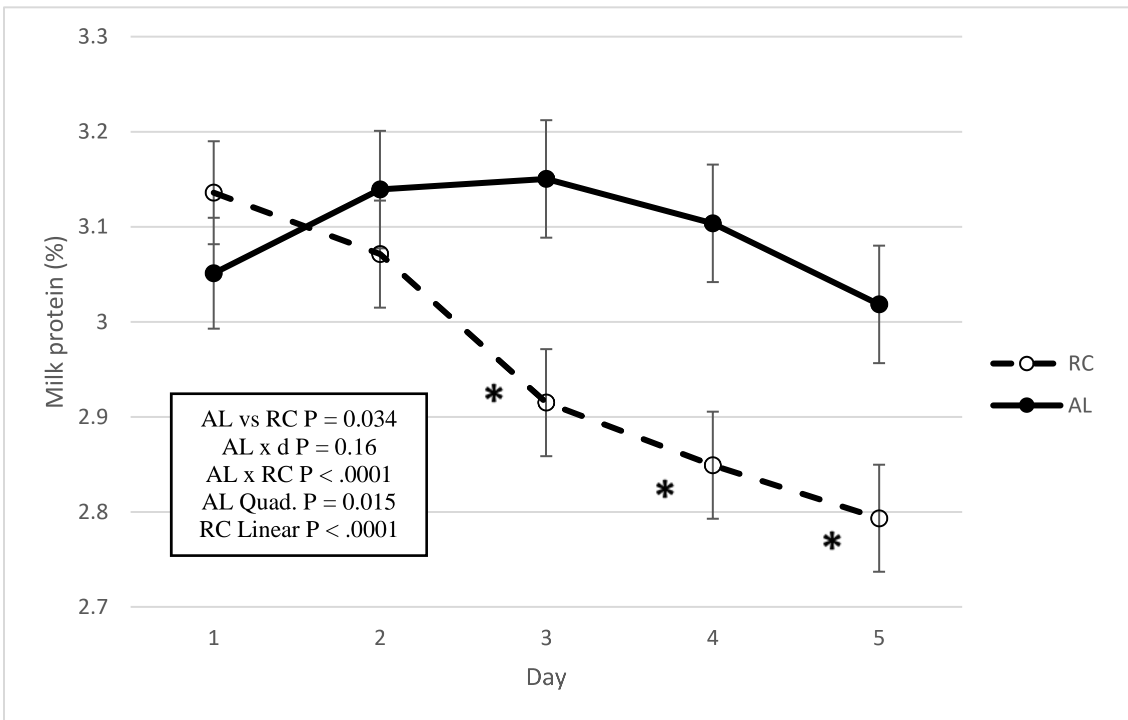

Figure 5 **Daily average milk protein percentage (%) of RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

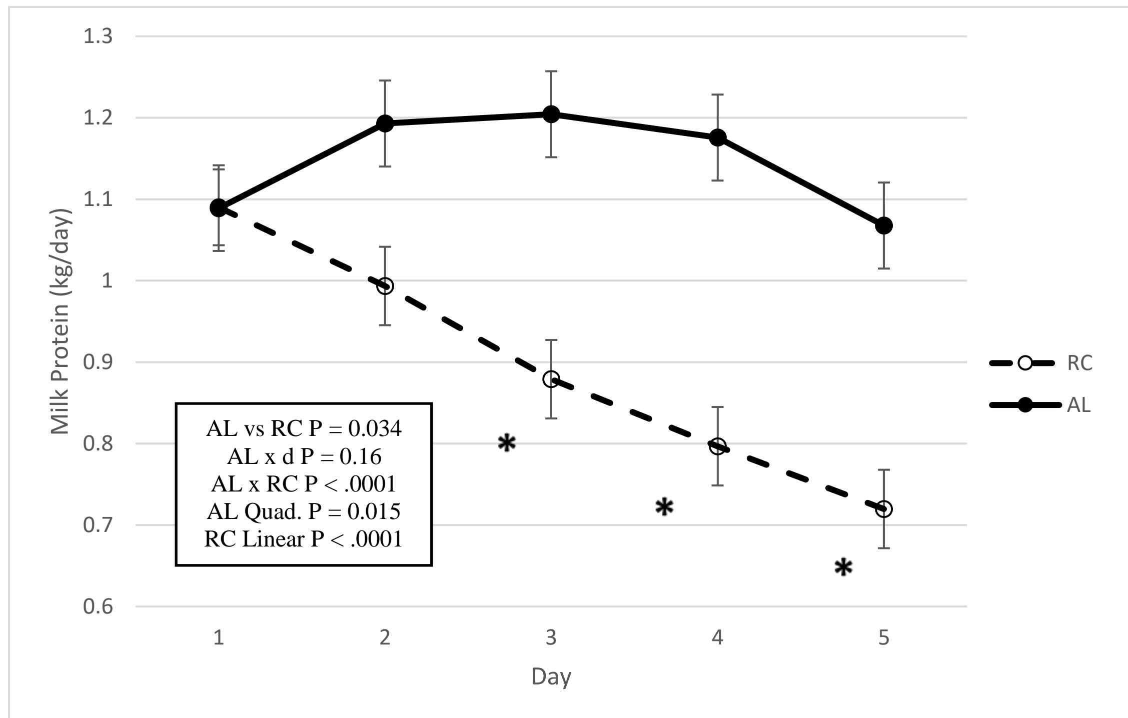

Figure 6 **Daily average of milk protein yield (kg /day) of RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

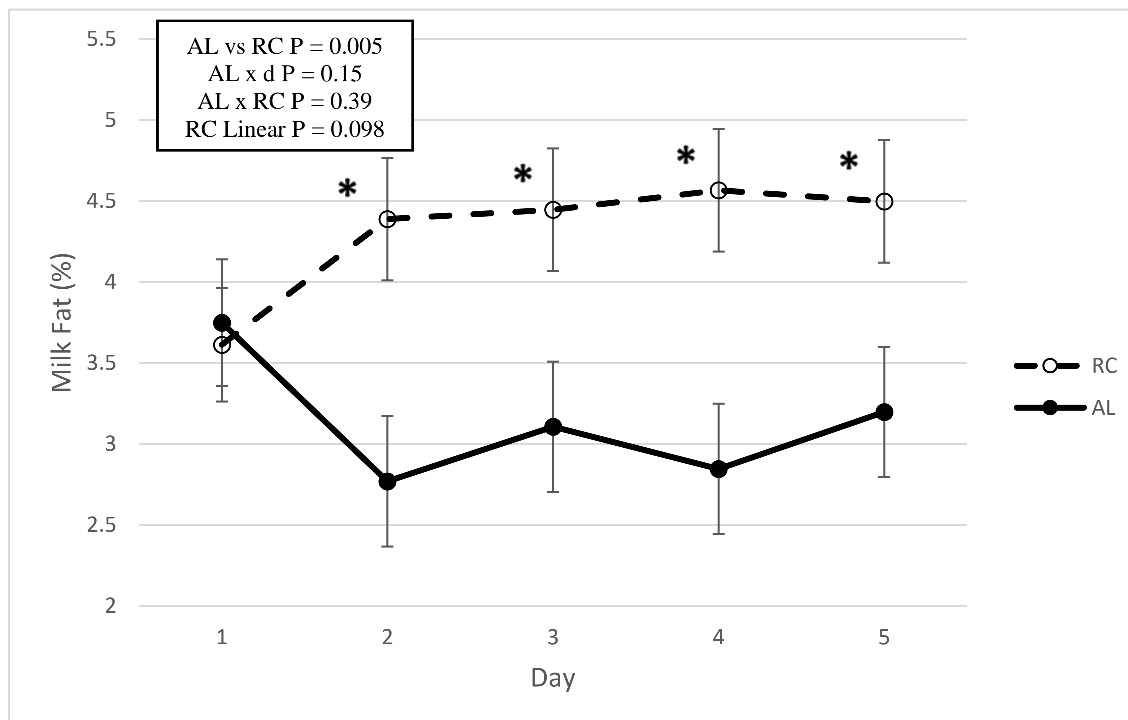

Figure 7 **Daily average of milk fat percentage (%) of RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

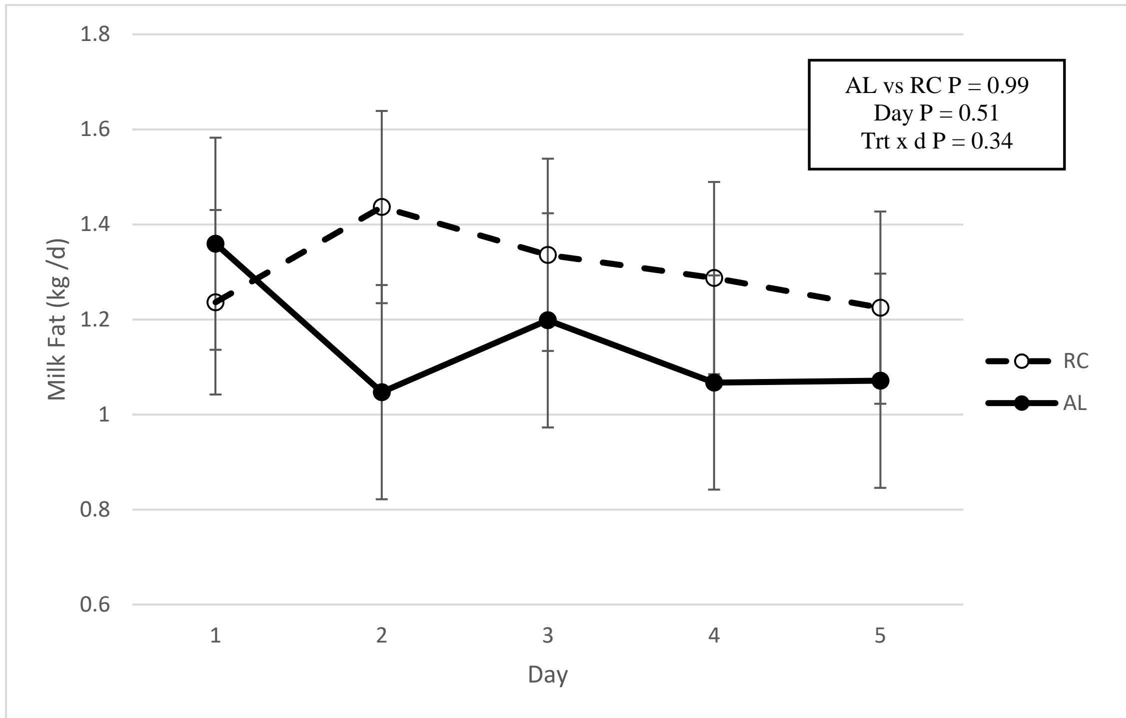

Figure 8 **Daily average of milk fat yield (kg/day) of RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

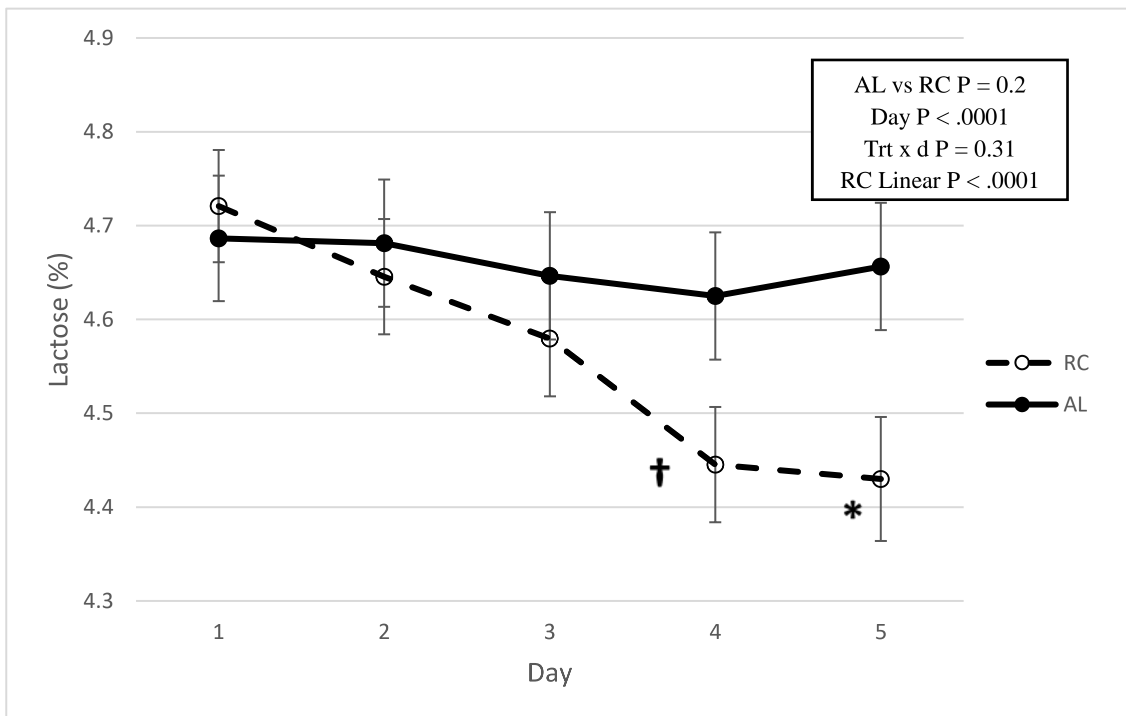

Figure 9 **Daily average of milk lactose percentage (%) of RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

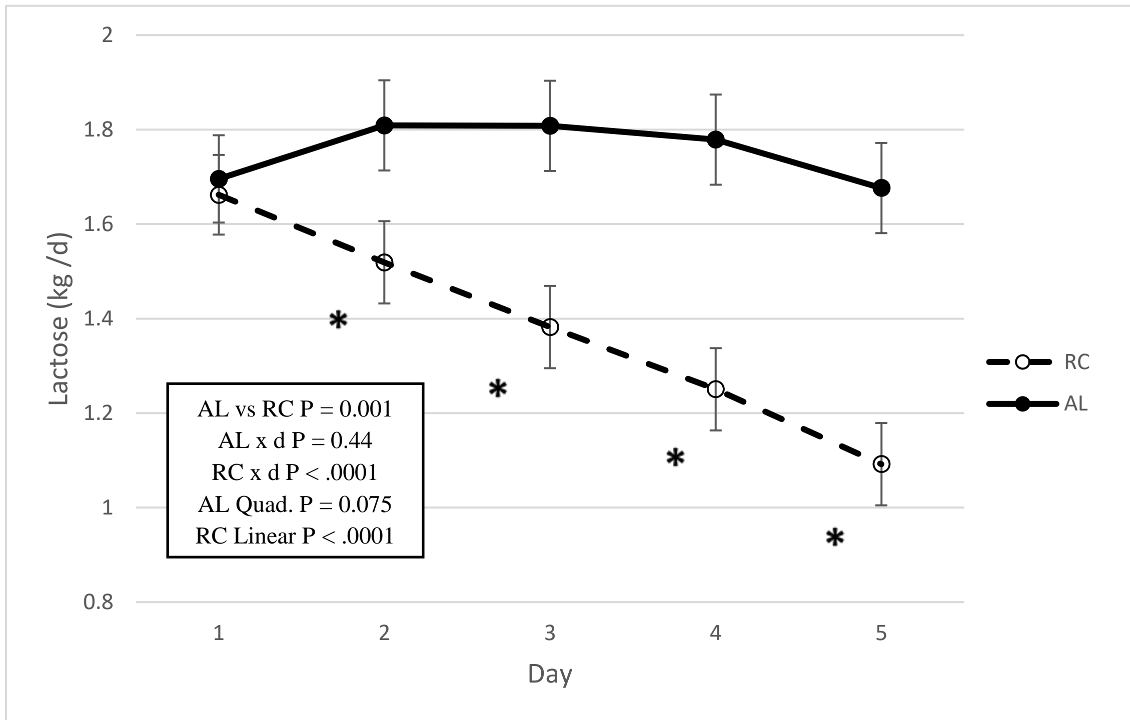

Figure 10 **Daily average of milk lactose yield (kg /day) of RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

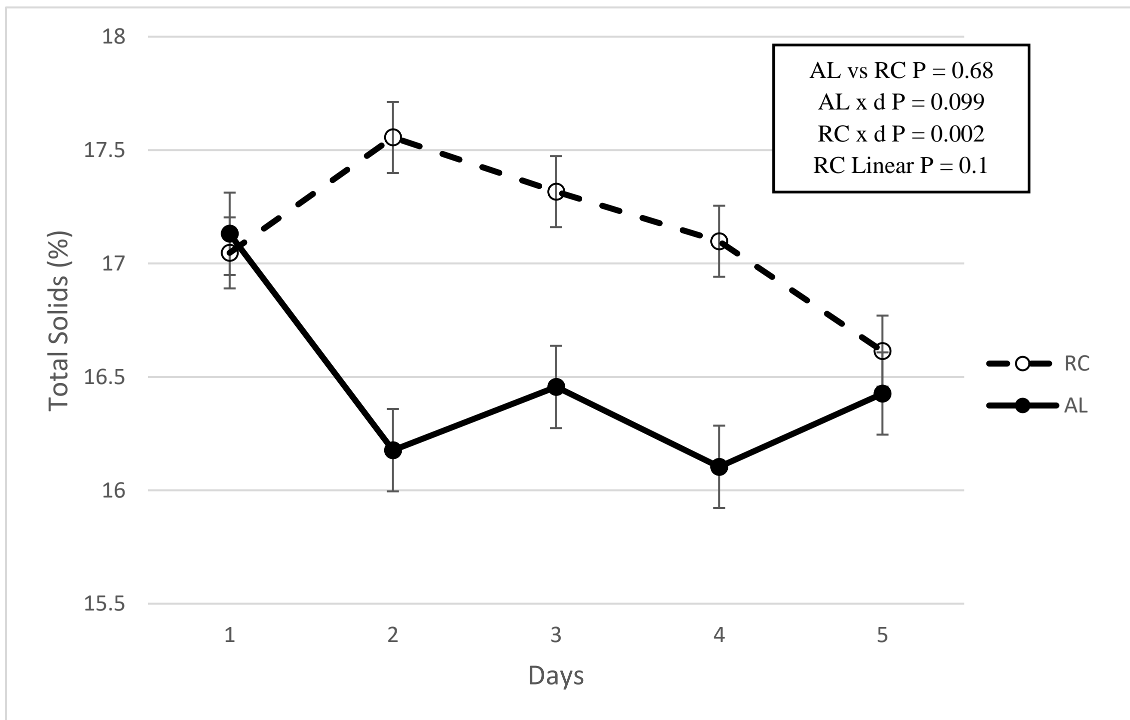

Figure 11 **Daily average of Total Solids in milk (%) in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

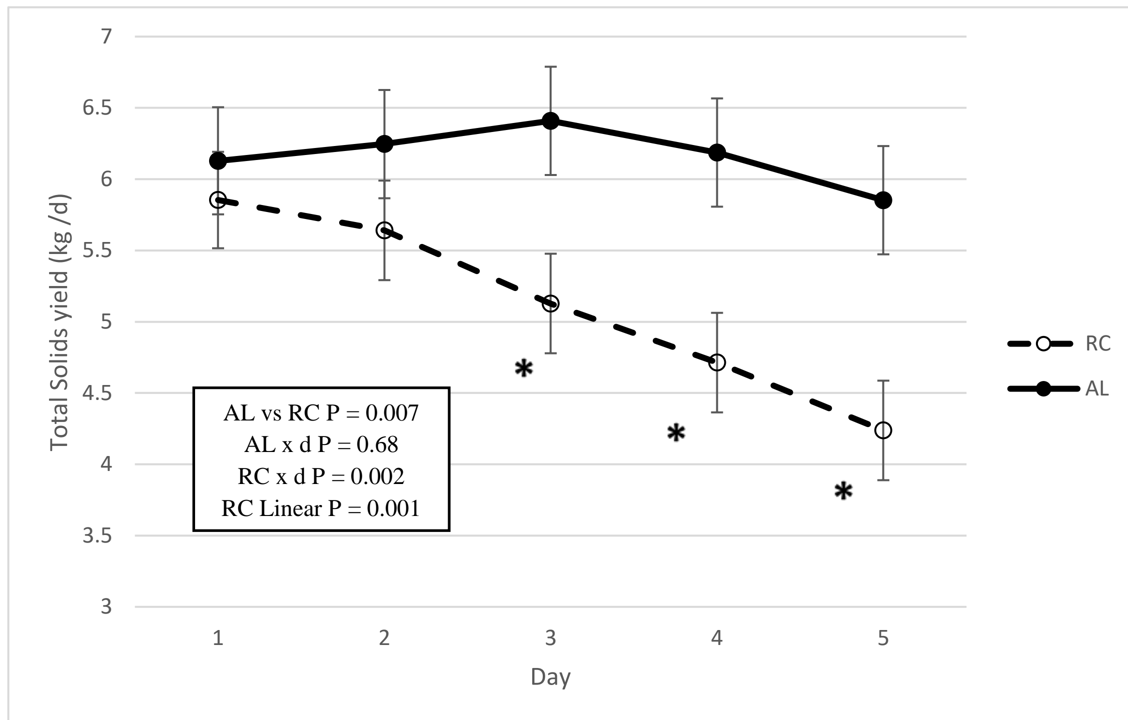

Figure 12 **Daily average of milk Total Solids yield (kg /d)) in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

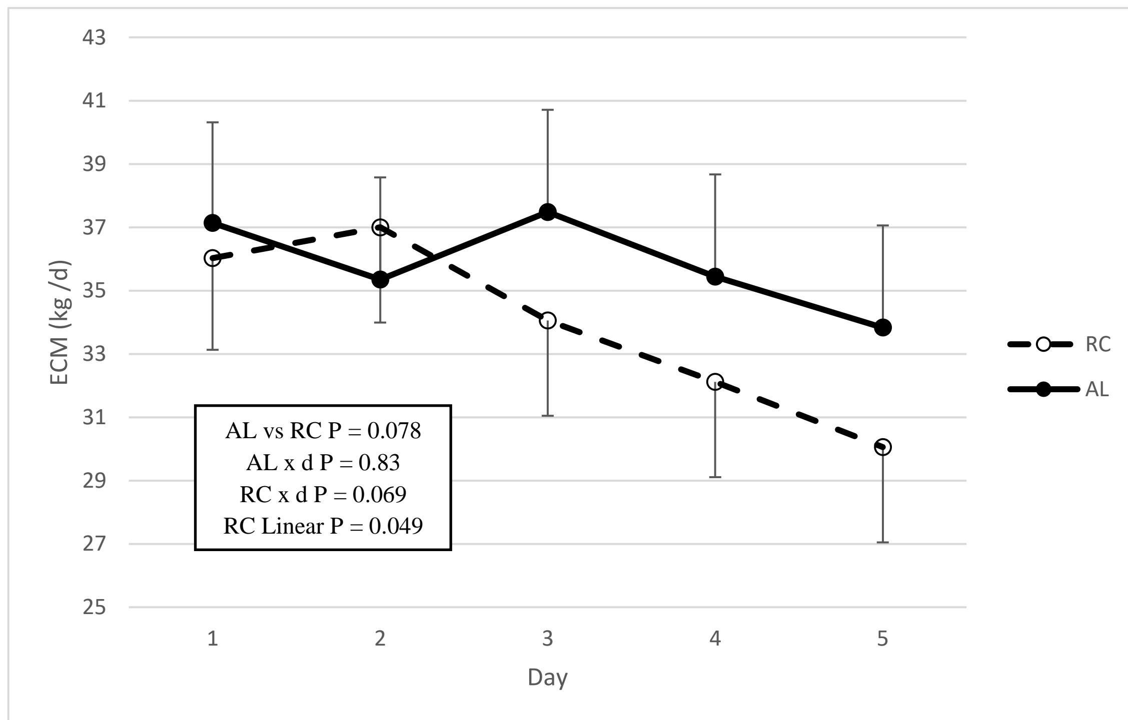

Figure 13 **Daily average of Energy Corrected Milk (kg /d)) in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

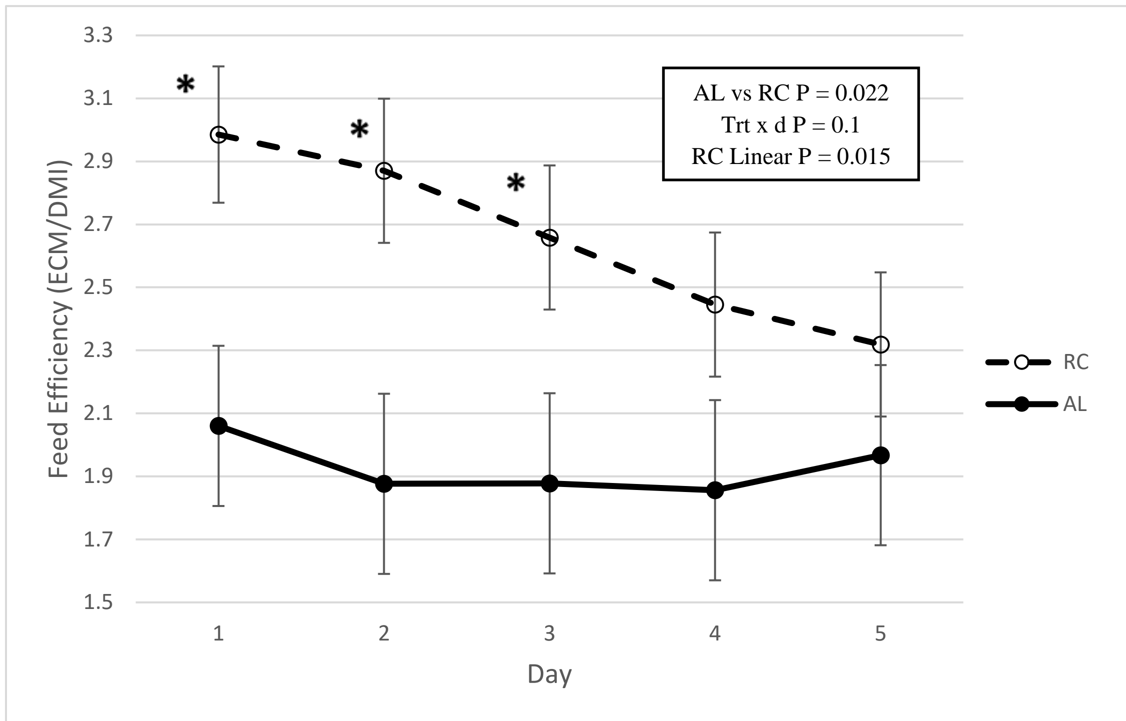

Figure 14 **Daily Feed efficiency (ECM/DMI) of RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (**P **< 0.10) and * (**P **< 0.05).**

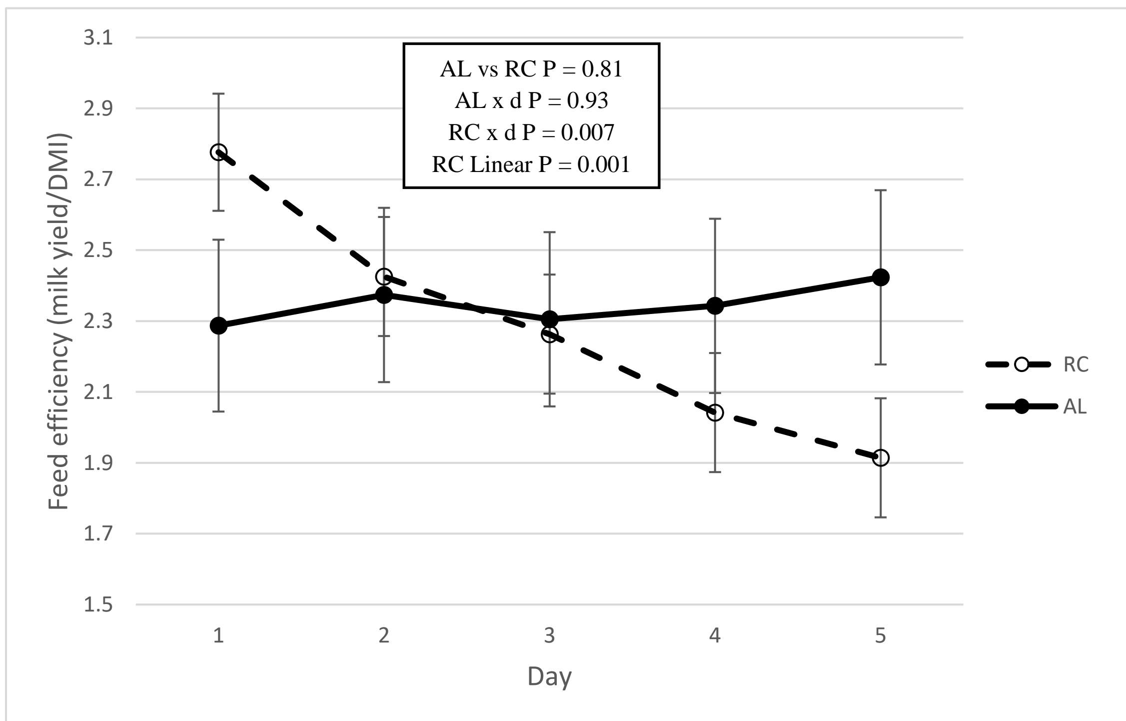

Figure 15 **Daily Feed efficiency (Milk yield/DMI) of RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (**P **< 0.10) and * (**P **< 0.05)**

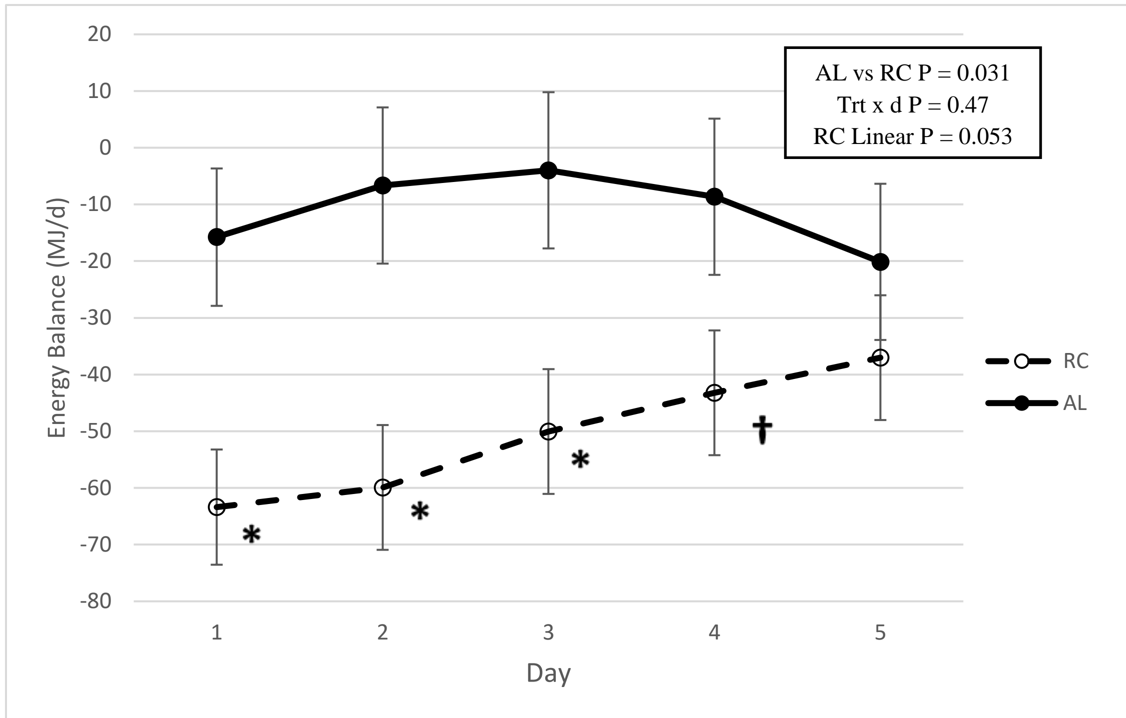

Figure 16 **Daily Energy Balance (MJ/d) of RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (**P **< 0.10) and * (**P **< 0.05)**

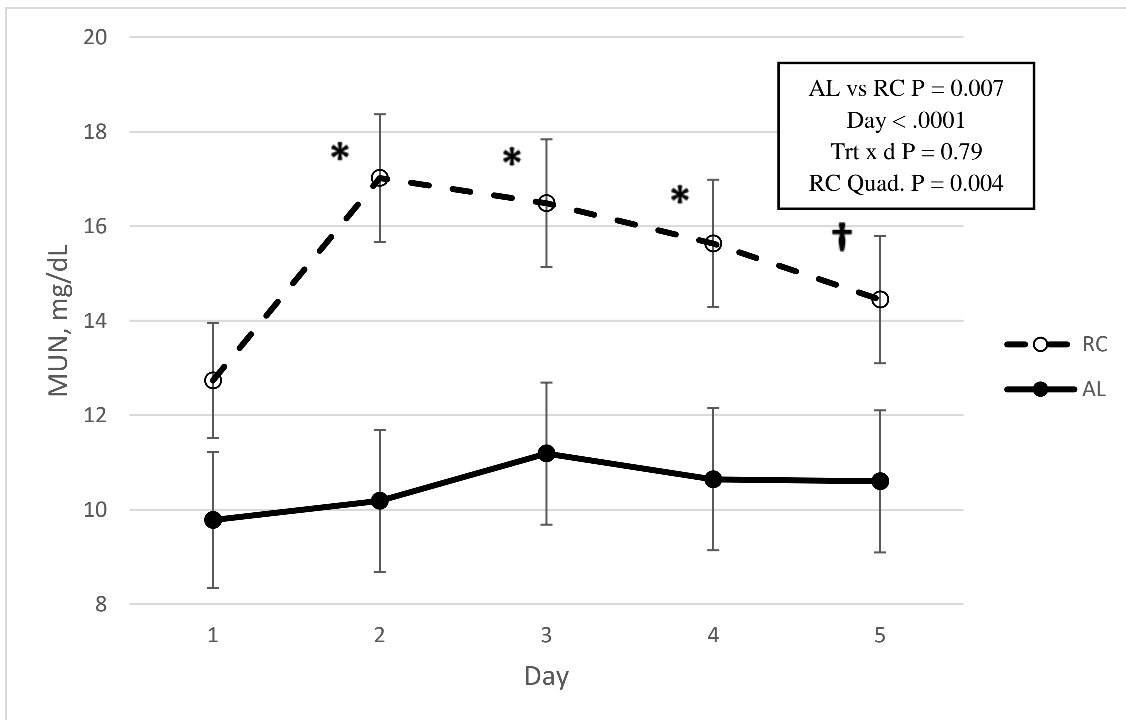

Figure 17 **Daily average of milk urea nitrogen (mg/dL) in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (**P **< 0.10) and * (**P **< 0.05).**

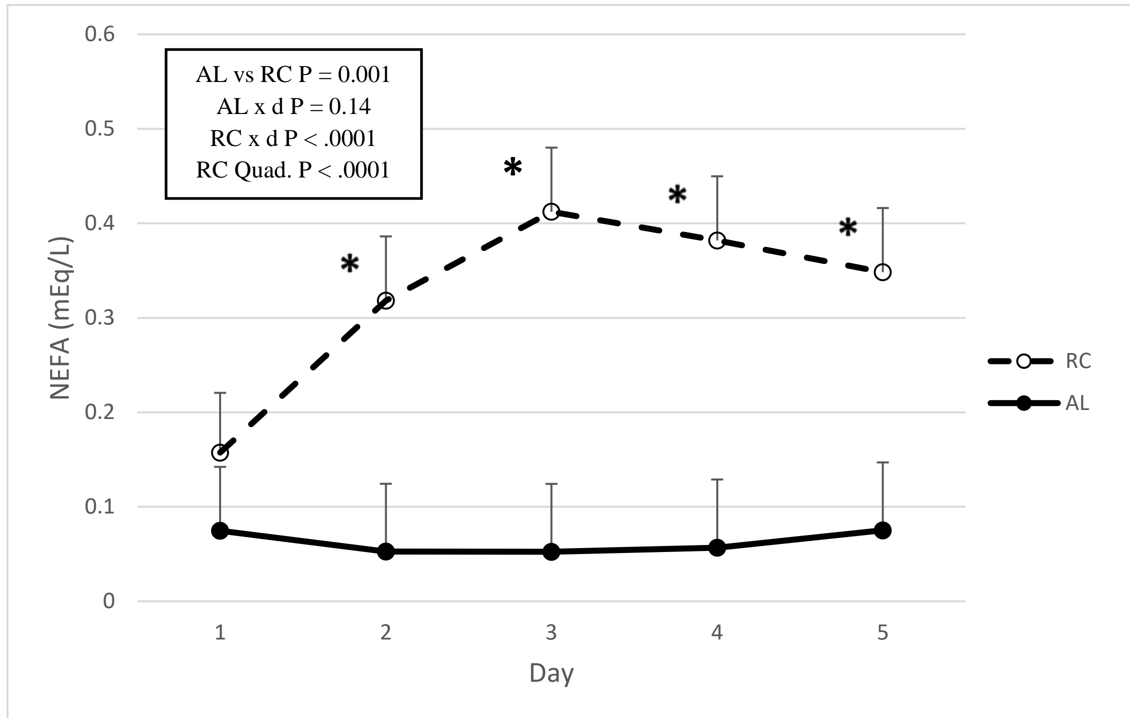

Figure 18 **Daily average plasma concentrations of Non Esterified Fatty Acids (NEFA) in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

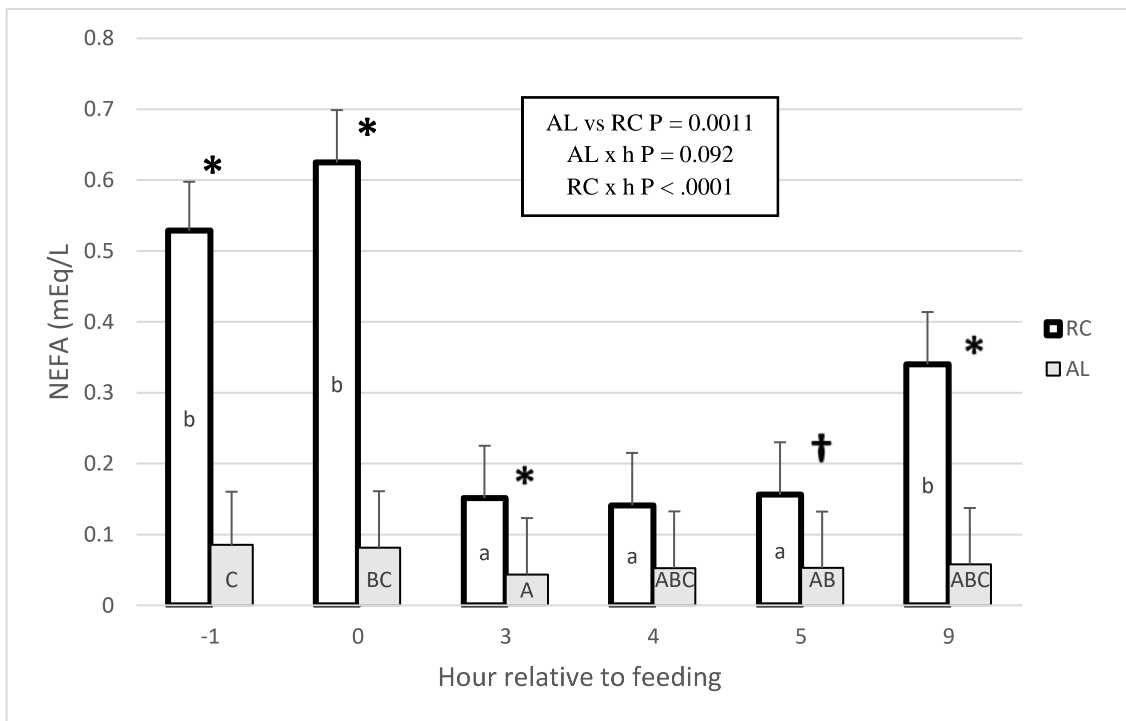

Figure 19 **NEFA concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

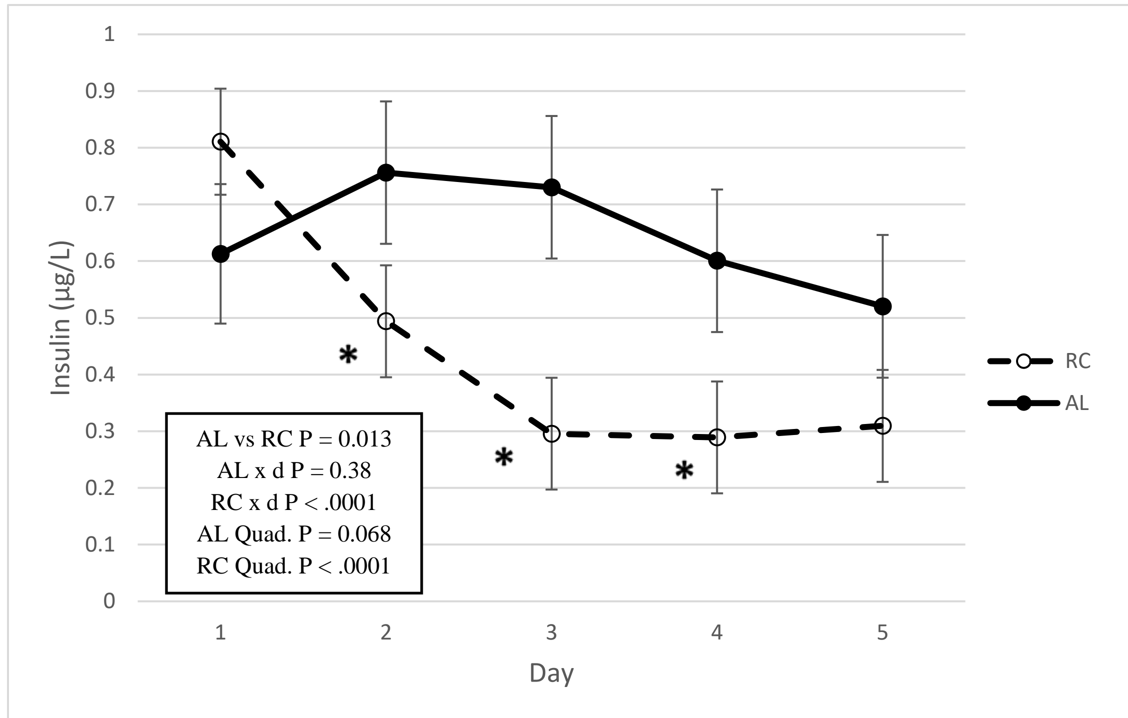

Figure 20 **Daily average plasma concentrations of Insulin in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P ≤ 0.10) and * (P ≤ 0.05).**

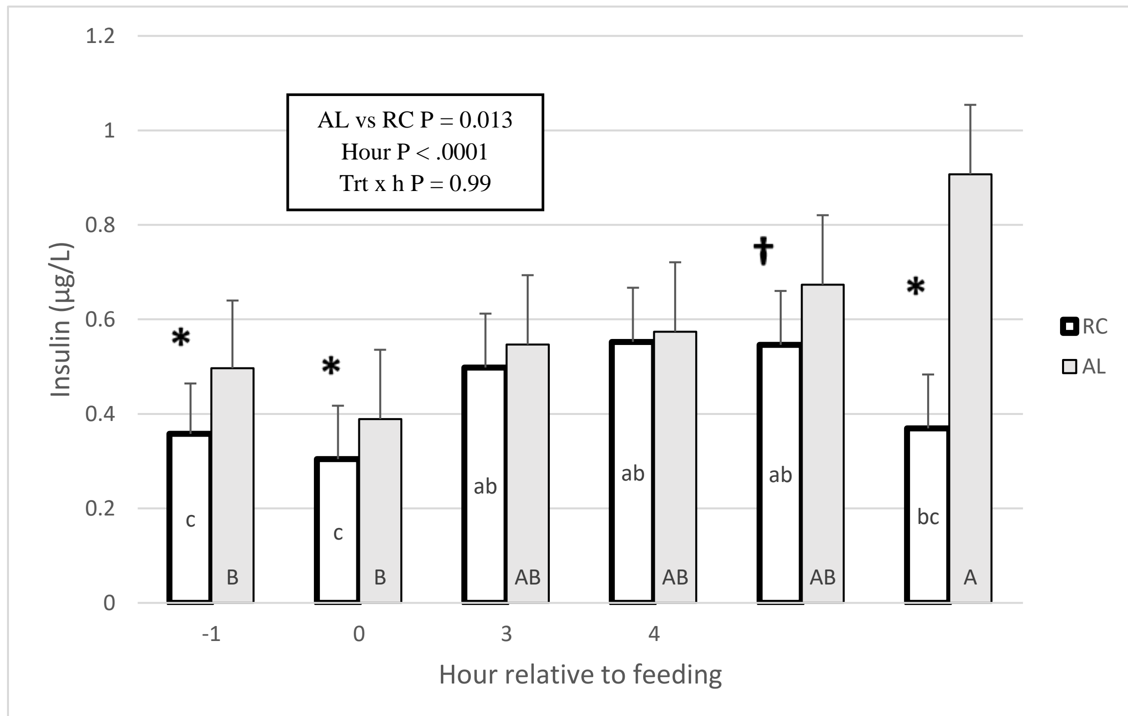

Figure 21 **Insulin concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P ≤ 0.10) and * (P ≤ 0.05).**

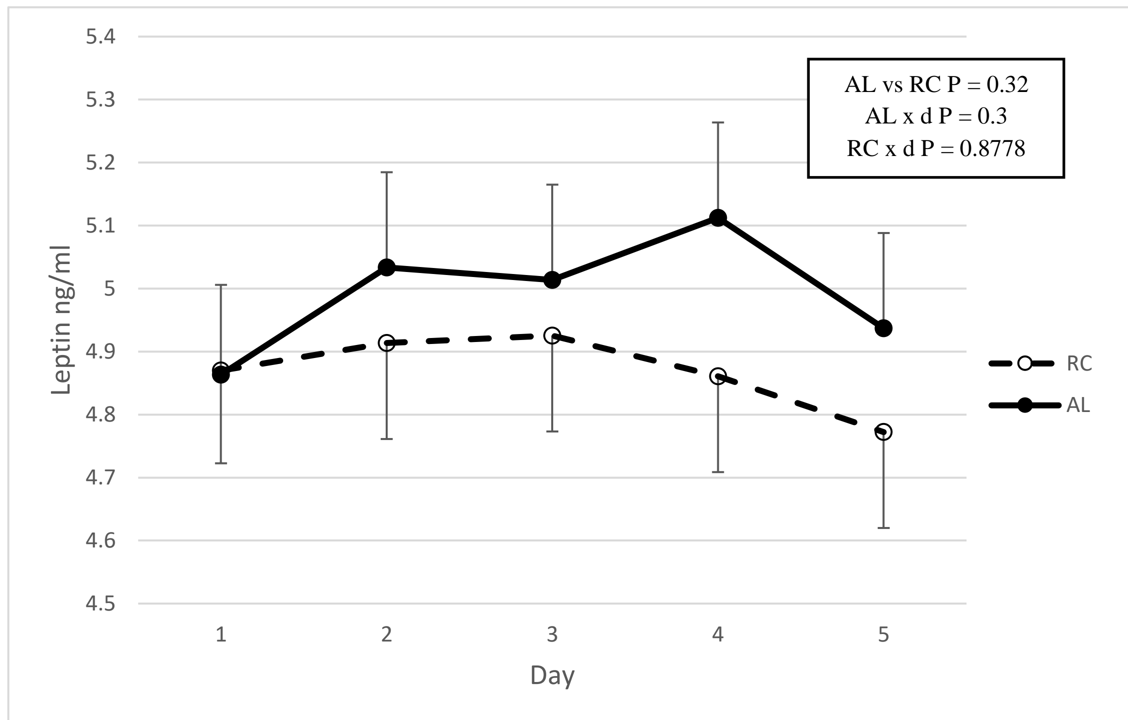

Figure 22 **Daily plasma concentrations pre-feeding of Leptin in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

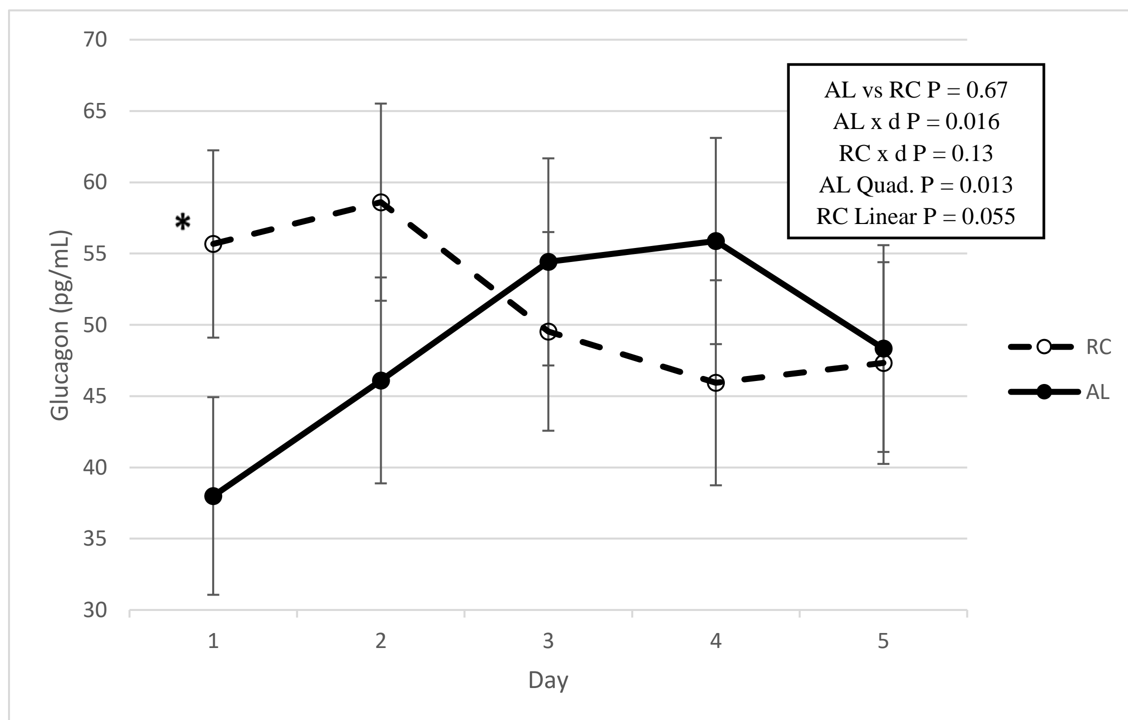

Figure 23 **Daily average plasma concentrations of Glucagon in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

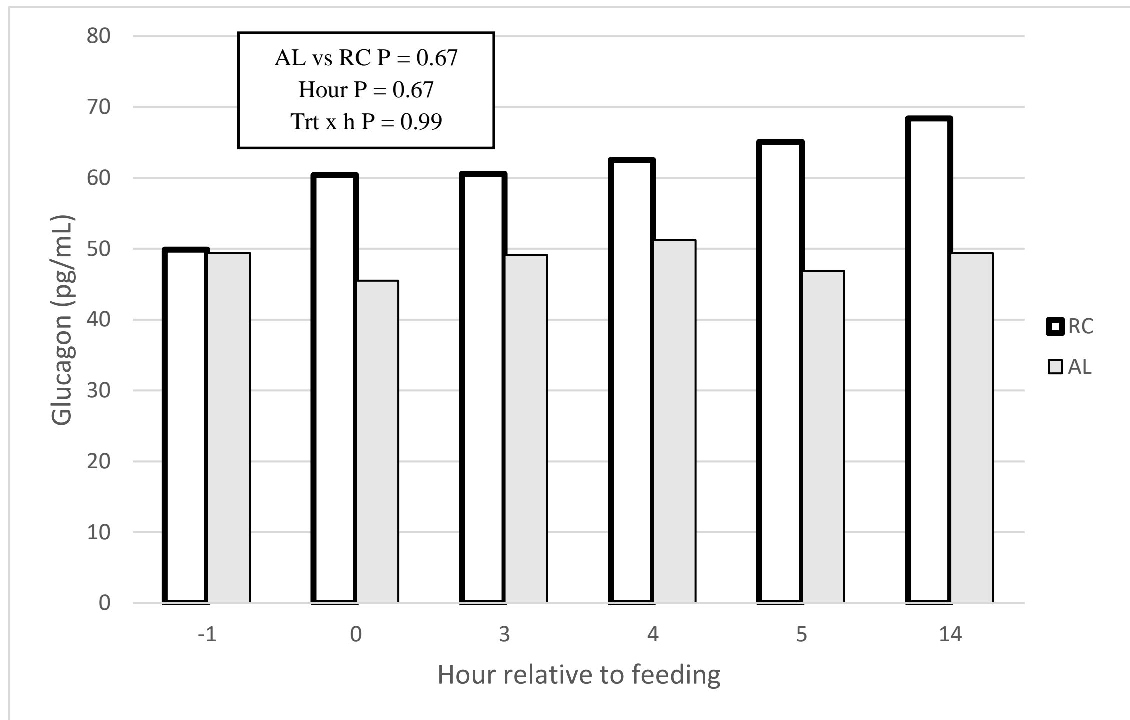

Figure 24 **Glucagon concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05**

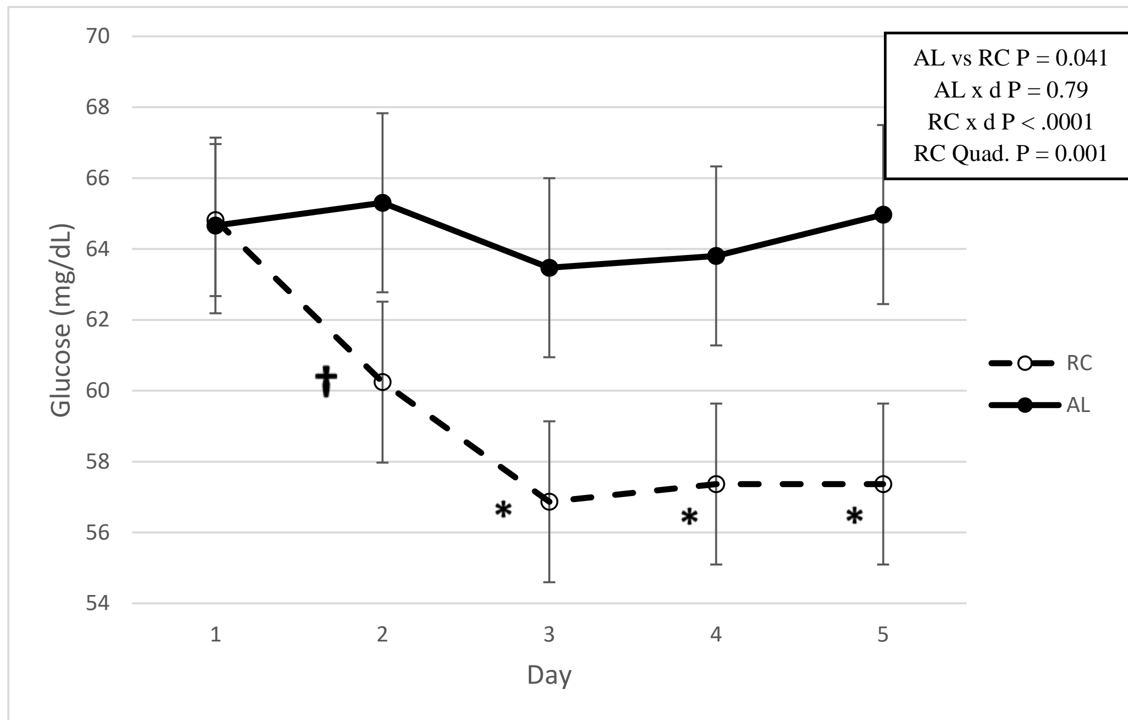

Figure 25 **Daily average plasma concentrations of Glucose in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

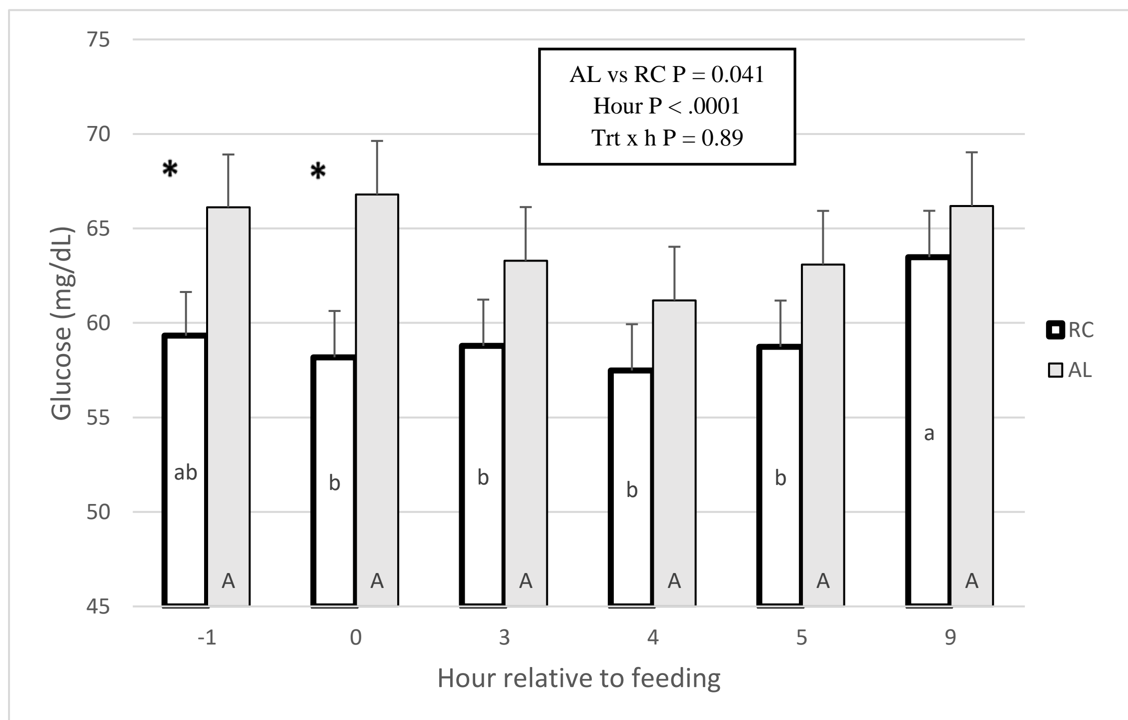

Figure 26 **Glucose concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05**

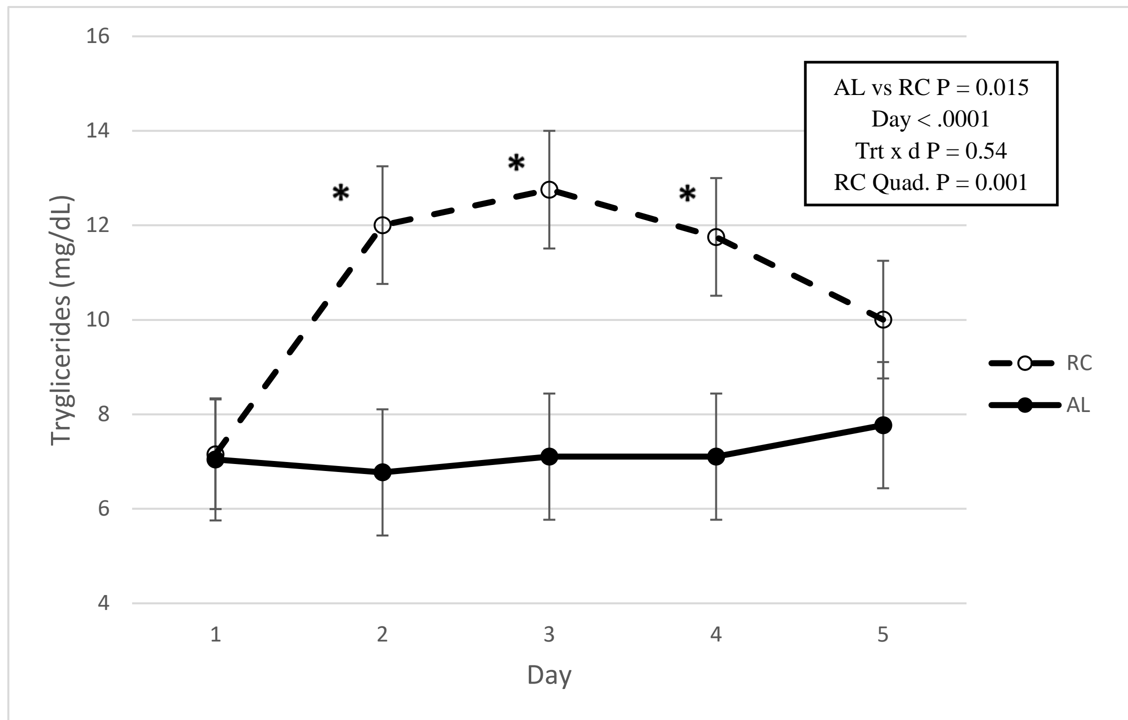

Figure 27 **Daily plasma concentrations pre-feeding of Triglycerides in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

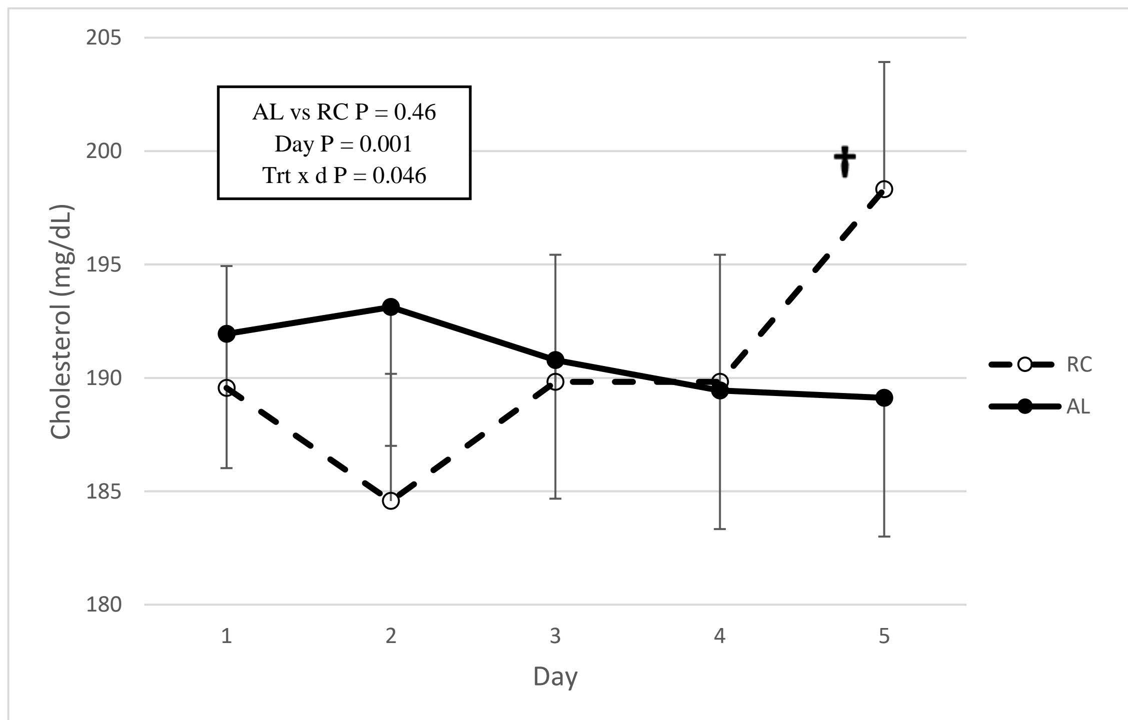

Figure 28 **Daily plasma concentrations pre-feeding of Cholesterol in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

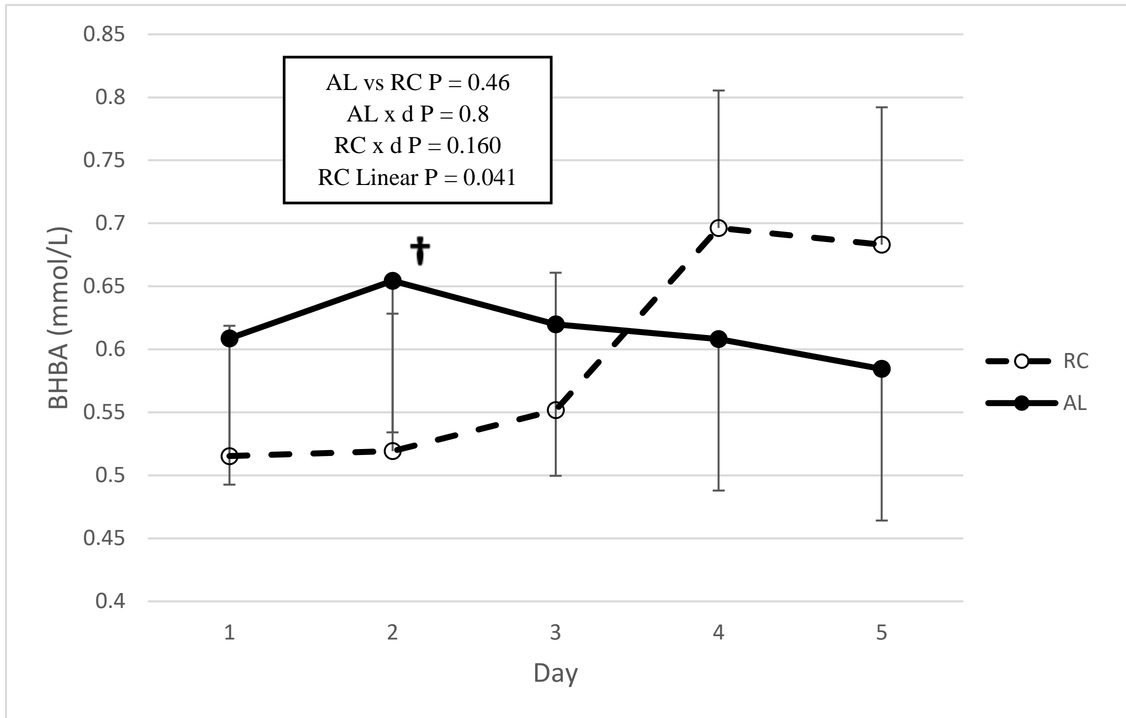

Figure 29 **Daily average plasma concentrations of β-hydroxybutyrate (BHBA) in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

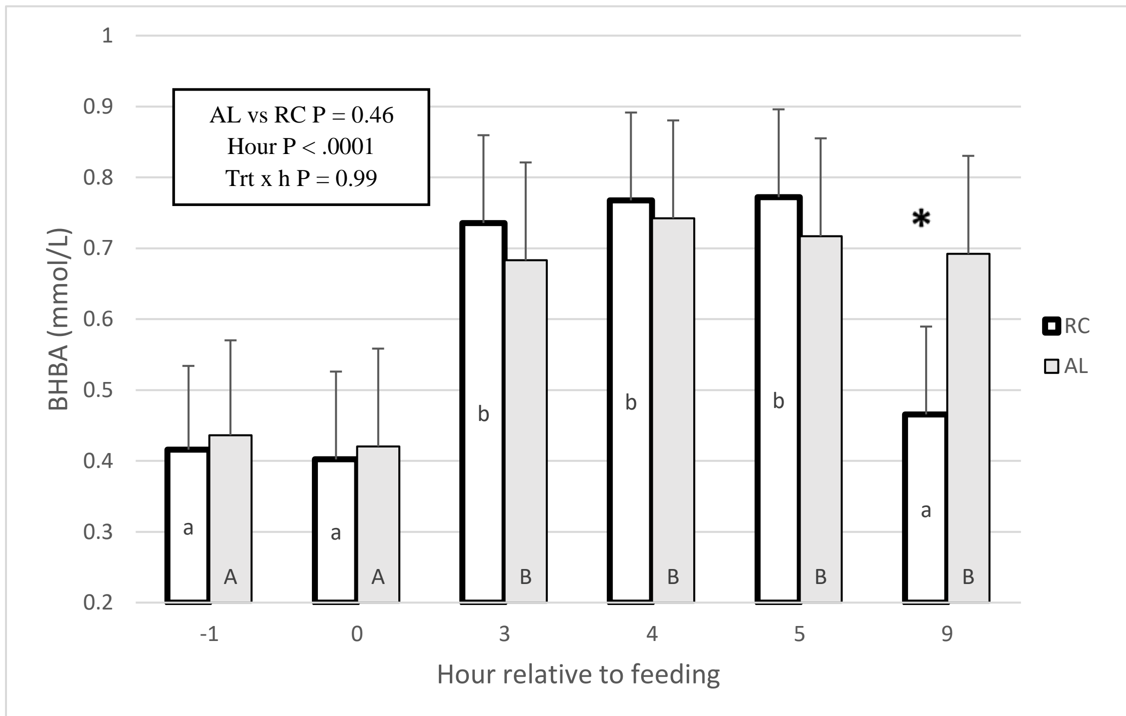

Figure 30 **BHBA concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

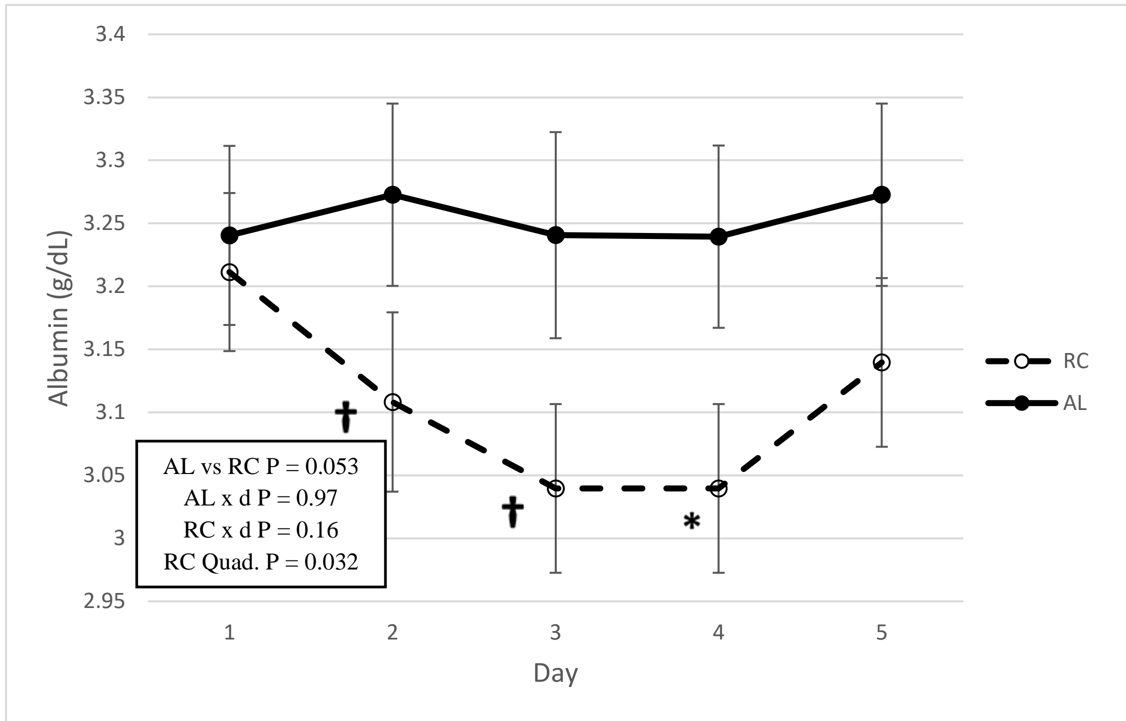

Figure 31 **Daily plasma concentrations pre-feeding of Albumin in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

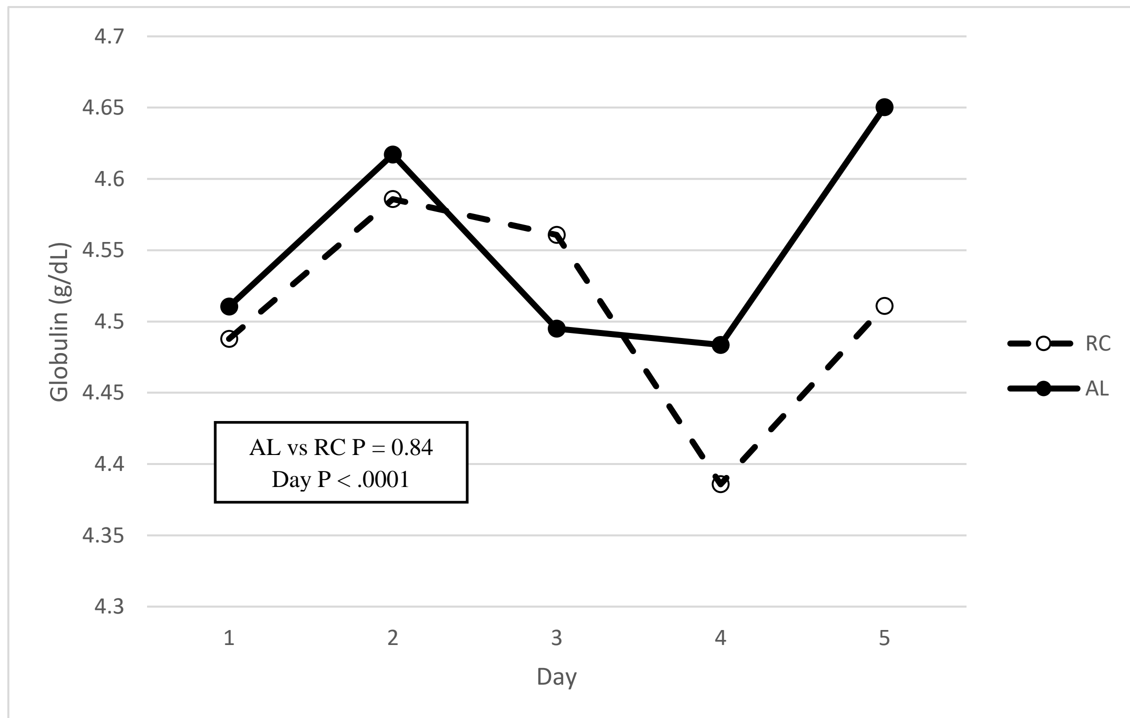

Figure 32 **Daily plasma concentrations pre-feeding of Globulin in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

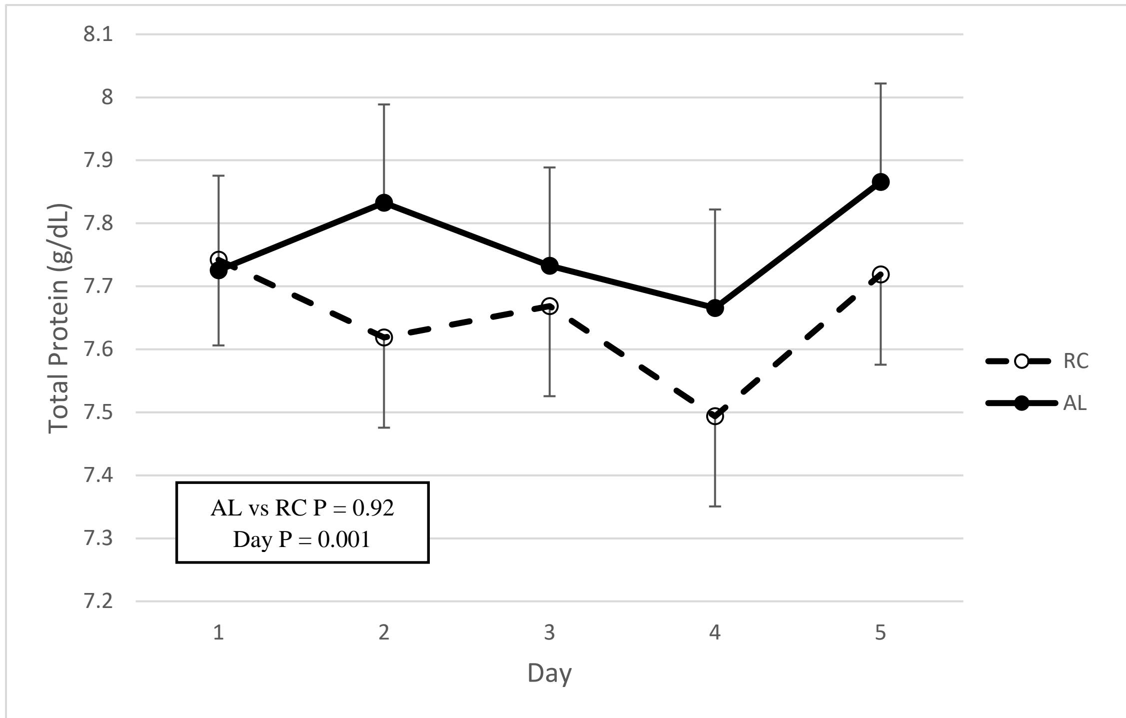

Figure 33 **Daily plasma concentrations pre-feeding of Total Protein in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

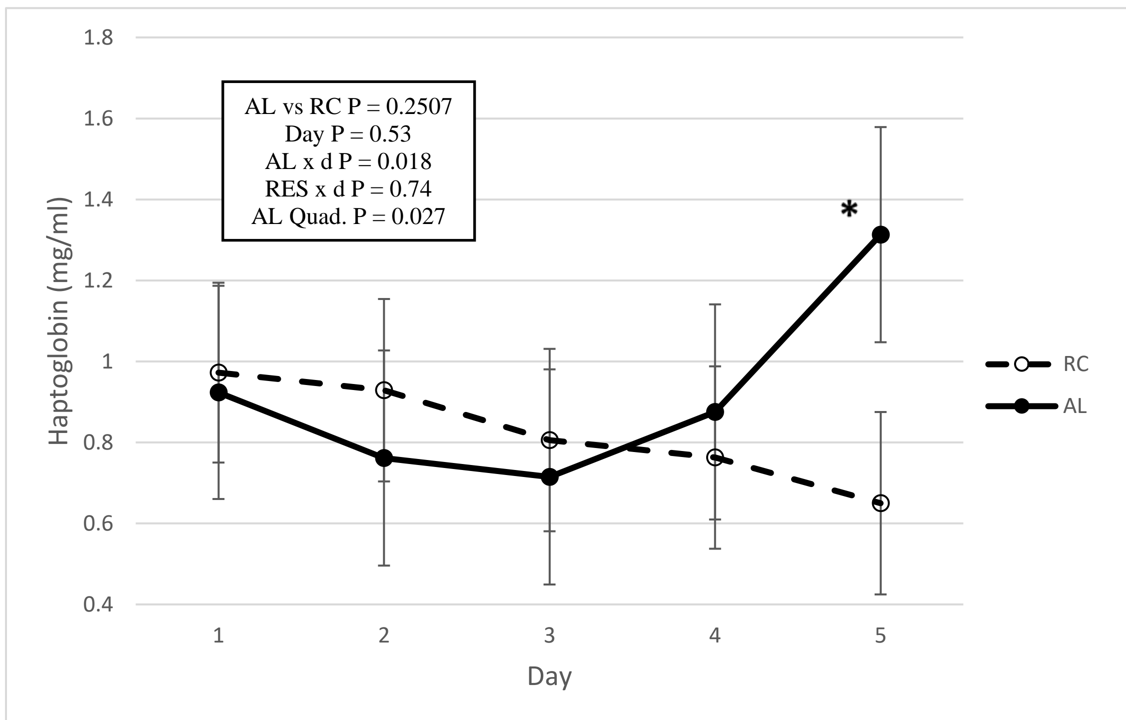

Figure 34 **Daily plasma concentrations pre-feeding of Haptoglobin in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

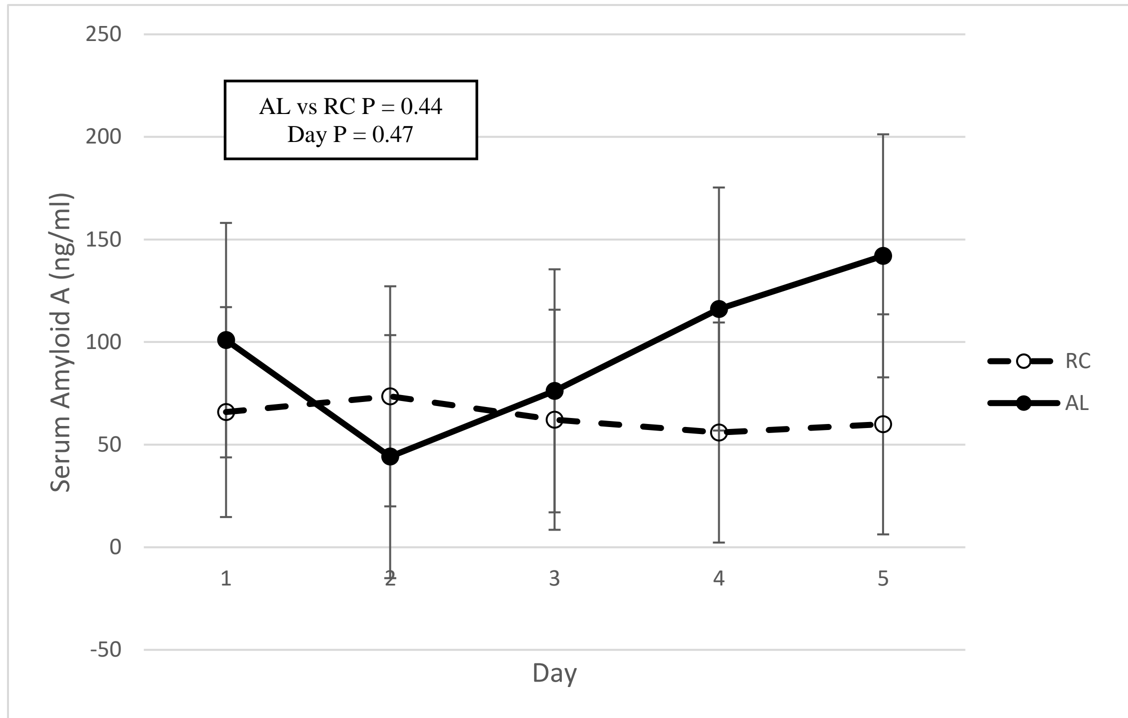

Figure 35 **Daily plasma concentrations pre-feeding of Serum Amyloid A in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

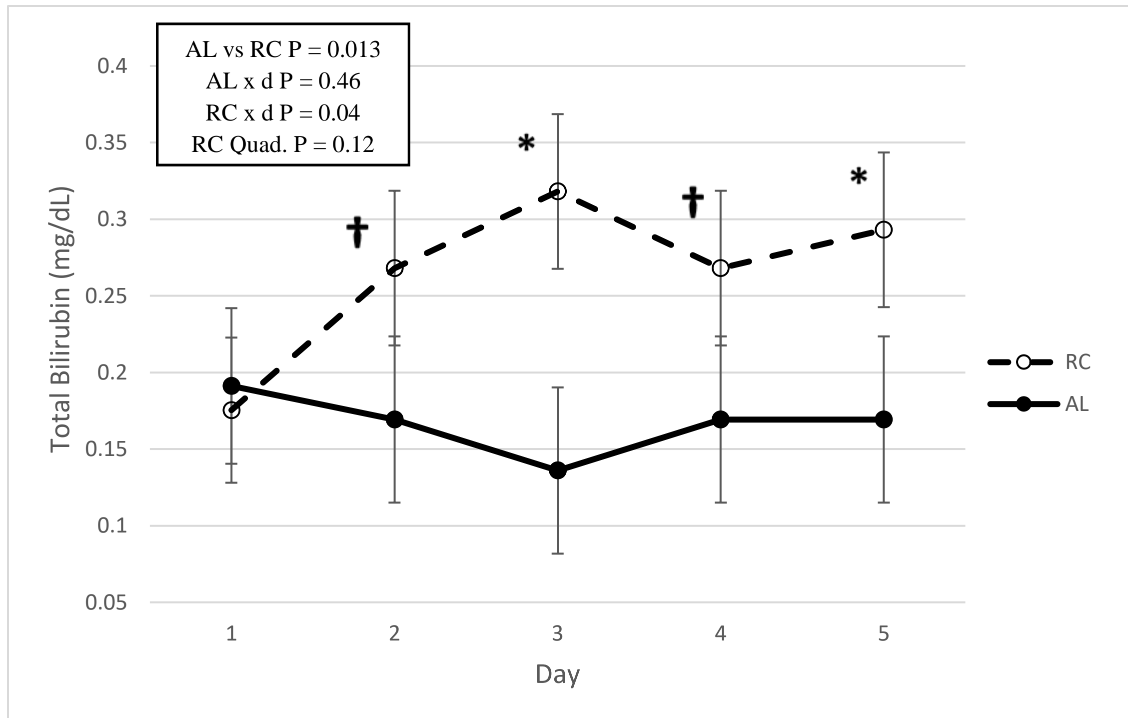

Figure 36 **Daily plasma concentrations pre-feeding of Total Bilirubin (TB) in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

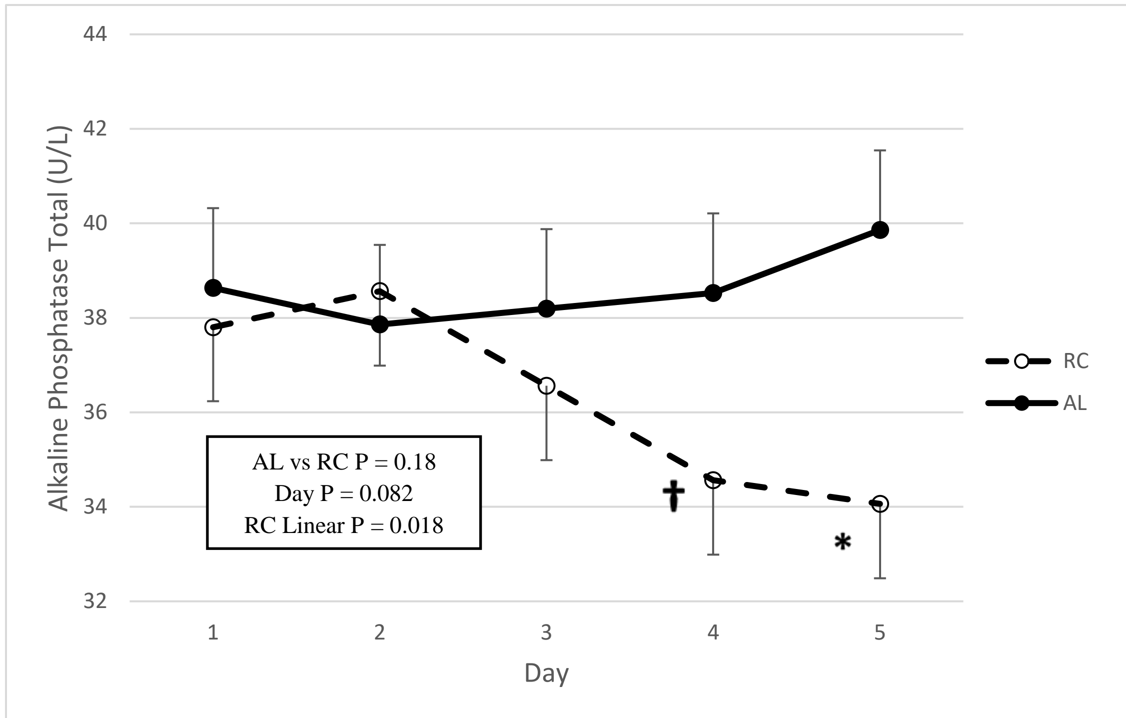

Figure 37 **Daily plasma concentrations pre-feeding of Alkaline Phosphatase Total (APT) in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

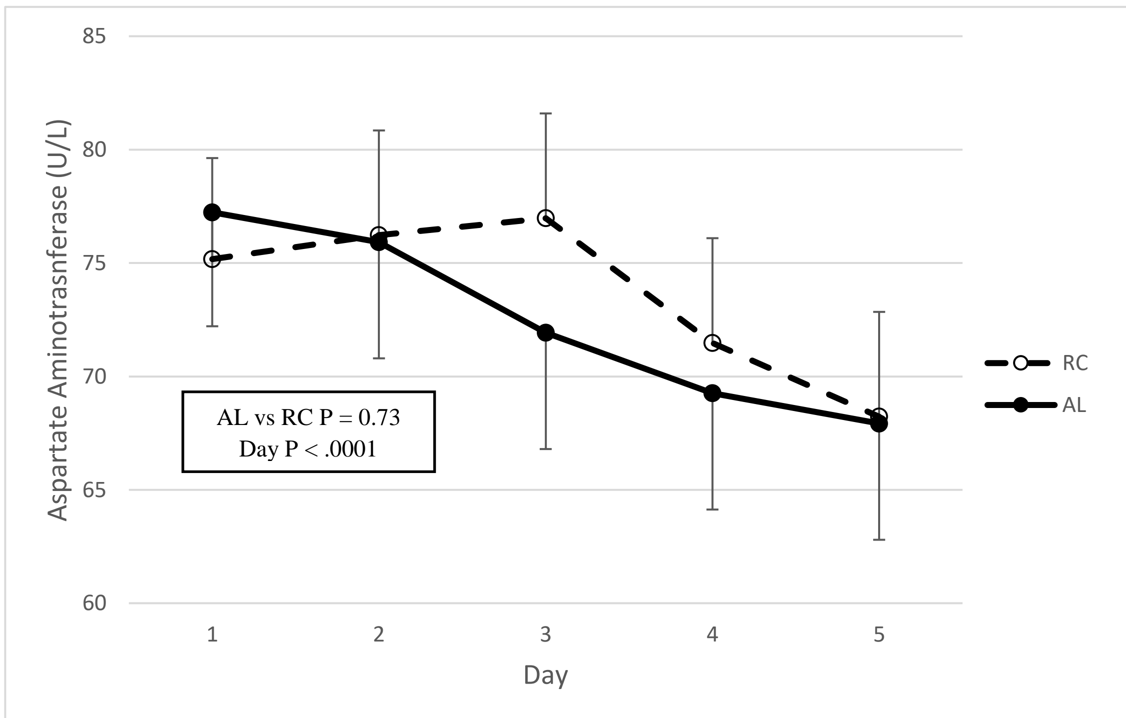

Figure 38 **Daily plasma concentrations pre-feeding of Aspartate Aminotransferase (AST) in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

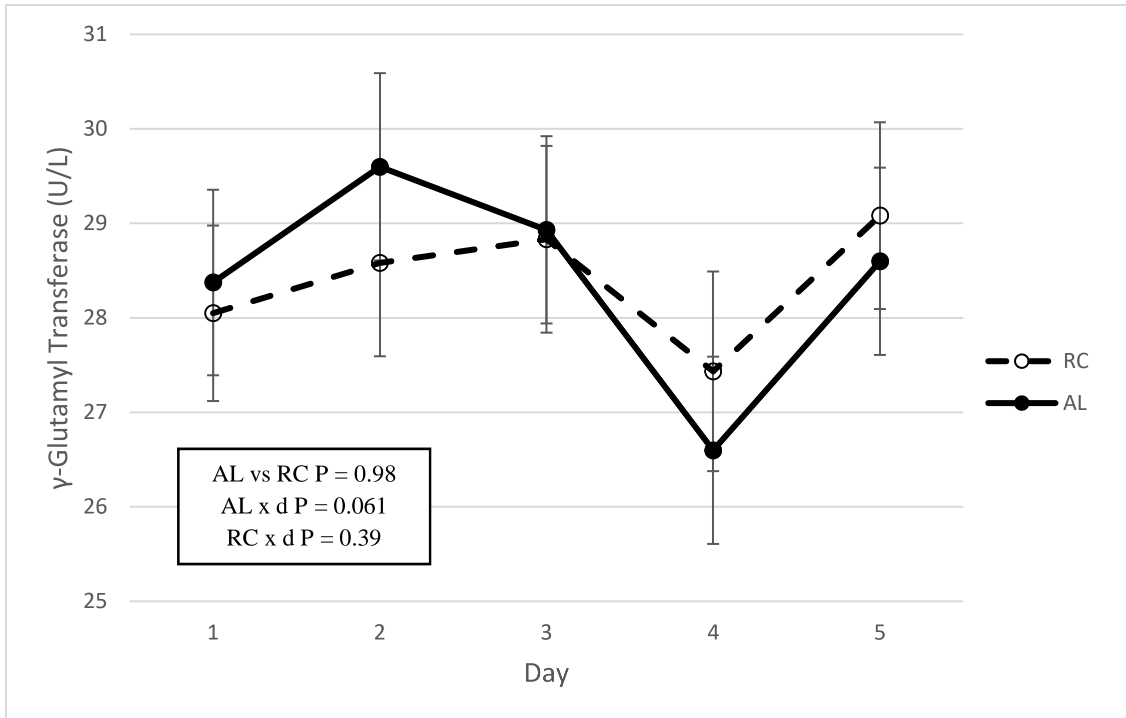

Figure 39 **Daily plasma concentrations pre-feeding of γ-Glutamyl Transferase (GGT) in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

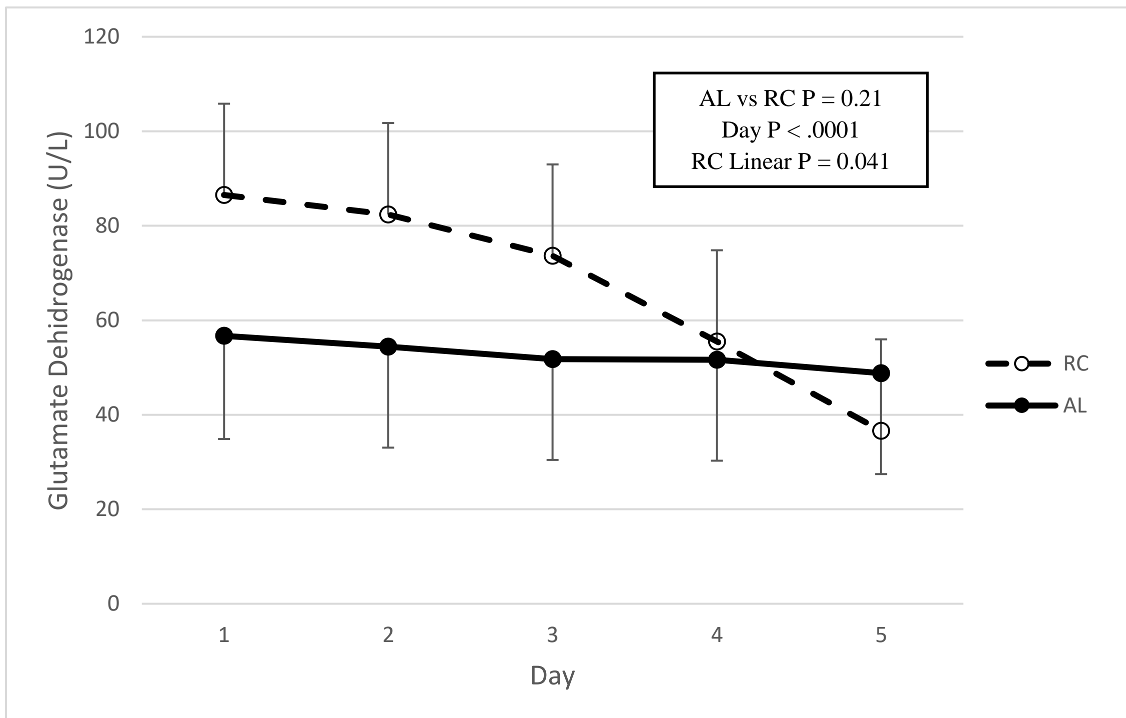

Figure 40 **Daily plasma concentrations pre-feeding of Glutamate Dehydrogenase (GLDH) in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

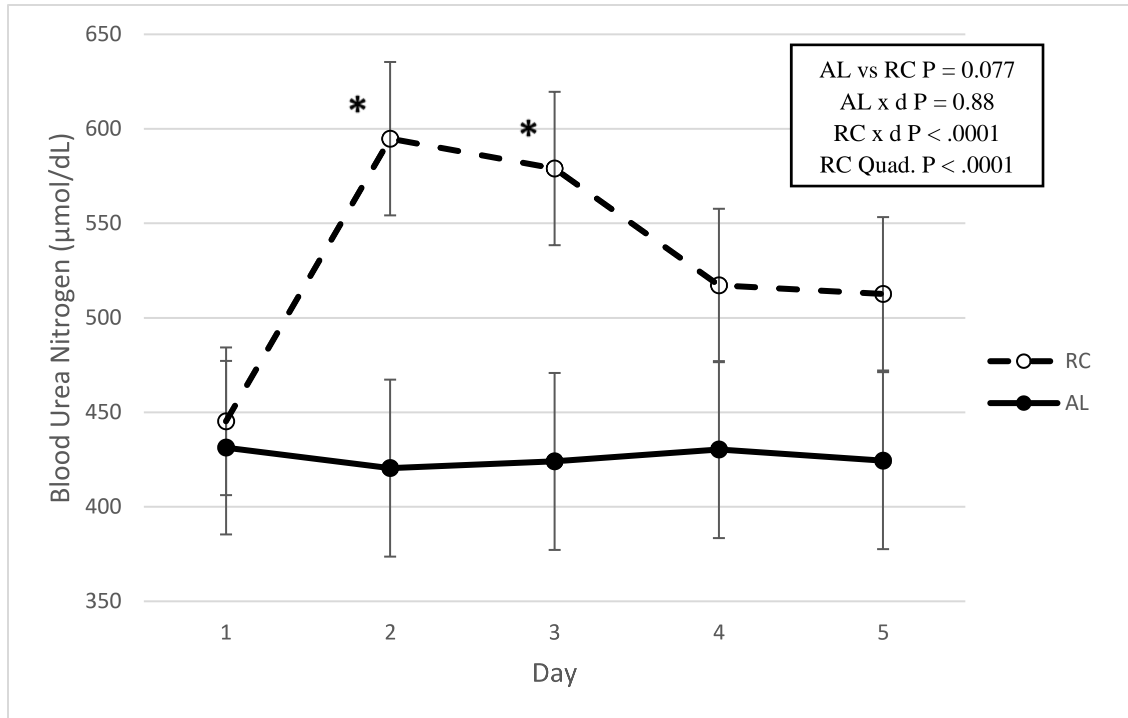

Figure 41 **Daily average plasma concentration of Blood Urea Nitrogen (BUN) in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

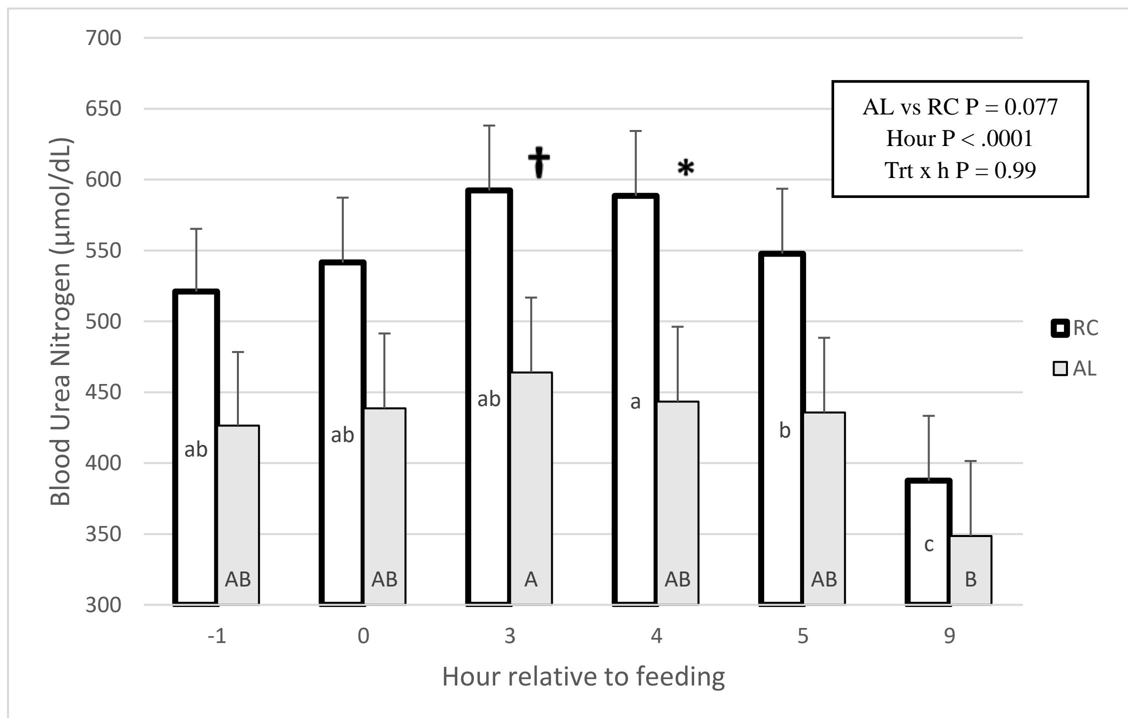

Figure 42 **Blood Urea Nitrogen (BUN) concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

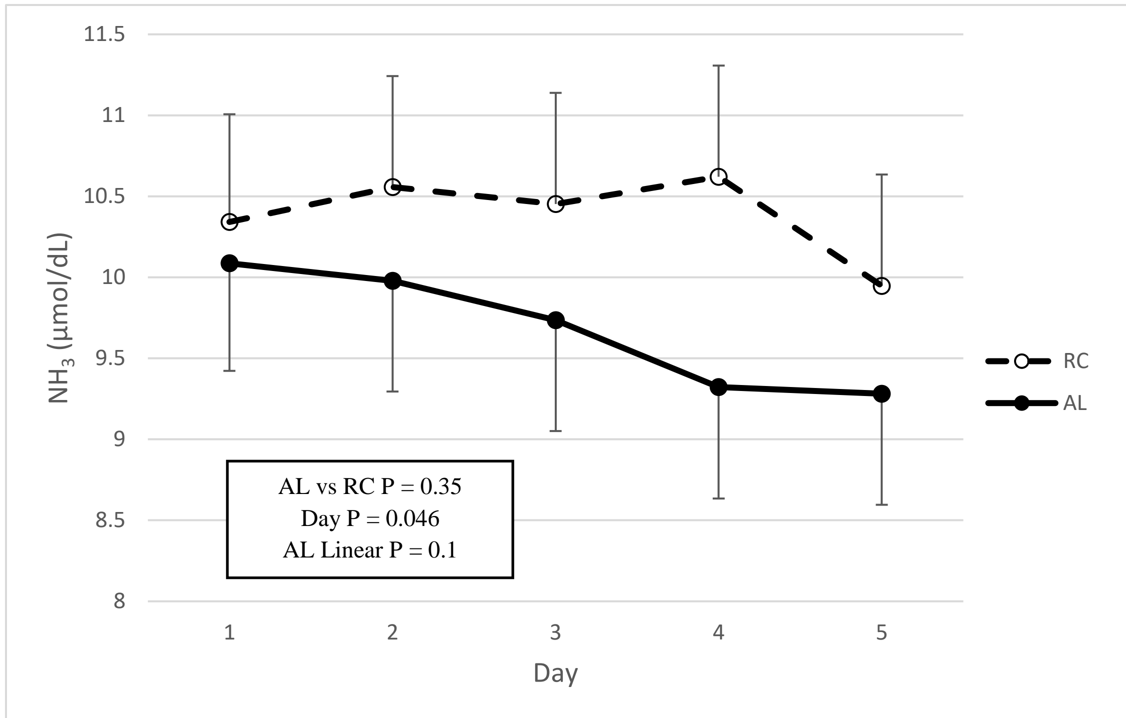

Figure 43 **Daily average plasma concentrations of Ammonia (NH_3) in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

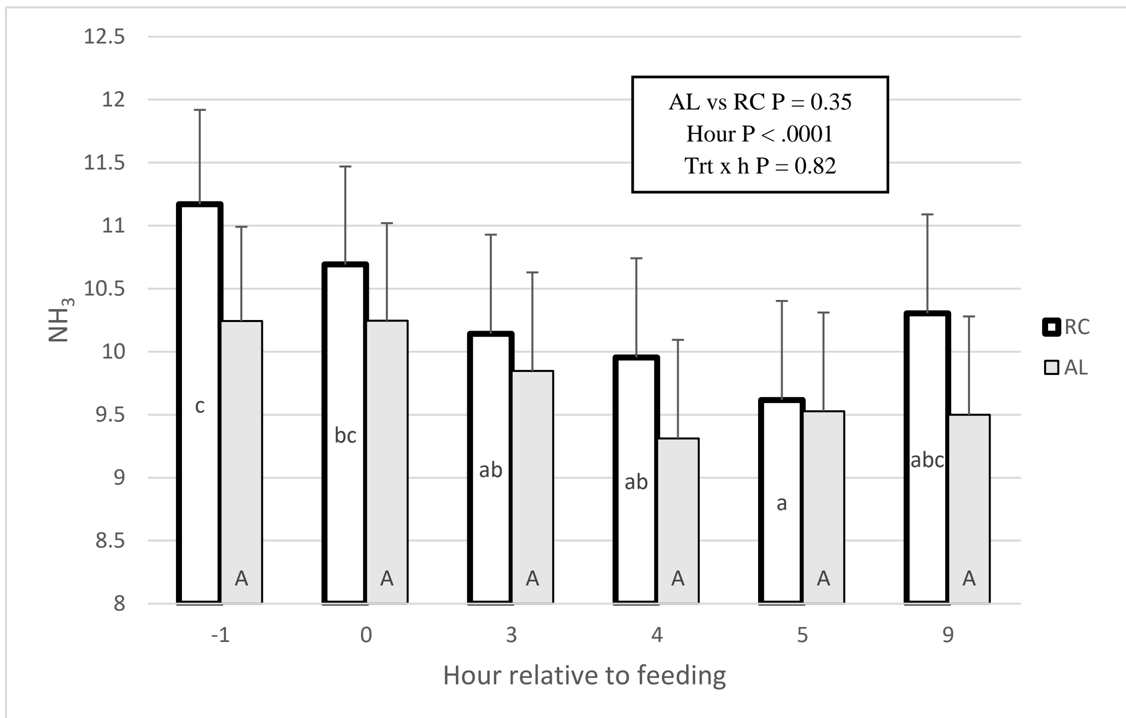

Figure 44 **Ammonia (NH_3) concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

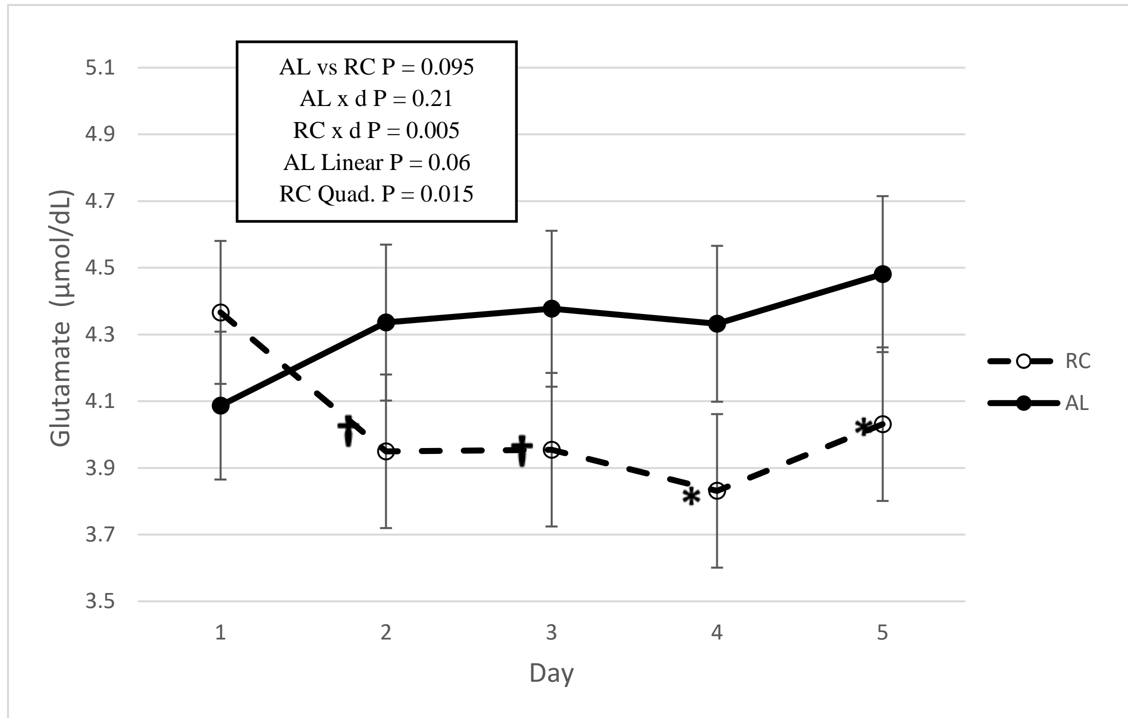

Figure 45 **Daily average plasma concentrations of Glutamate in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

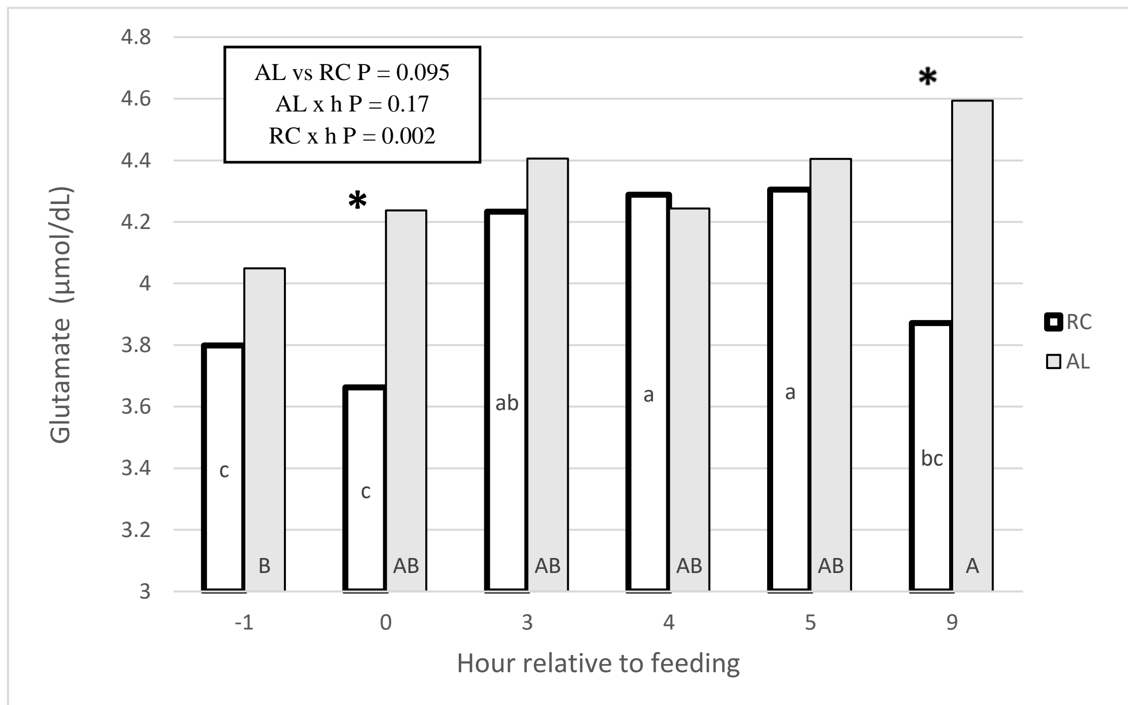

Figure 46 **Glutamate concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

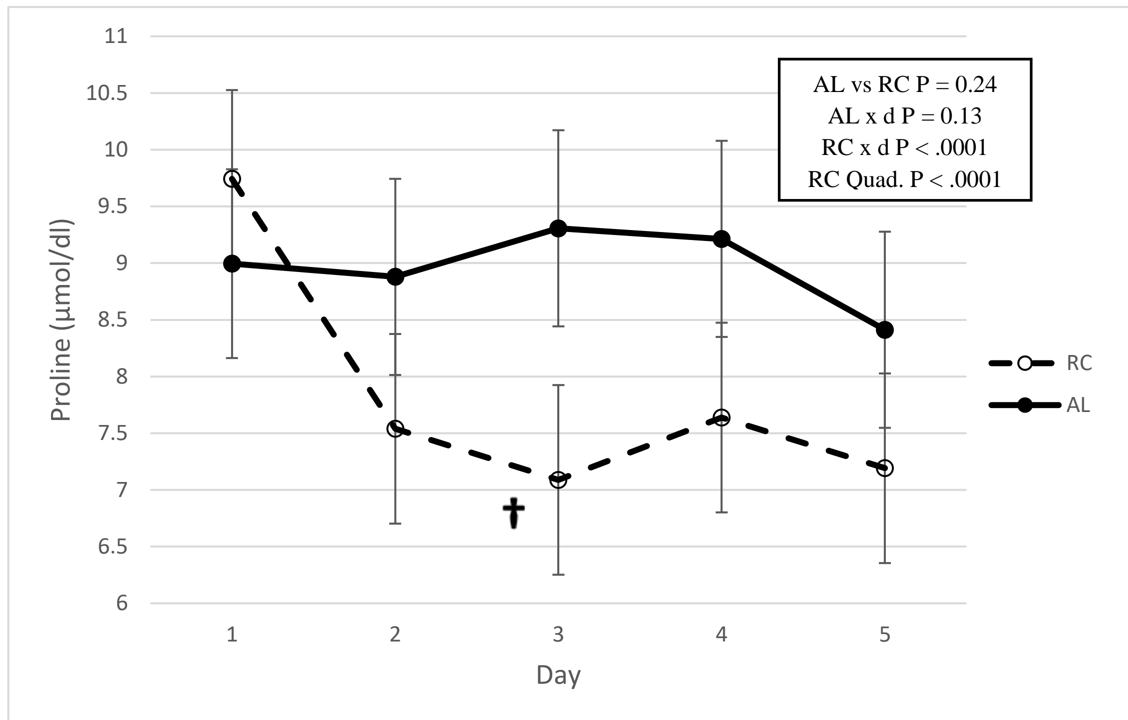

Figure 47 **Daily average plasma concentrations of Proline in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

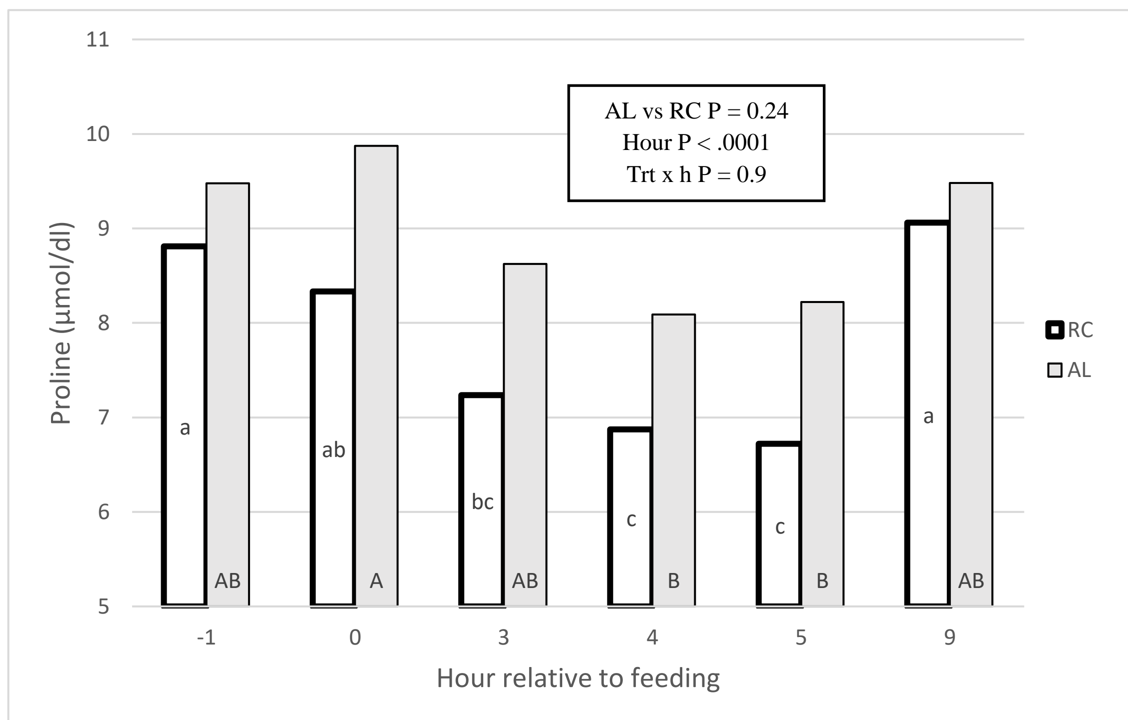

Figure 48 Proline concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).

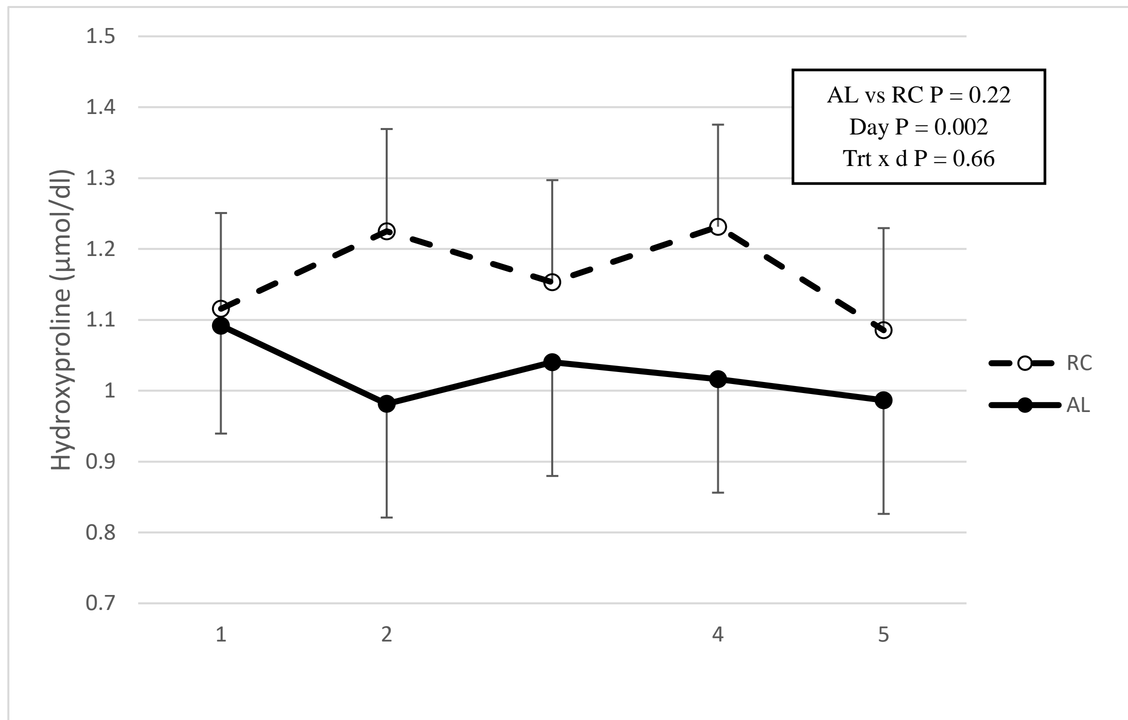

Figure 49 **Daily average plasma concentrations of Hydroxyproline in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

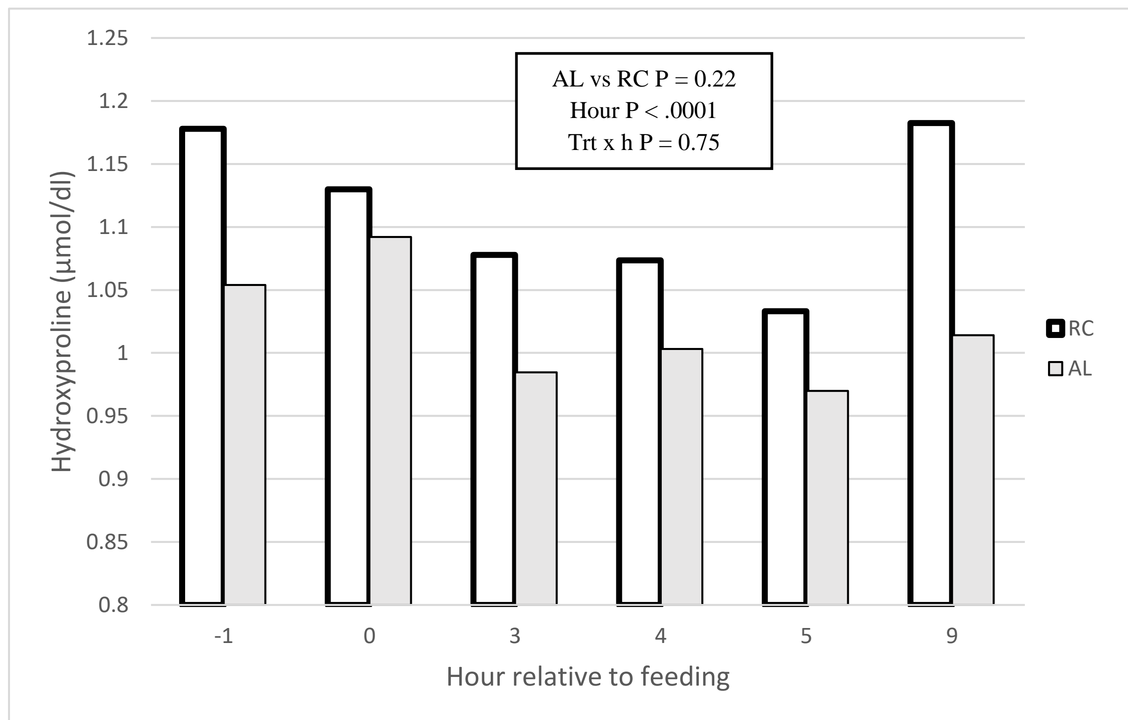

Figure 50 **Hydroxyproline concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

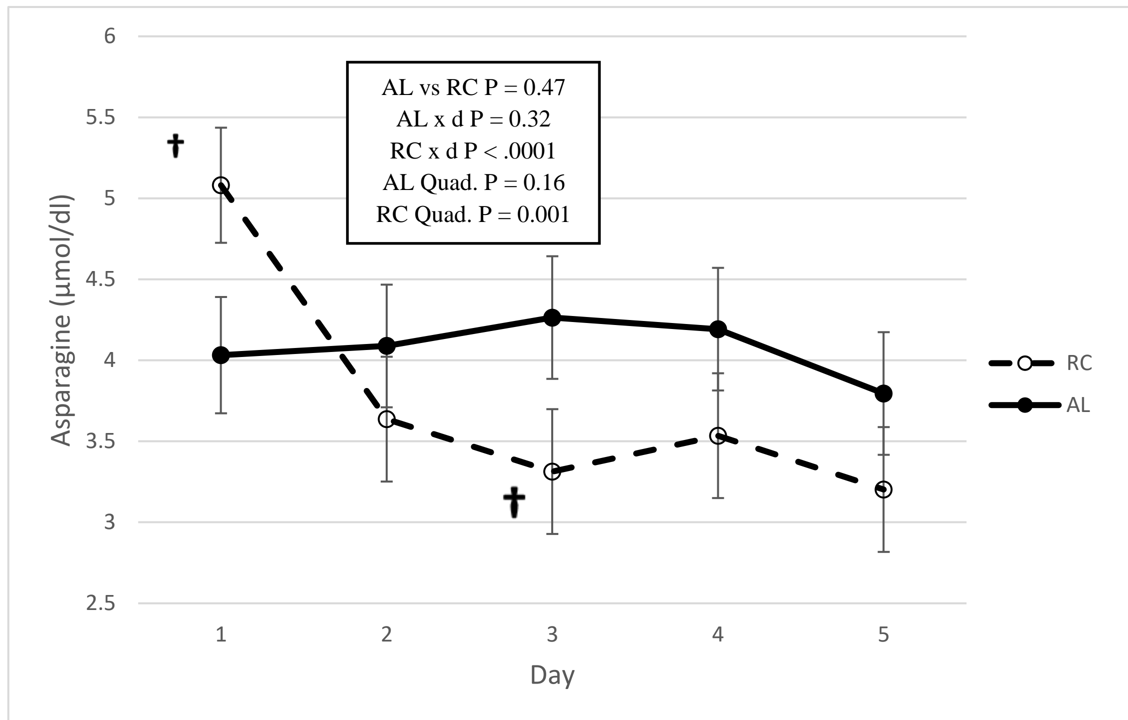

Figure 51 Daily average plasma concentrations of Asparagine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).

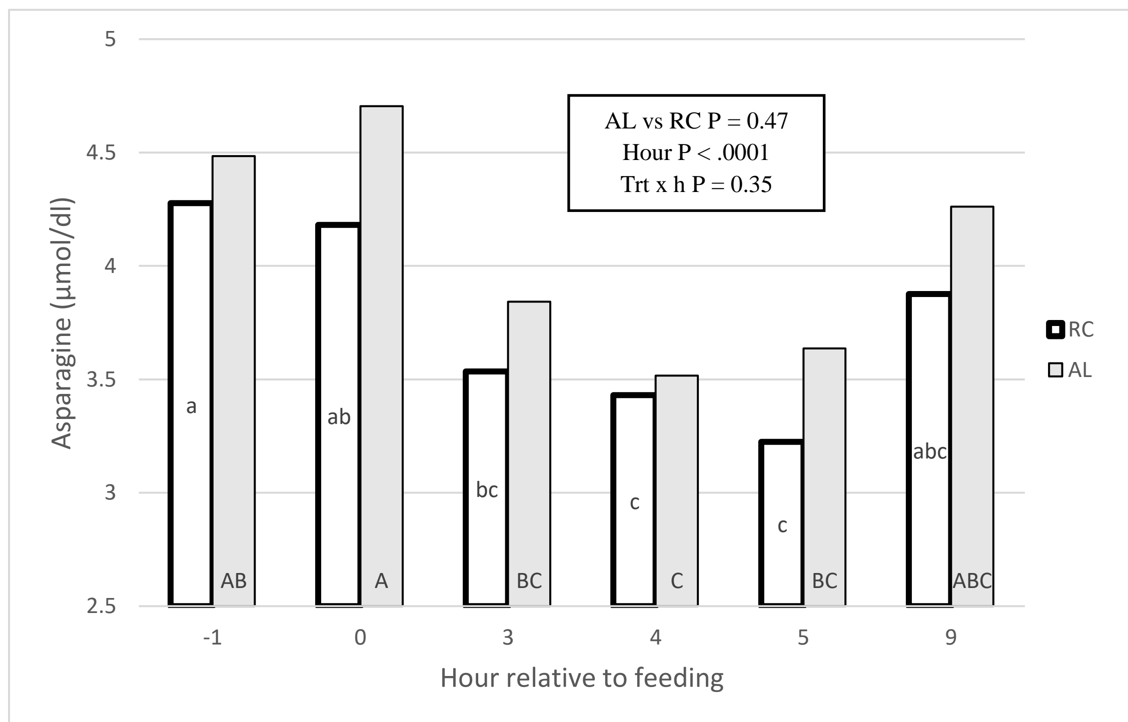

Figure 52 Asparagine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).

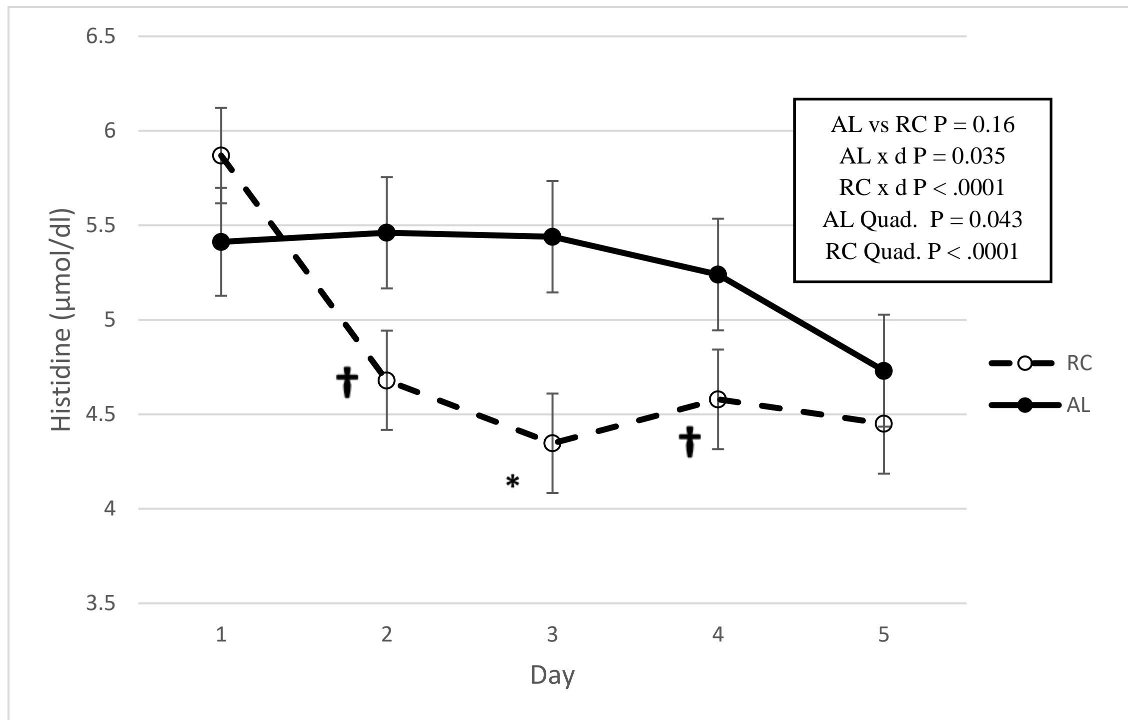

Figure 53 **Daily average plasma concentrations of Histidine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

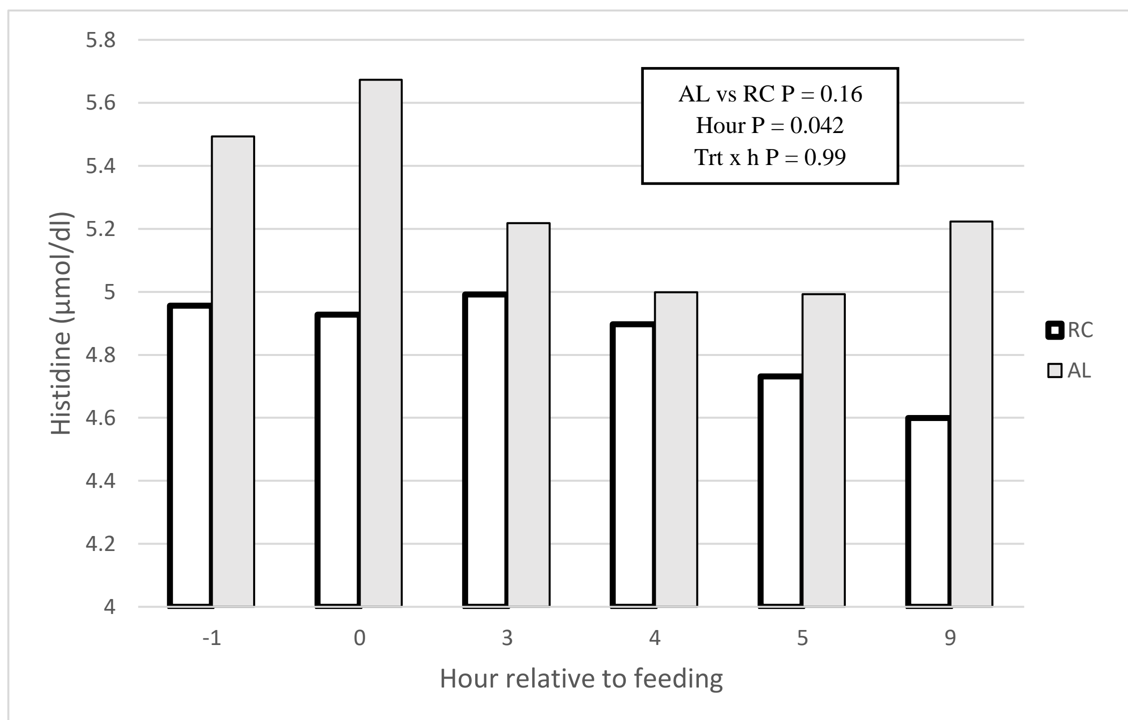

Figure 54 **Histidine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

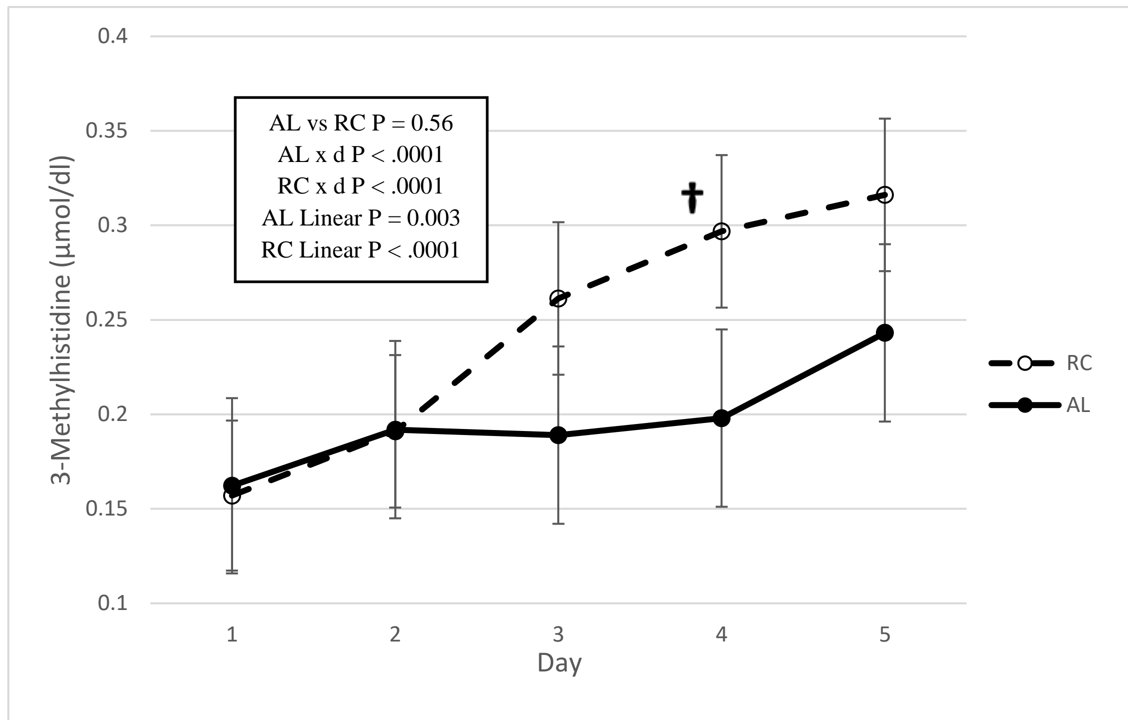

Figure 55 **Daily average plasma concentrations of 3-Methylhistidine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

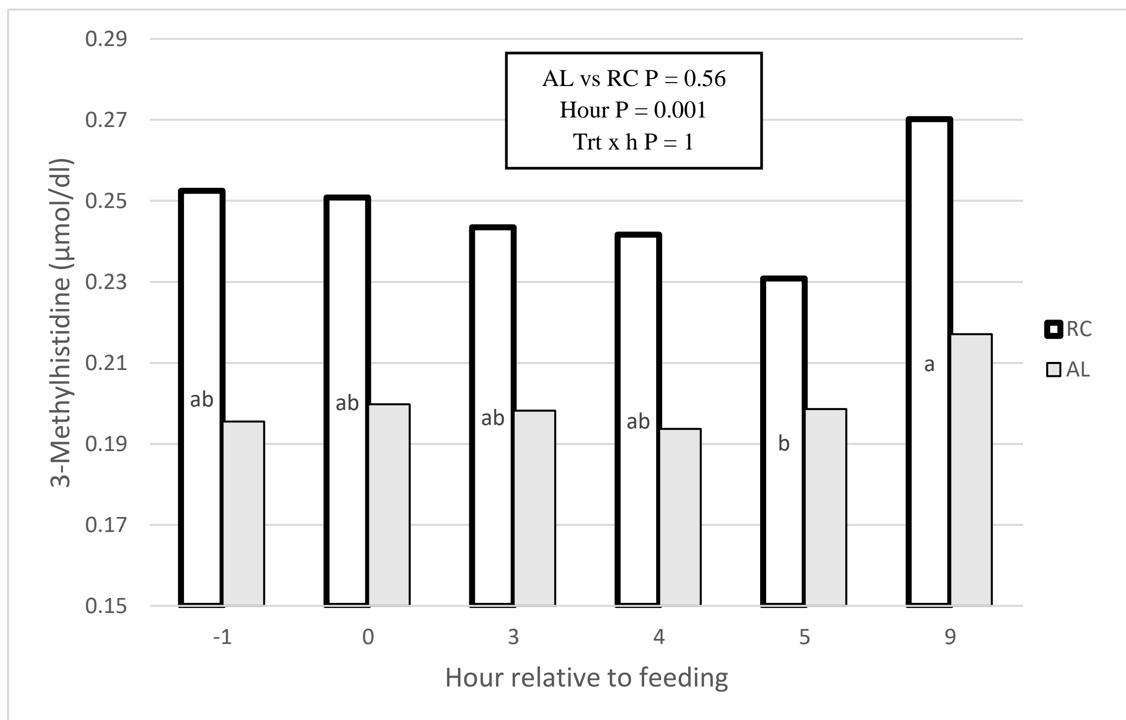

Figure 56 **3-Methylhistidine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

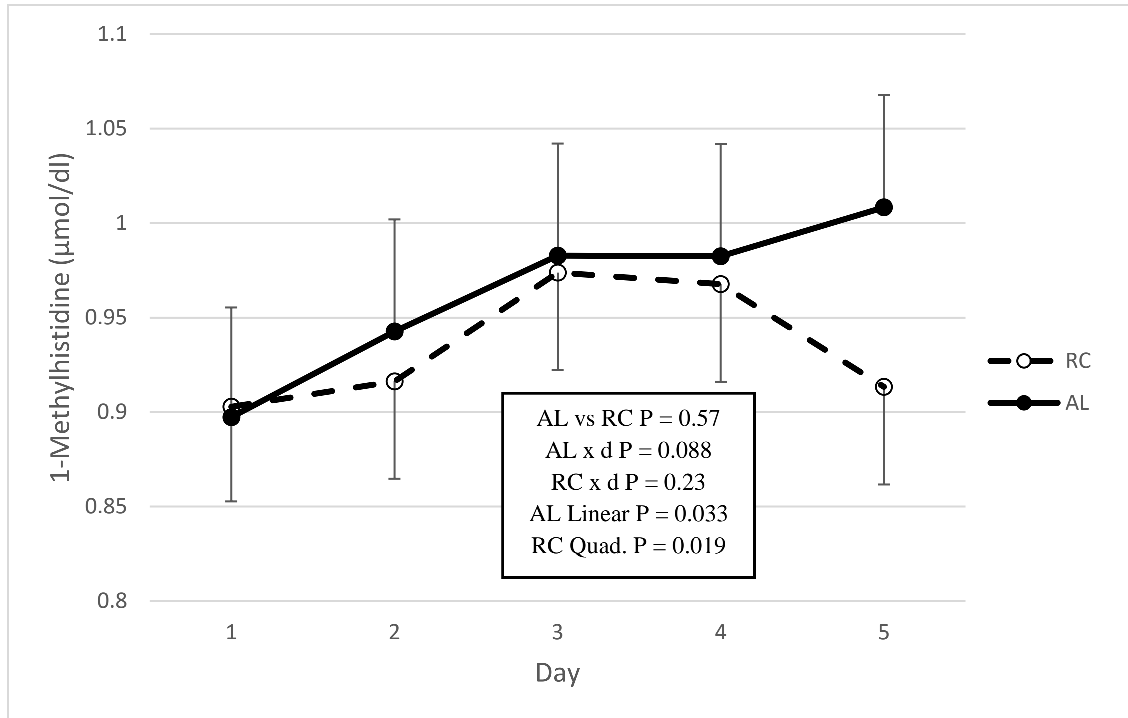

Figure 57 **Daily average plasma concentrations of 1-Methylhistidine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

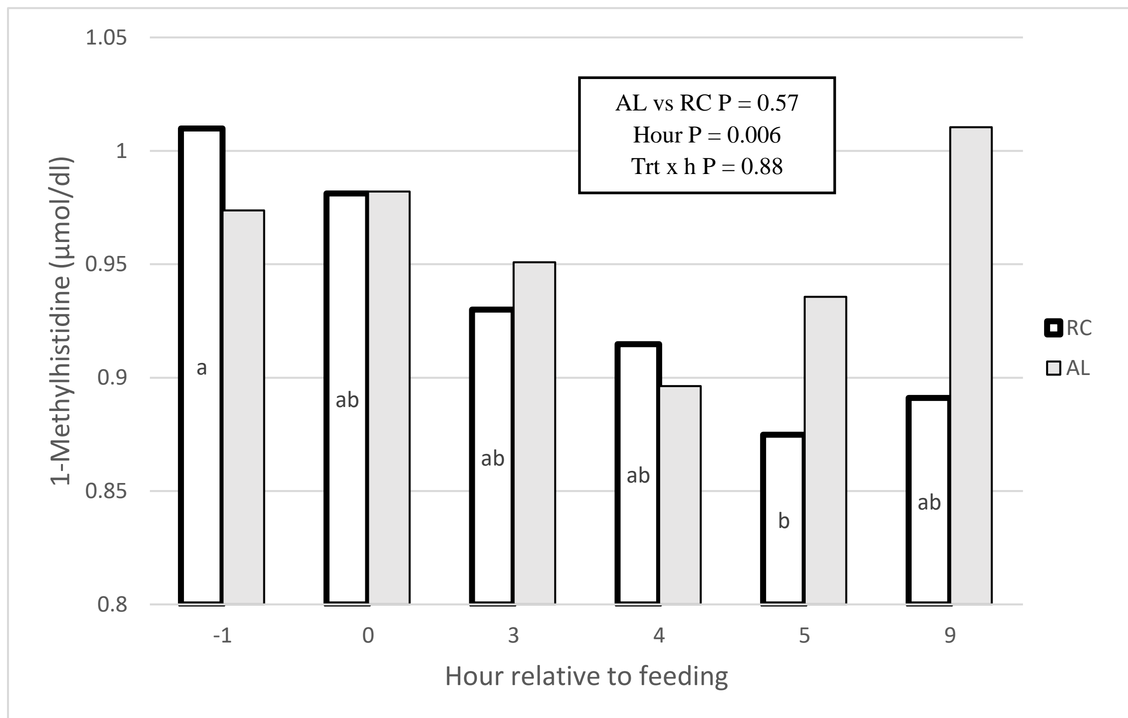

Figure 58 **1-Methylhistidine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

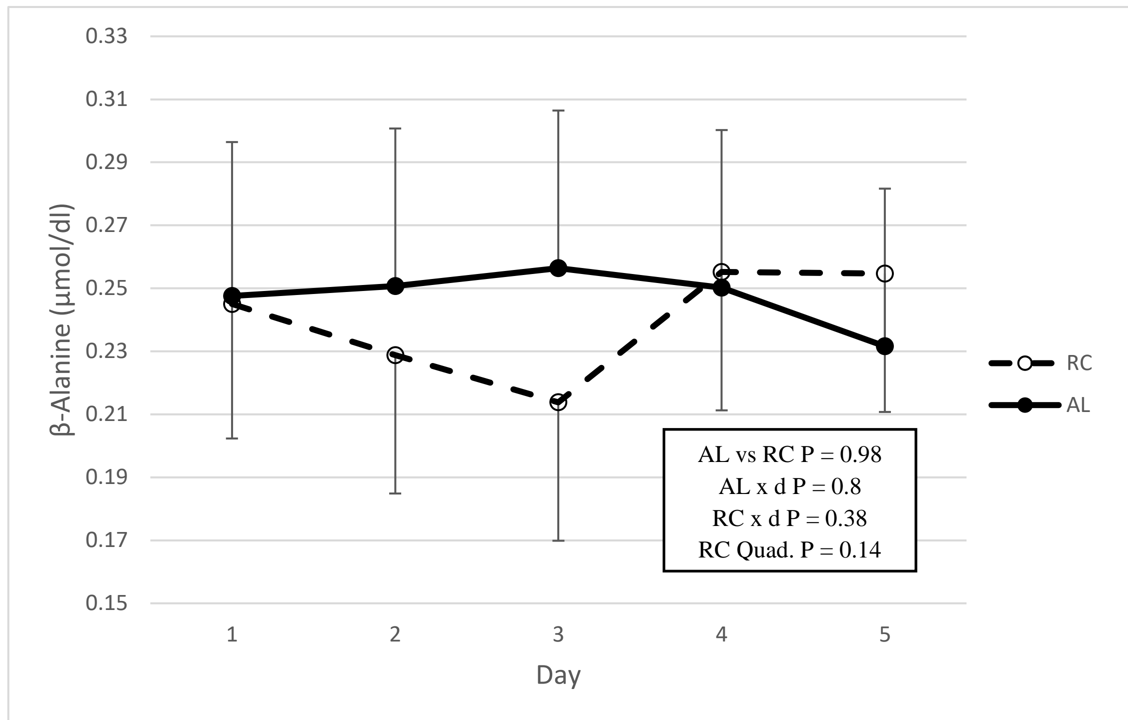

Figure 59 **Daily average plasma concentrations of β-Alanine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

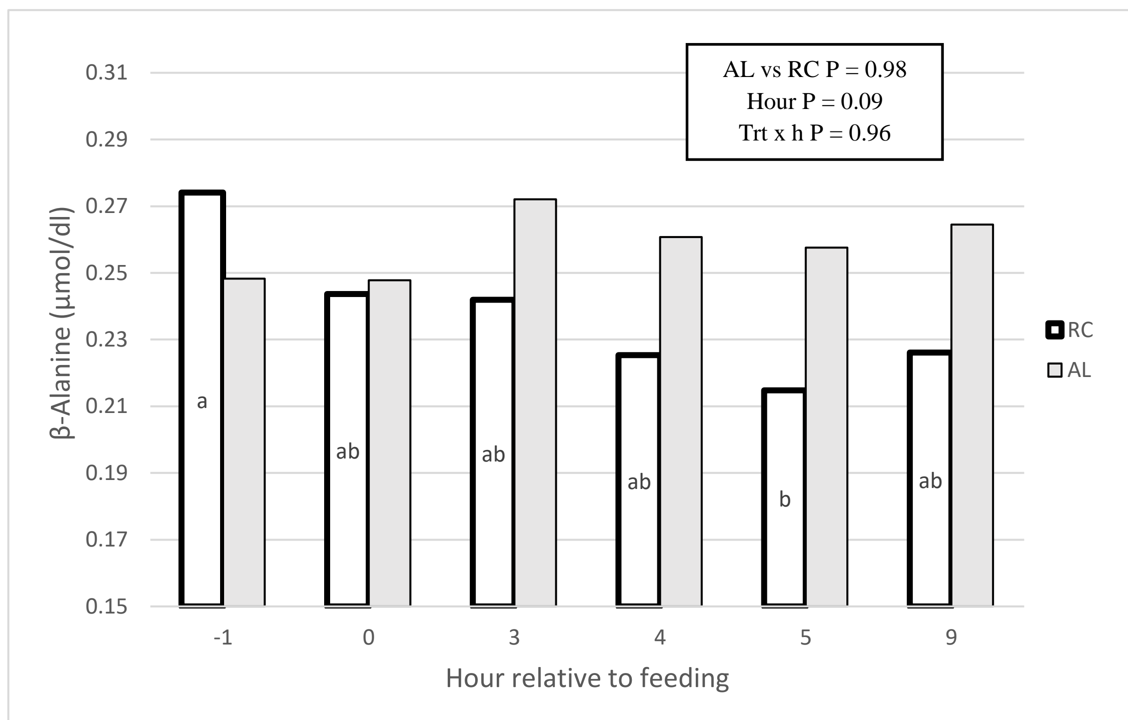

Figure 60 **β-Alanine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

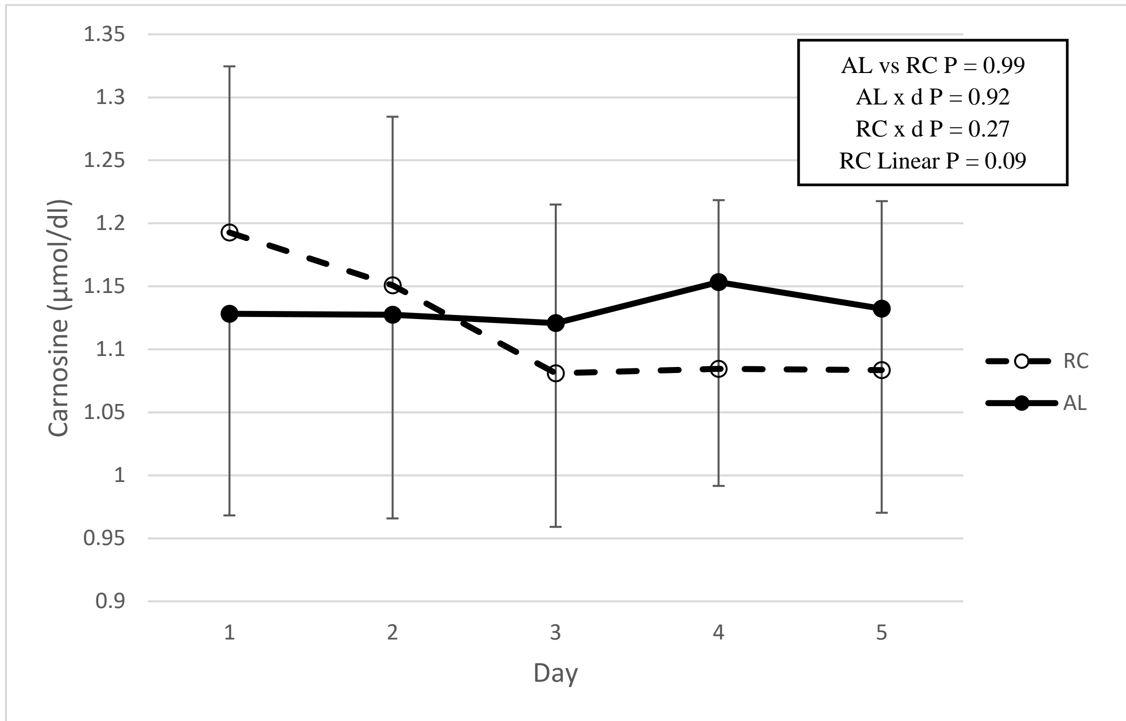

Figure 61 **Daily average plasma concentrations of Carnosine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

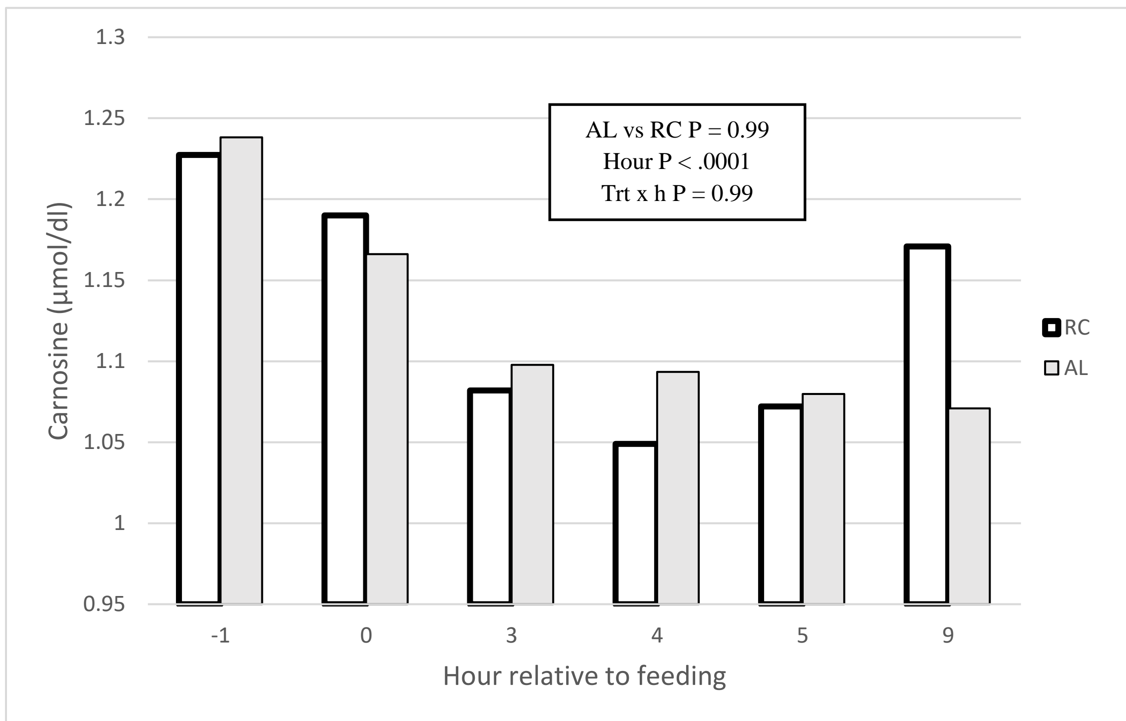

Figure 62 **Carnosine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

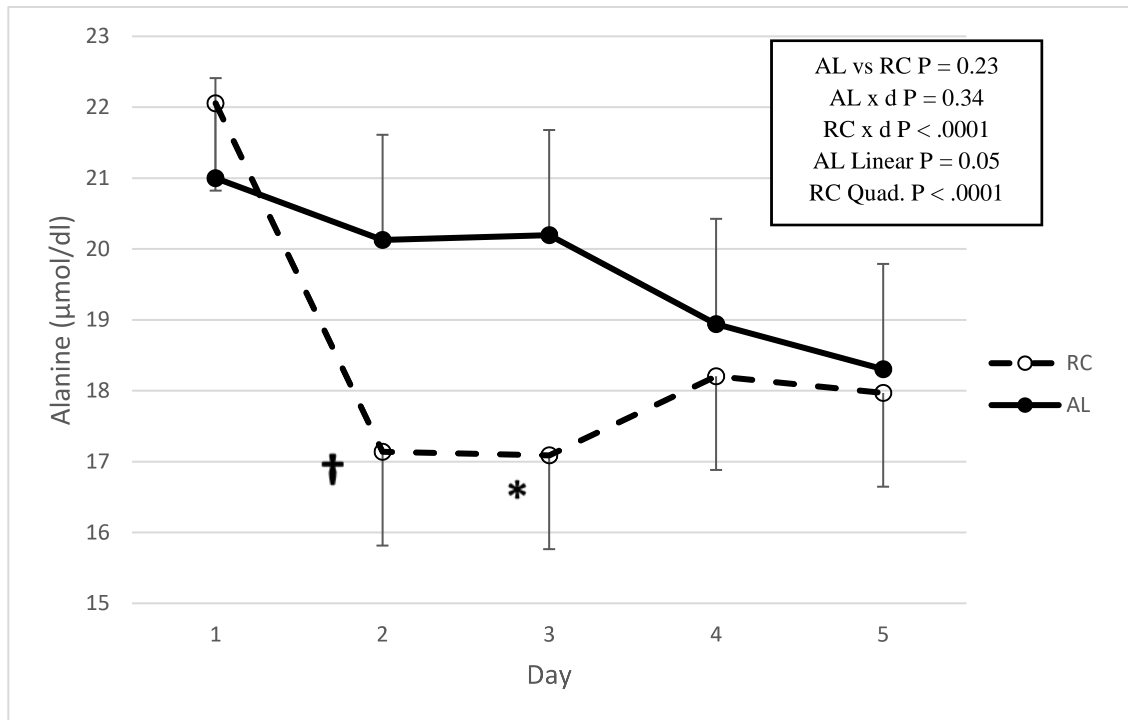

Figure 63 **Daily average plasma concentrations of Alanine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

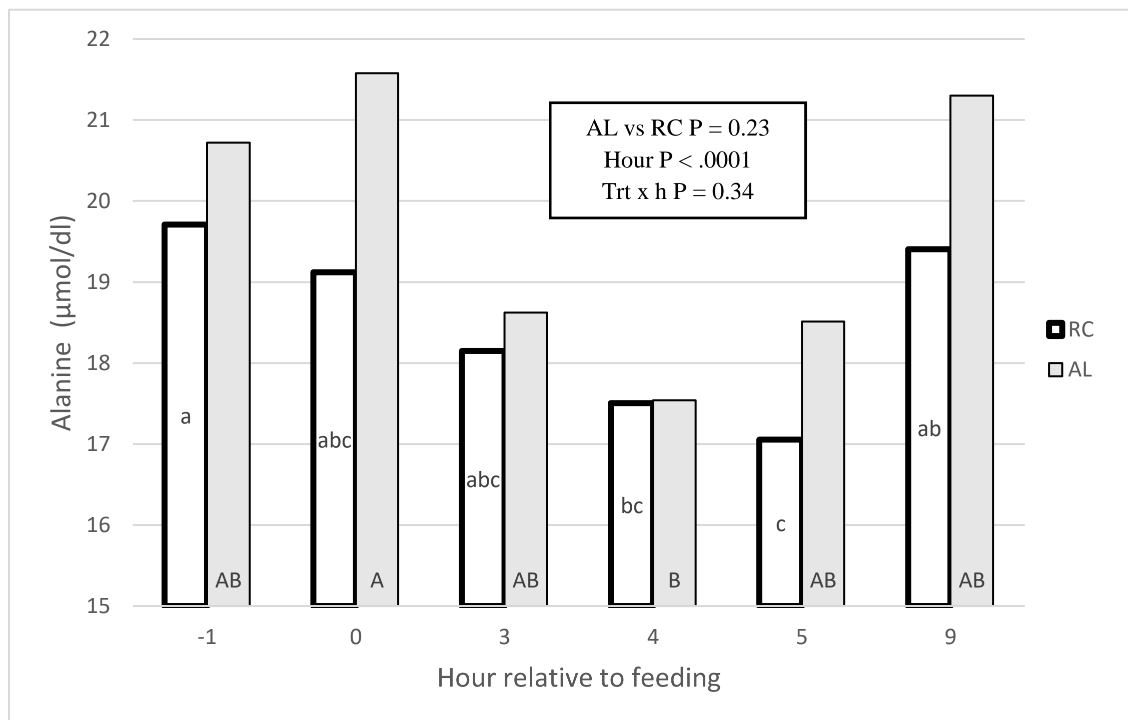

Figure 64 **Alanine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

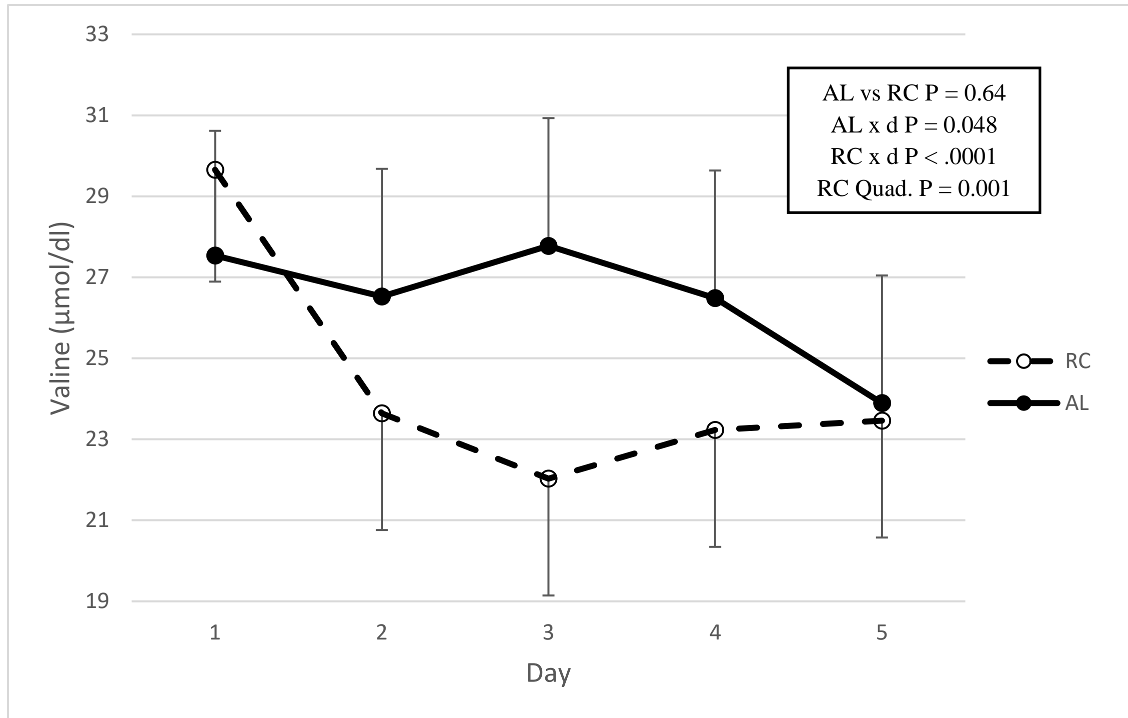

Figure 65 **Daily average plasma concentrations of Valine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

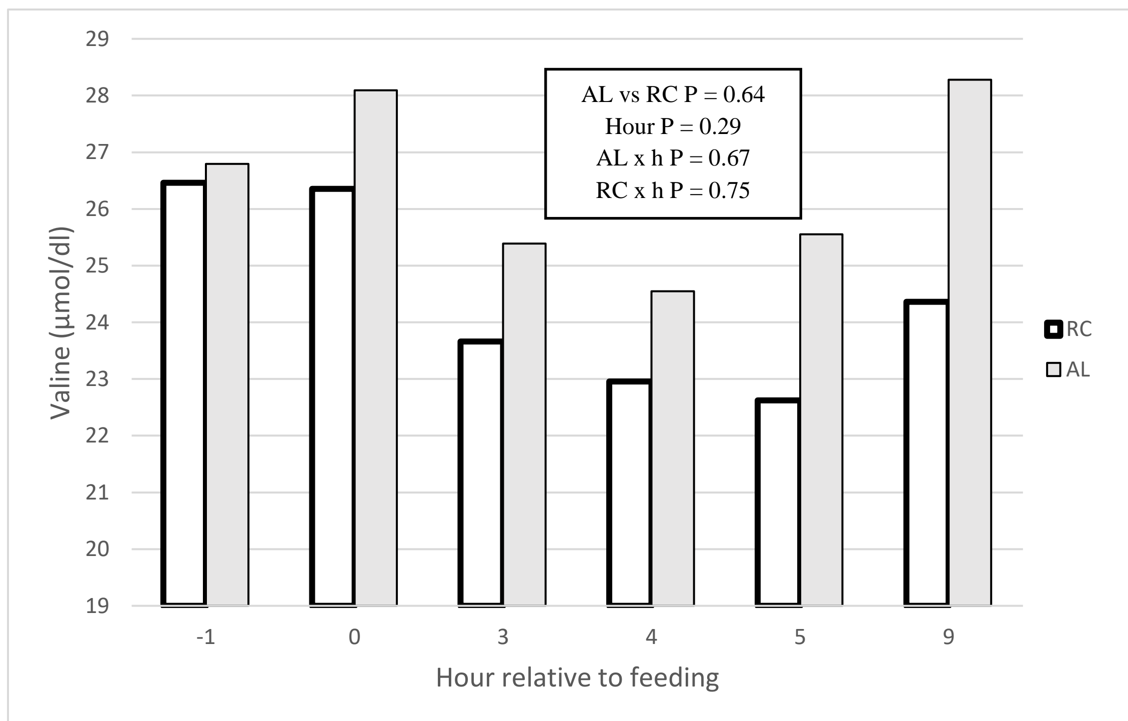

Figure 66 **Valine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

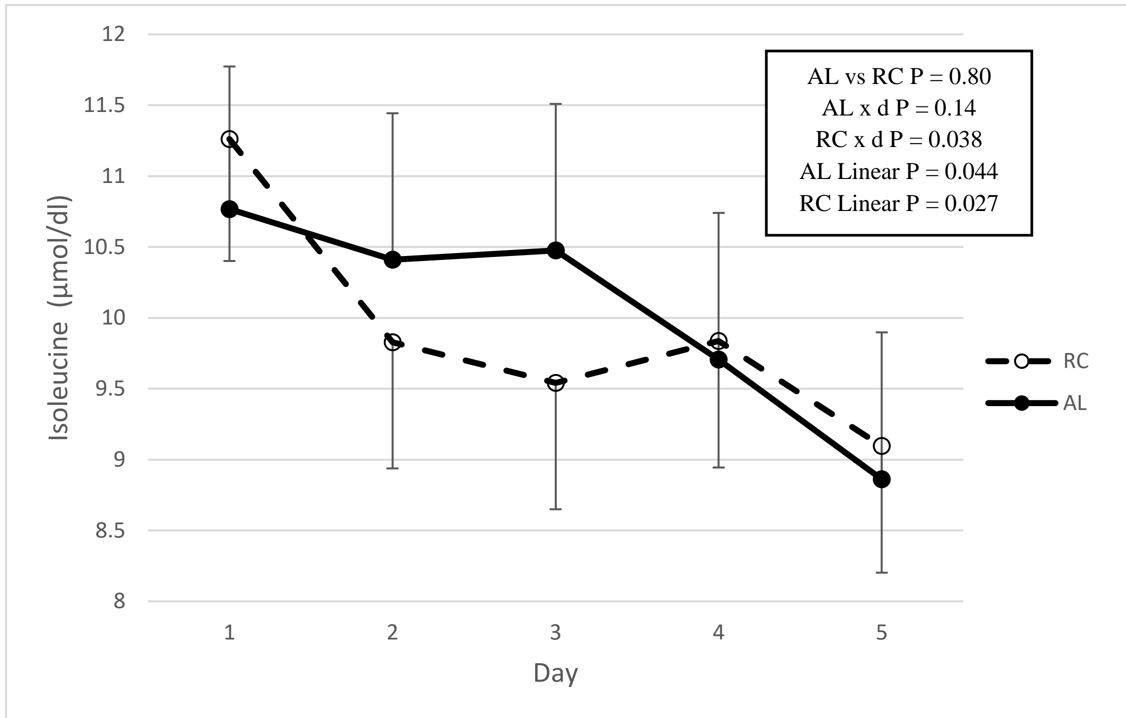

Figure 67 **Daily average plasma concentrations of Isoleucine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

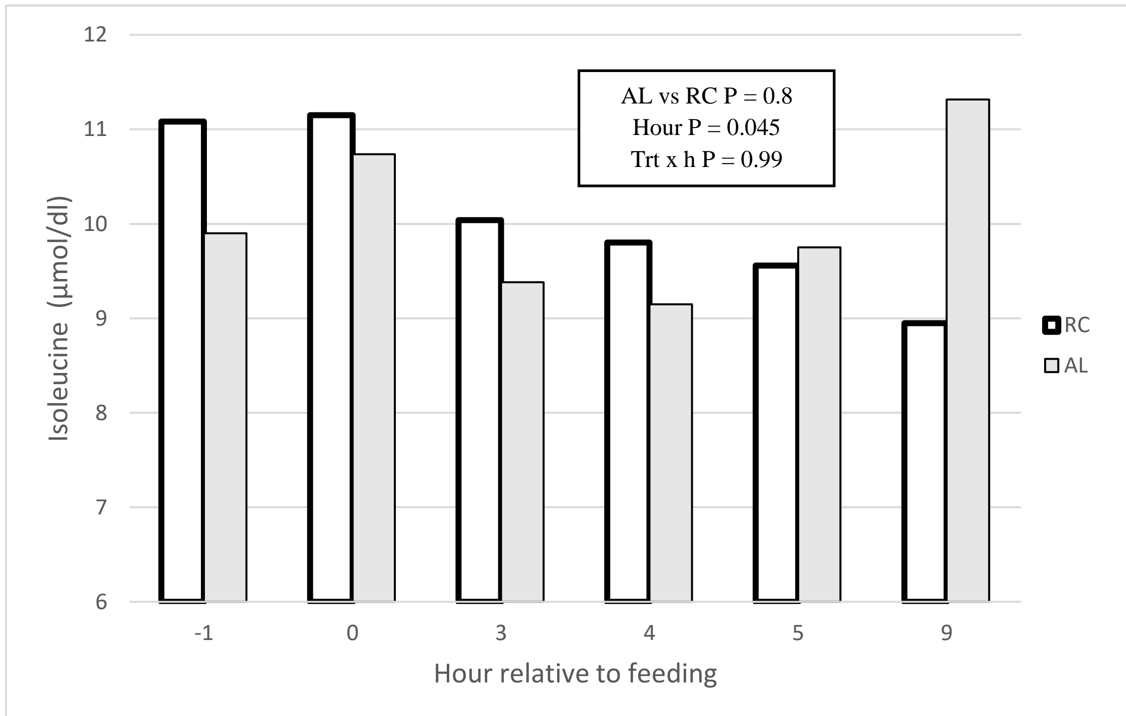

Figure 68 **Isoleucine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

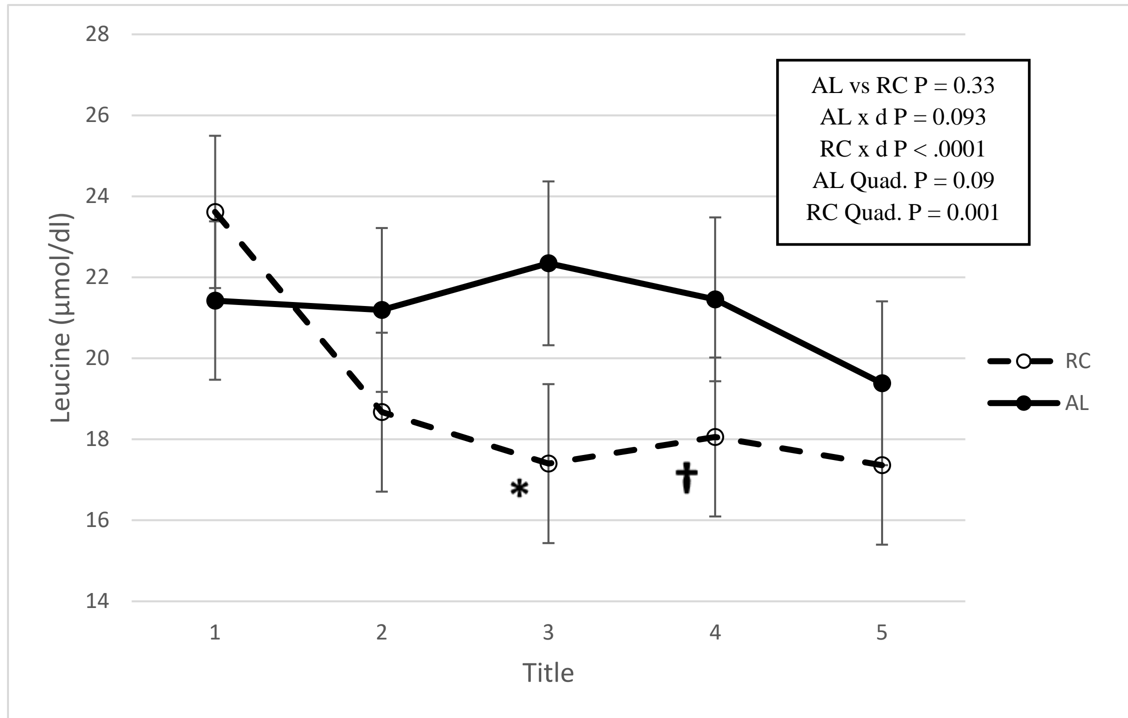

Figure 69 **Daily average plasma concentrations of Leucine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (**P **< 0.10) and * (**P **< 0.05).**

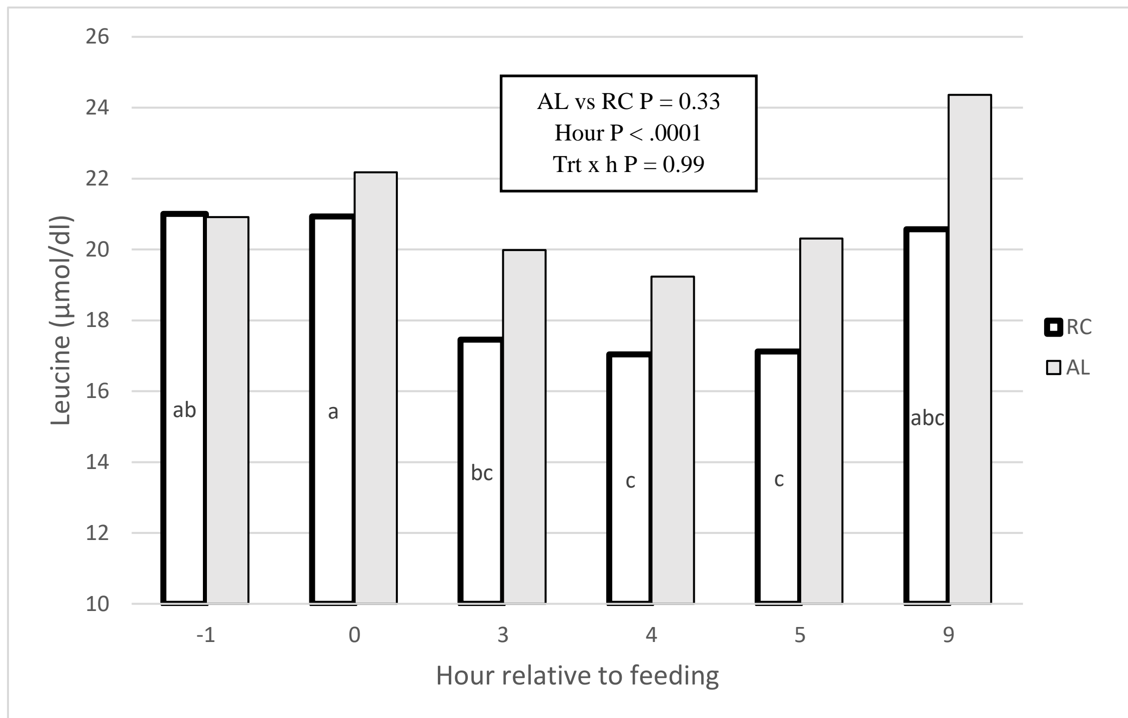

Figure 70 **Leucine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at** P**<0.05. Bars with same Upper case letter are not significant different at** P**<0.05. Differences between treatments (AL and RC) are marked with † (**P **< 0.10) and * (**P **< 0.05).**

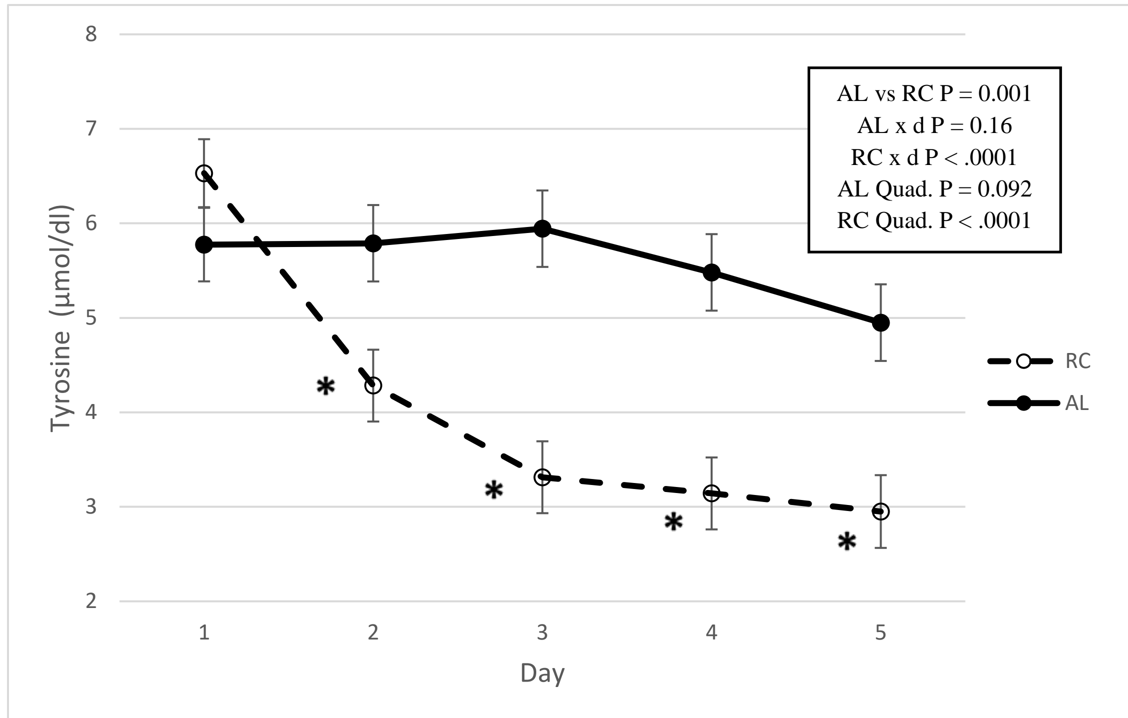

Figure 71 **Daily average plasma concentrations of Tyrosine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

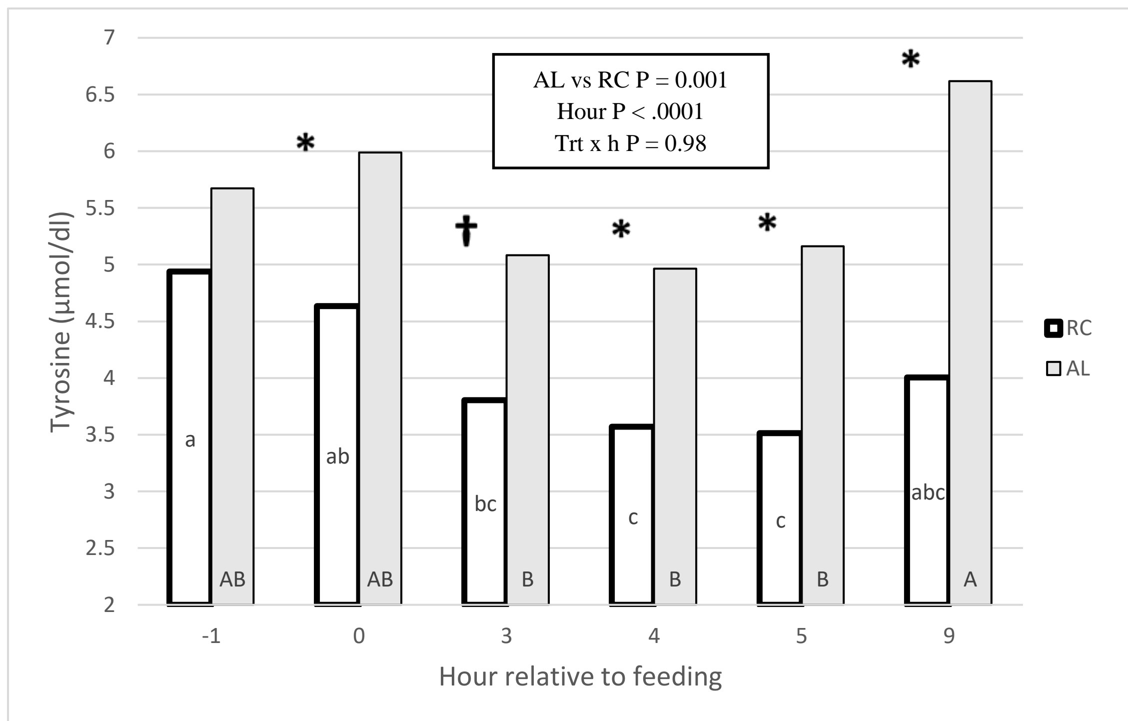

Figure 72 **Tyrosine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

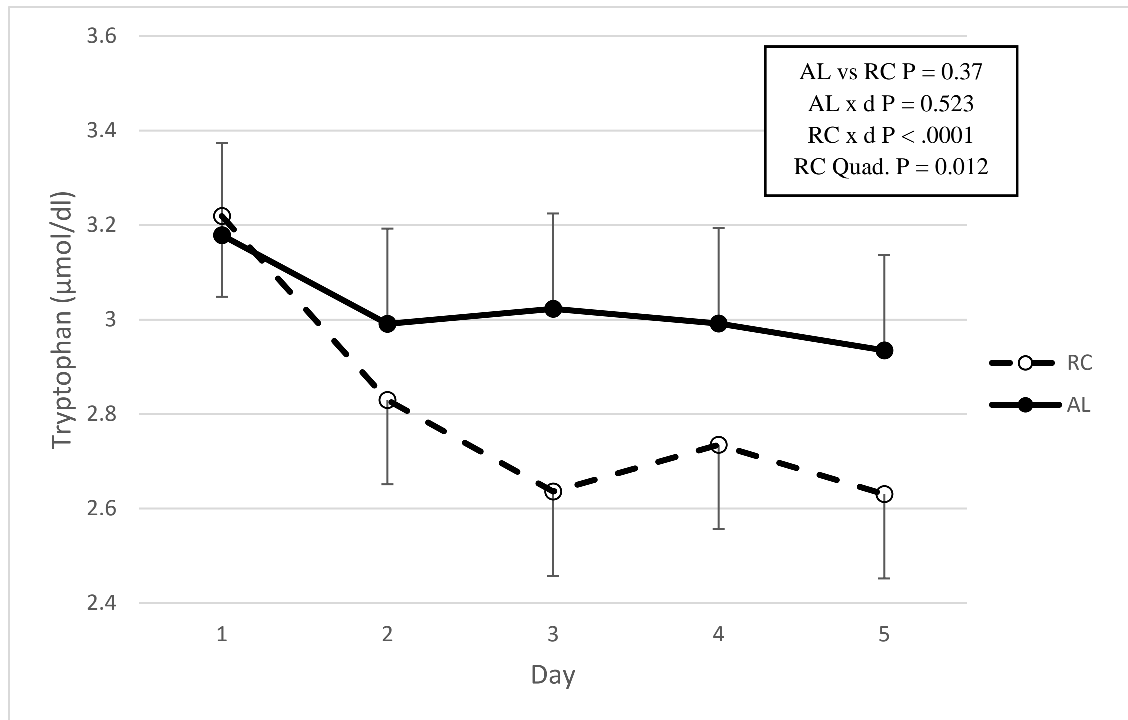

Figure 73 **Daily average plasma concentrations of Tryptophan in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

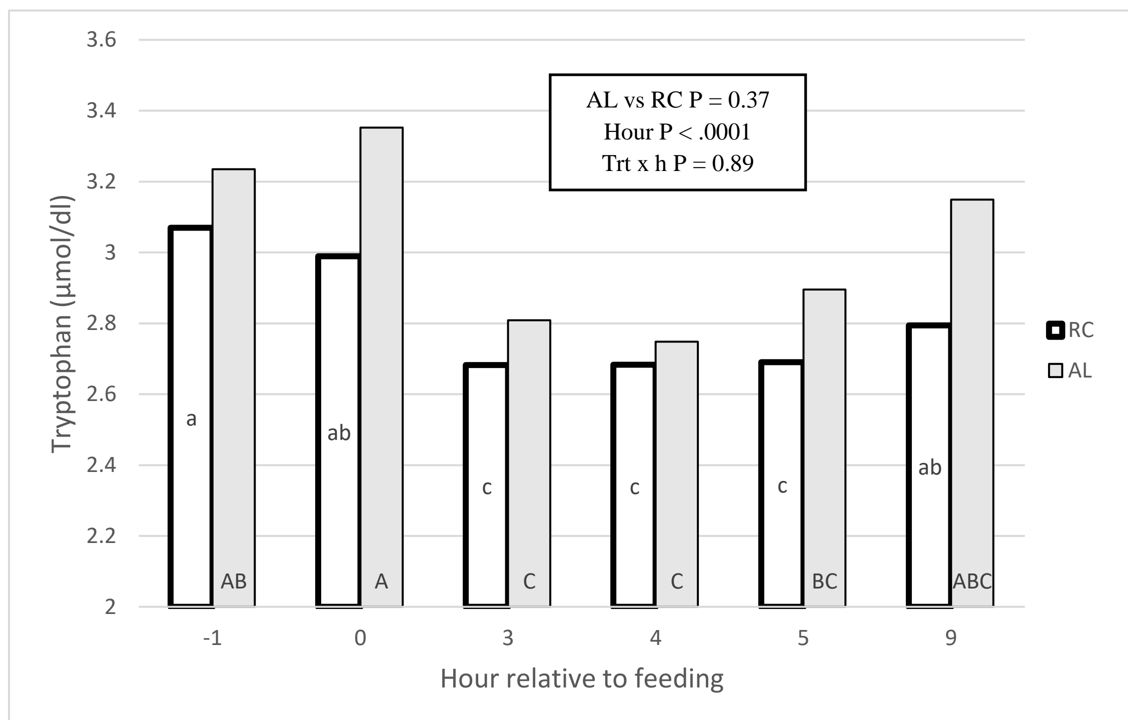

Figure 74 **Tryptophan concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

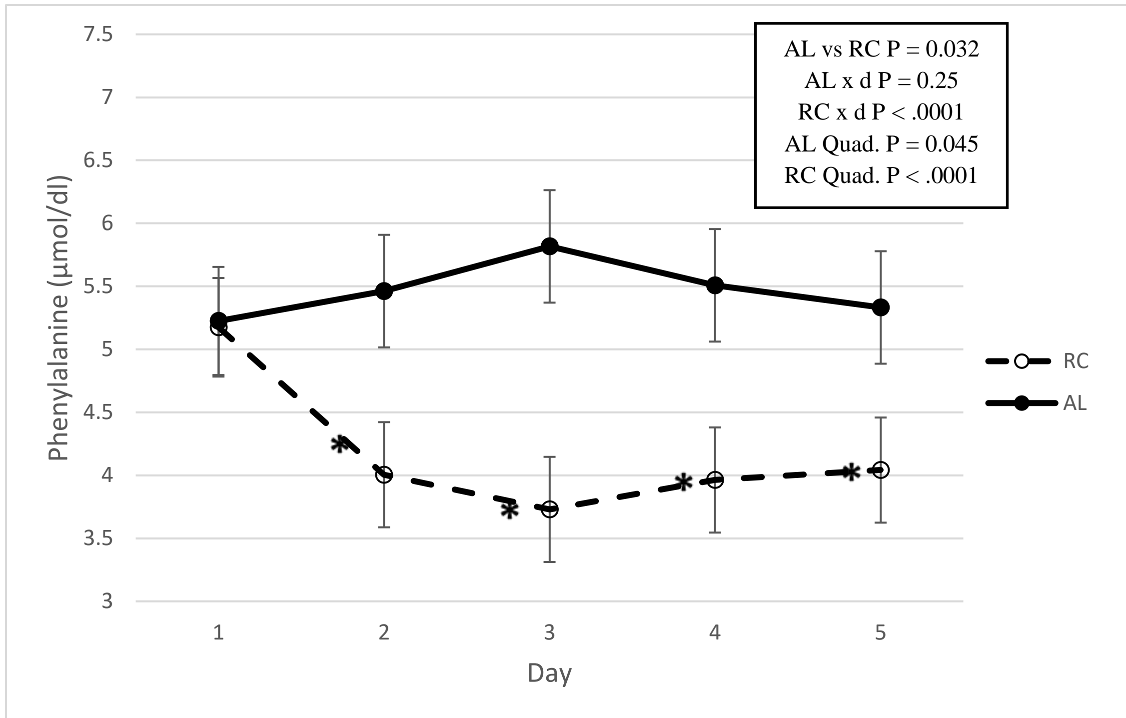

Figure 75 **Daily average plasma concentrations of Phenylalanine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

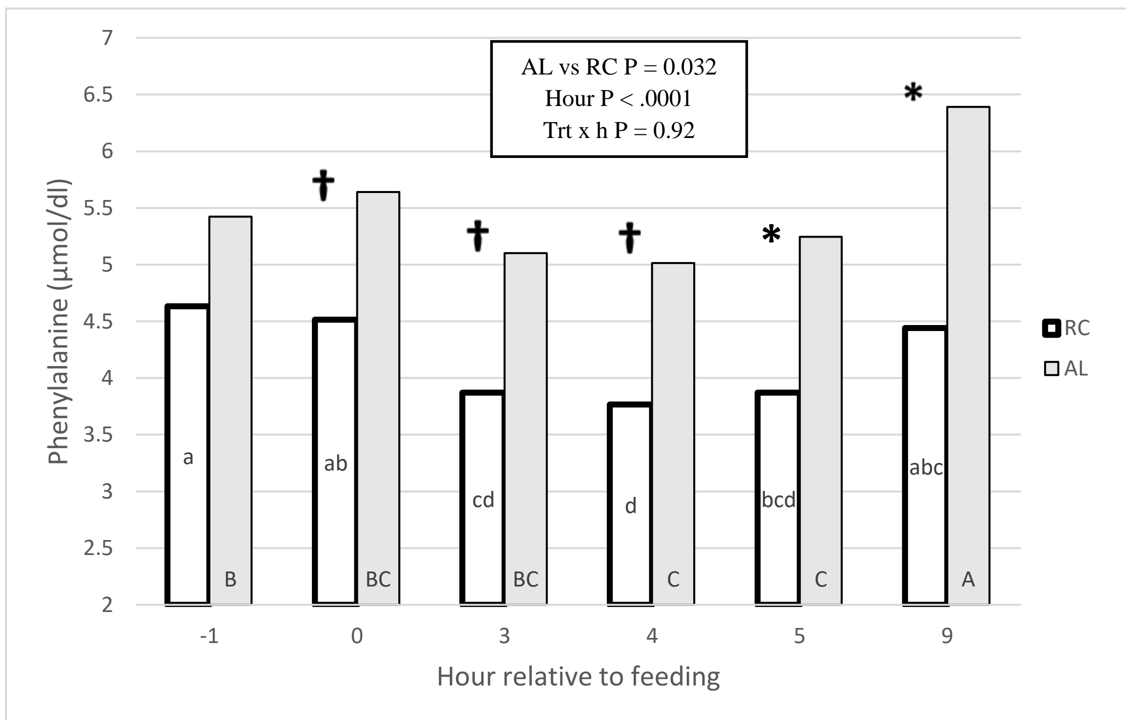

Figure 76 **Phenylalanine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

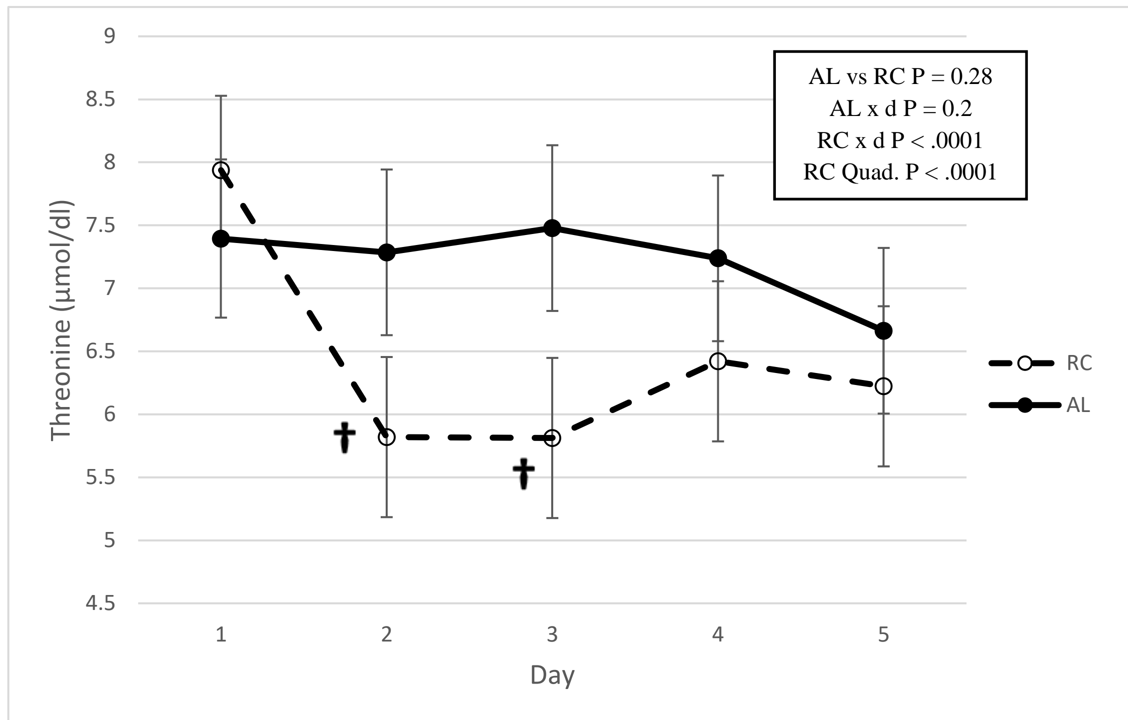

Figure 77 **Daily average plasma concentrations of Threonine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

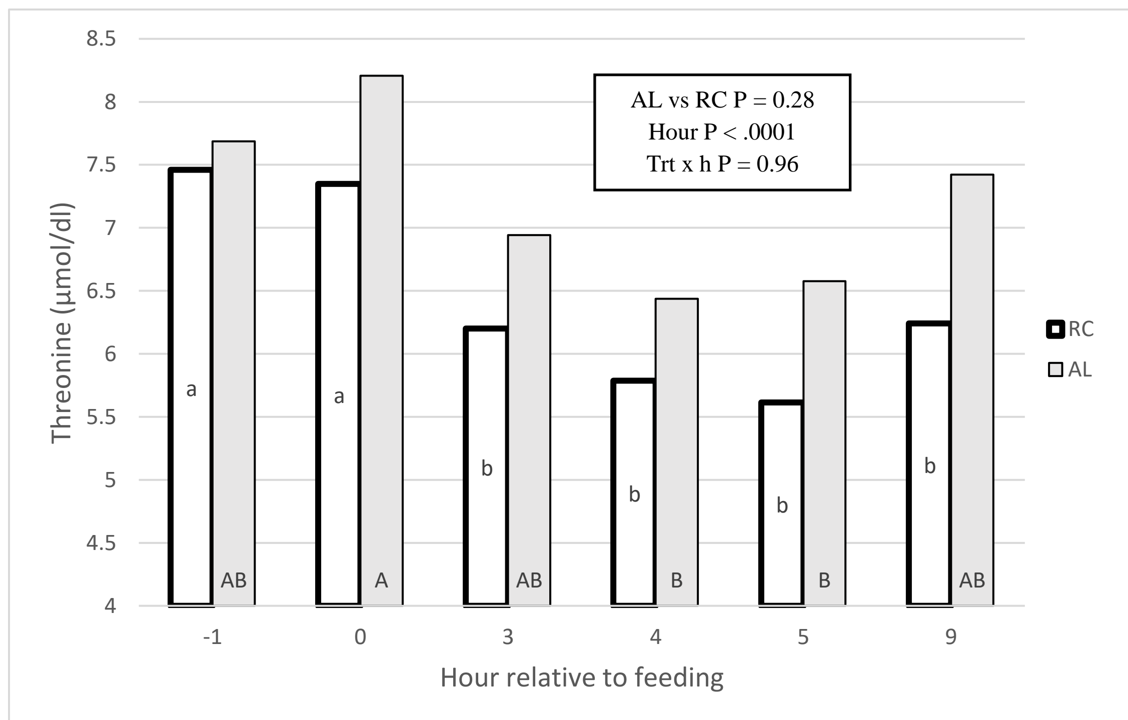

Figure 78 **Threonine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

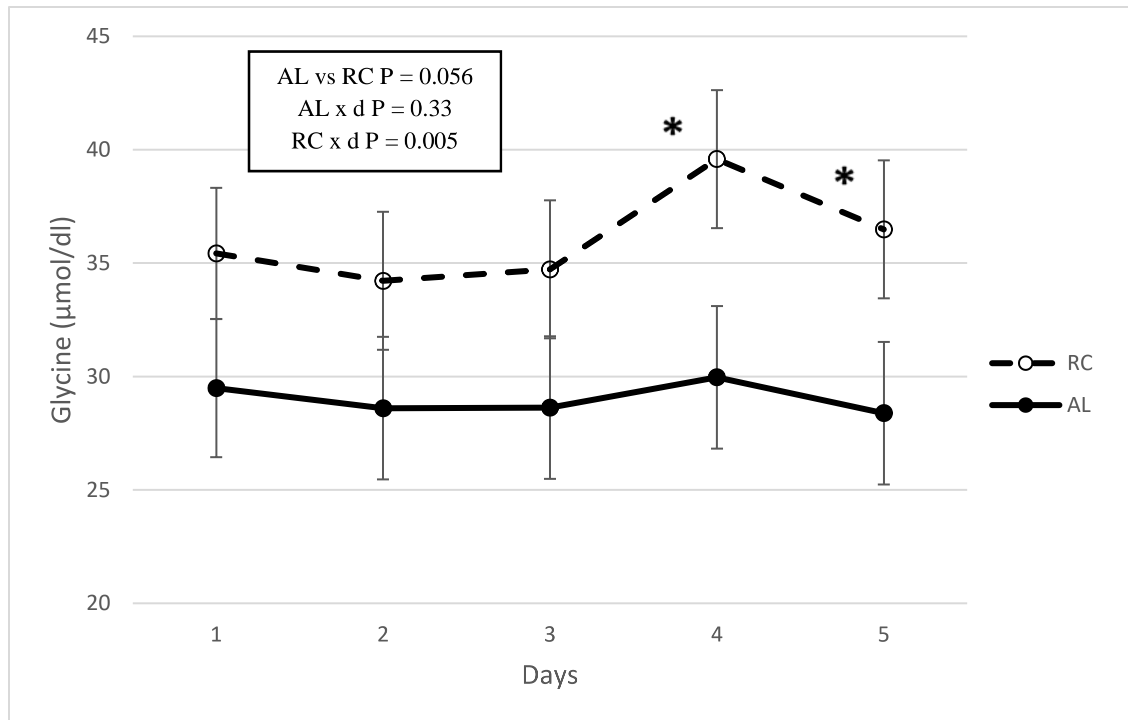

Figure 79 **Daily average plasma concentrations of Glycine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (**P **< 0.10) and * (**P **< 0.05).**

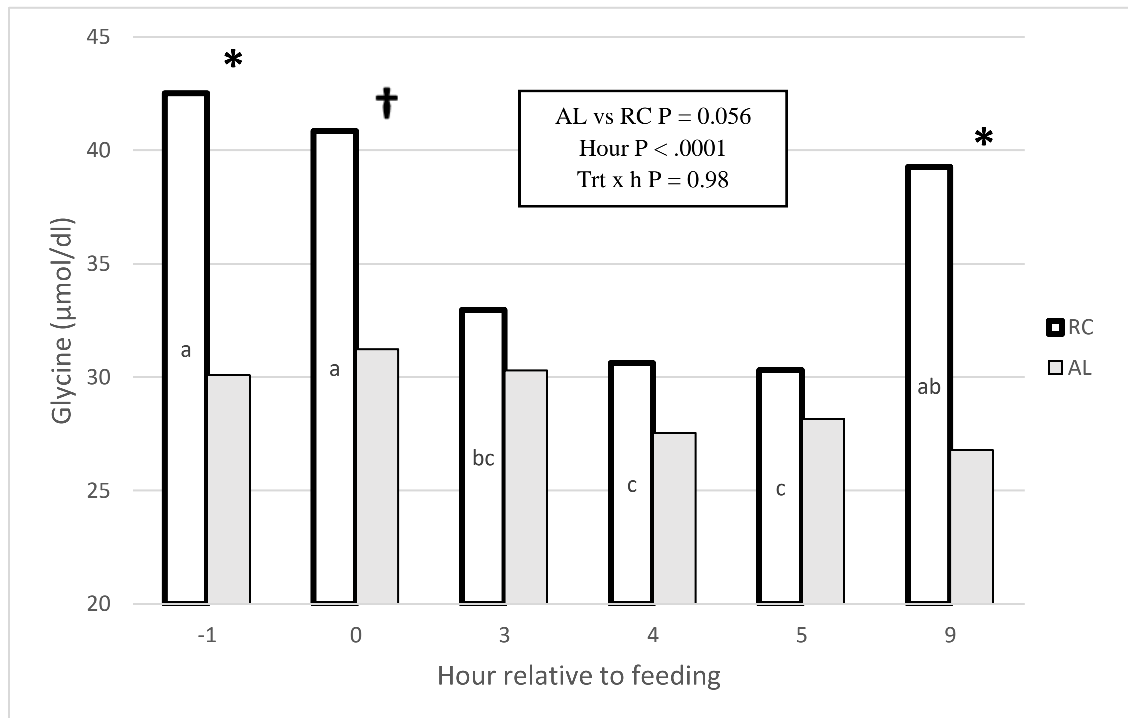

Figure 80 **Glycine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at **P**<0.05. Bars with same Upper case letter are not significant different at **P**<0.05. Differences between treatments (AL and RC) are marked with † (**P **< 0.10) and * (**P **< 0.05).**

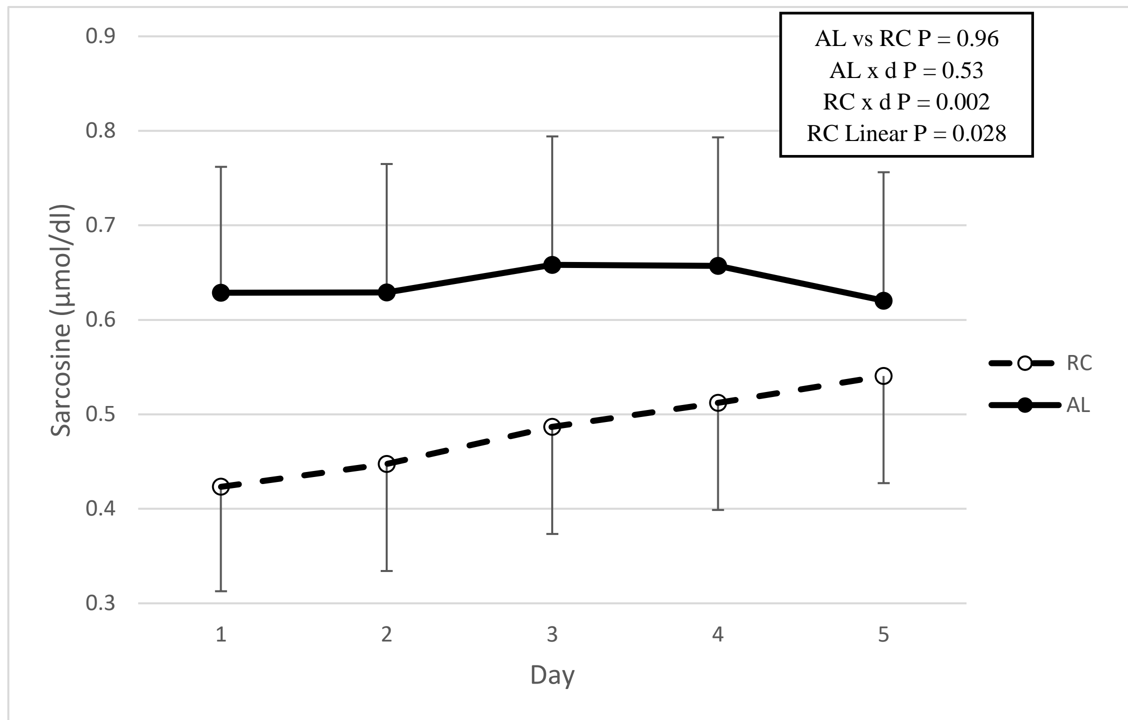

Figure 81 **Daily average plasma concentrations of Sarcosine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

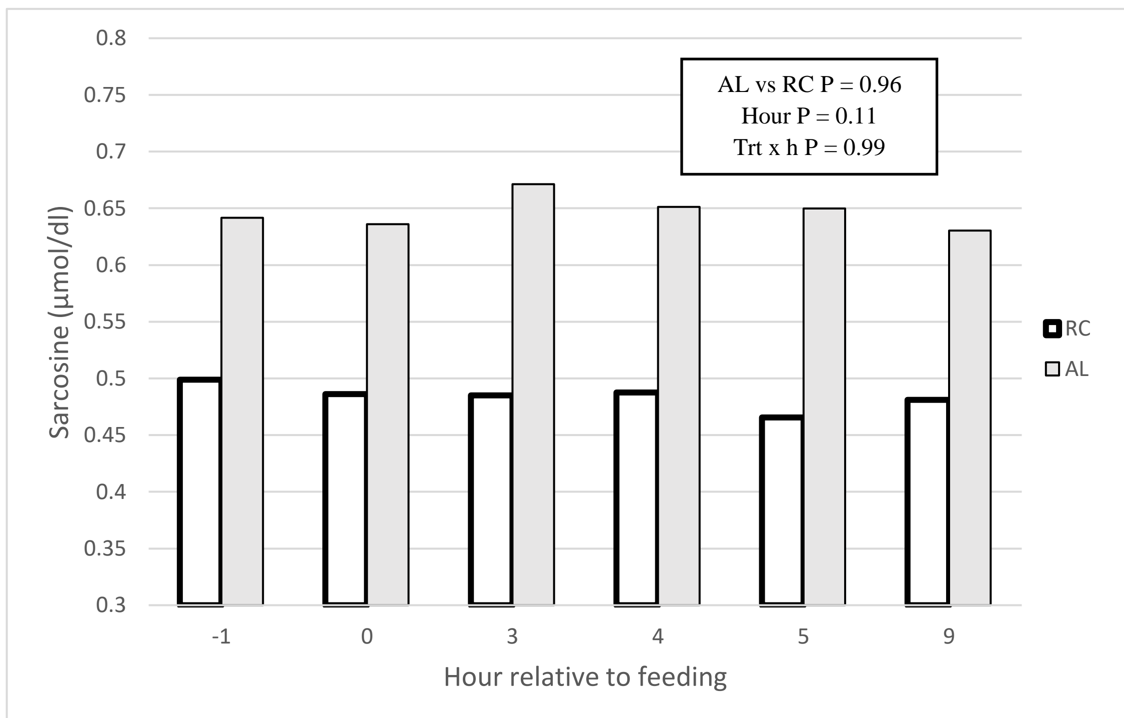

Figure 82 **Sarcosine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

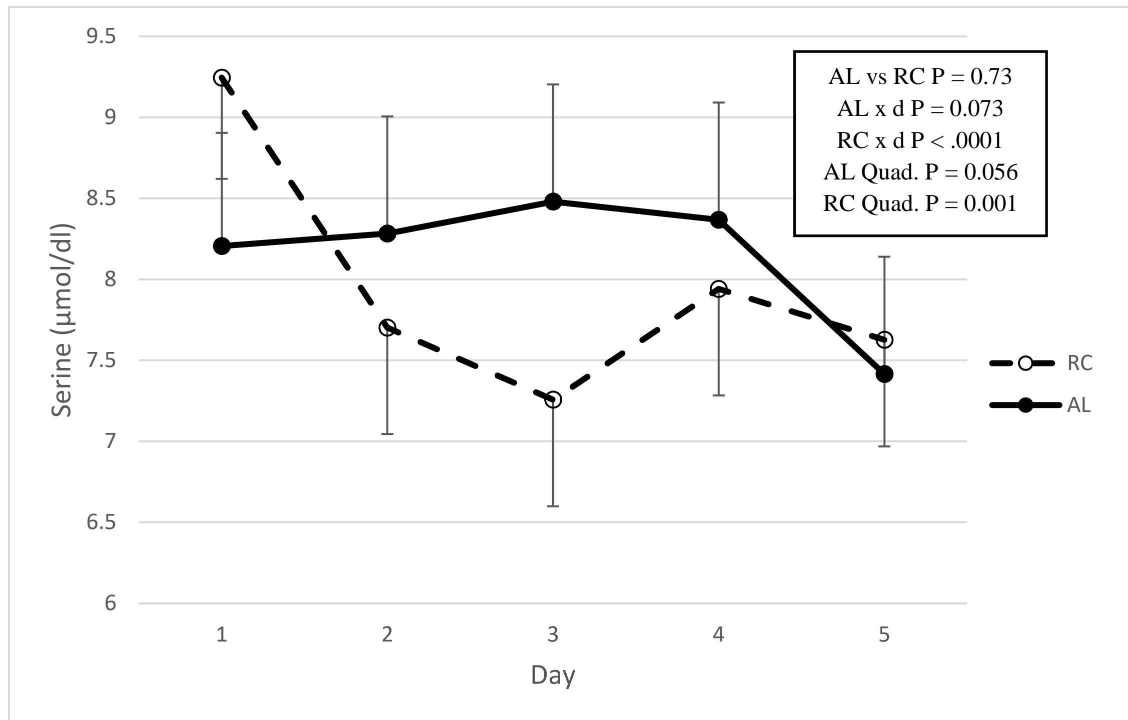

Figure 83 **Daily average plasma concentrations of Serine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

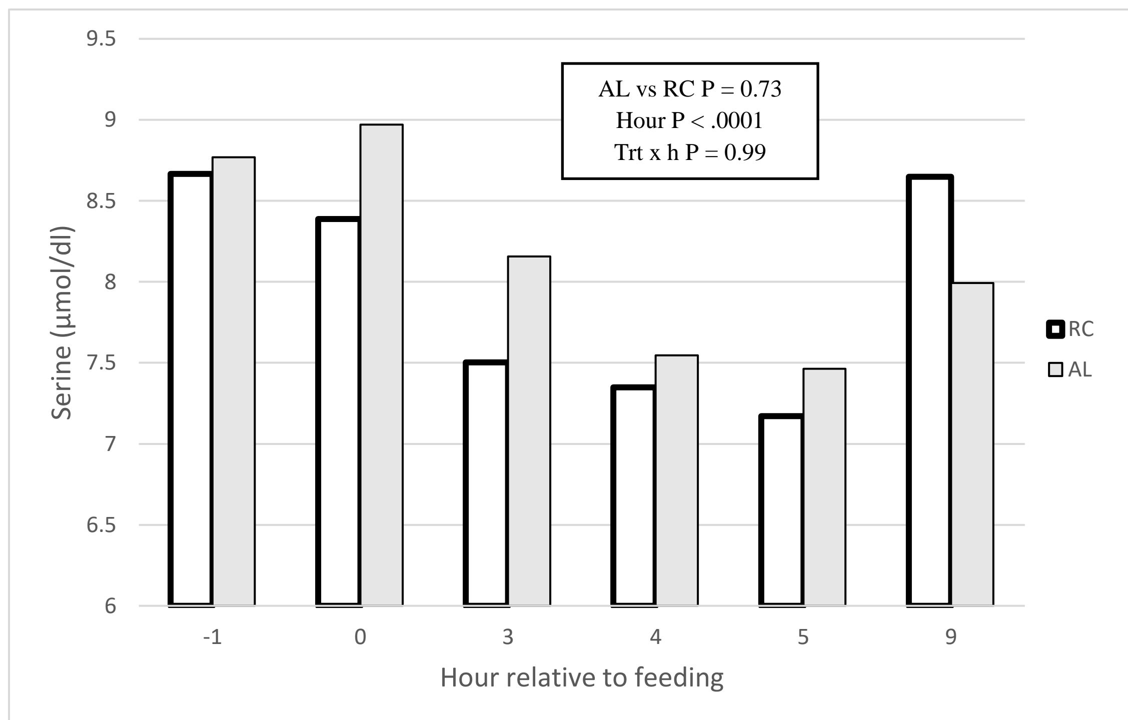

Figure 84 **Serine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

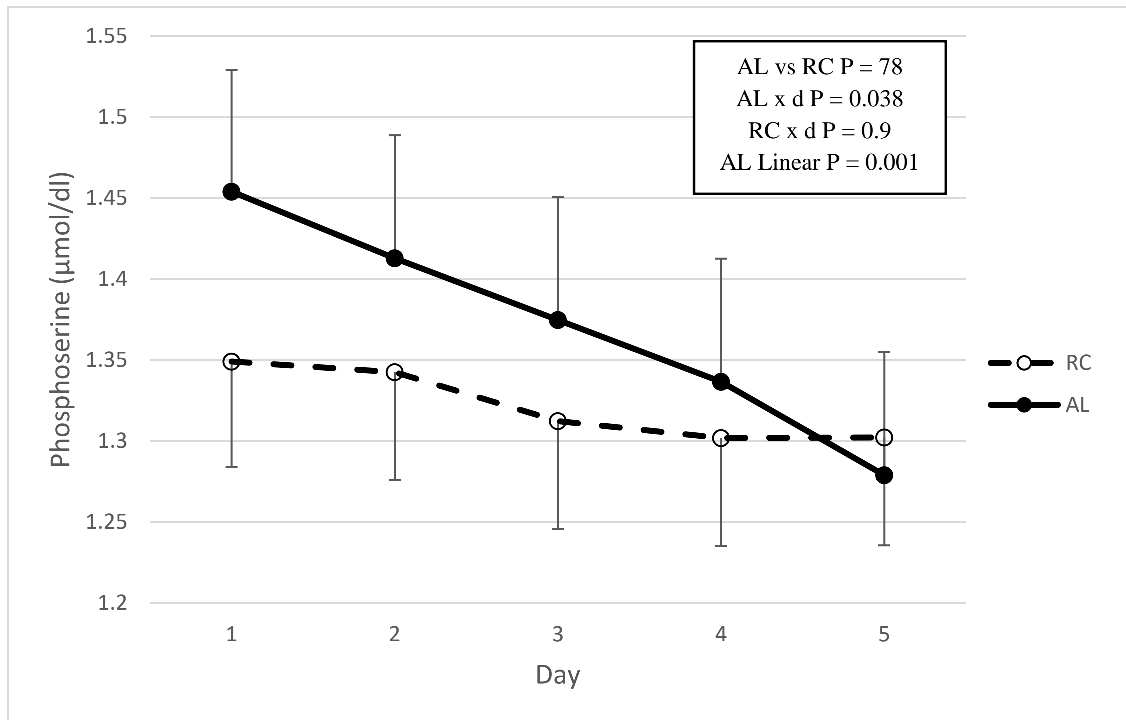

Figure 85 **Daily average plasma concentrations of Phosphoserine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

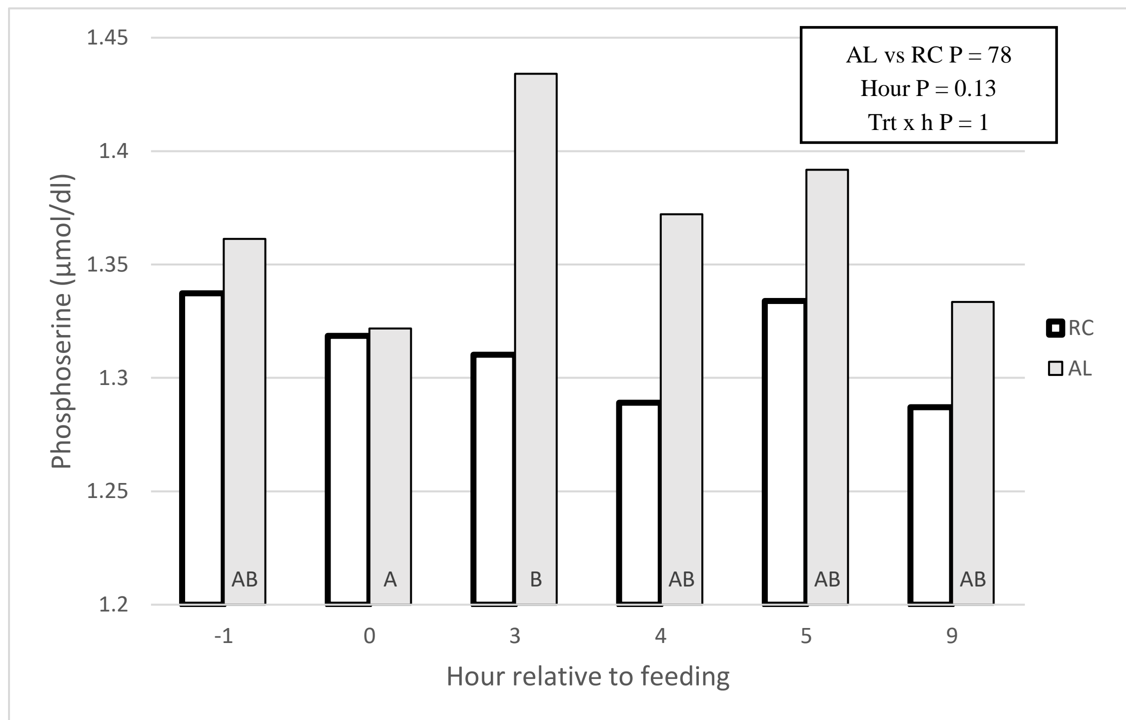

Figure 86 **Phosphoserine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

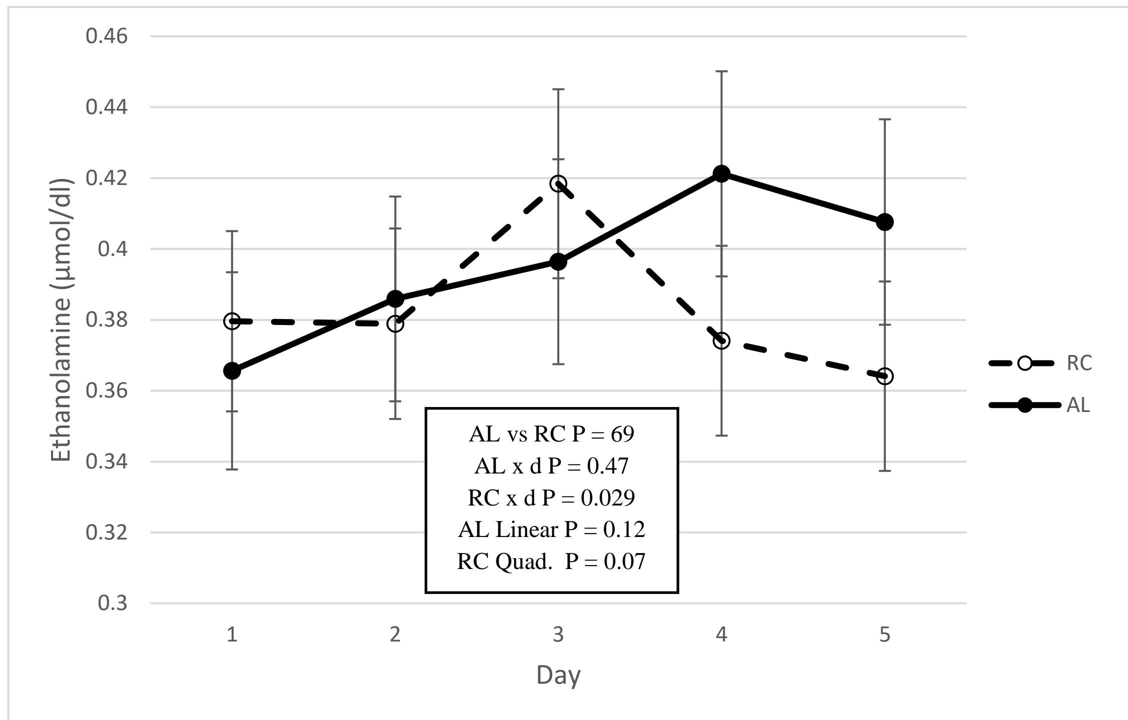

Figure 87 **Daily average plasma concentrations of Ethanolamine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

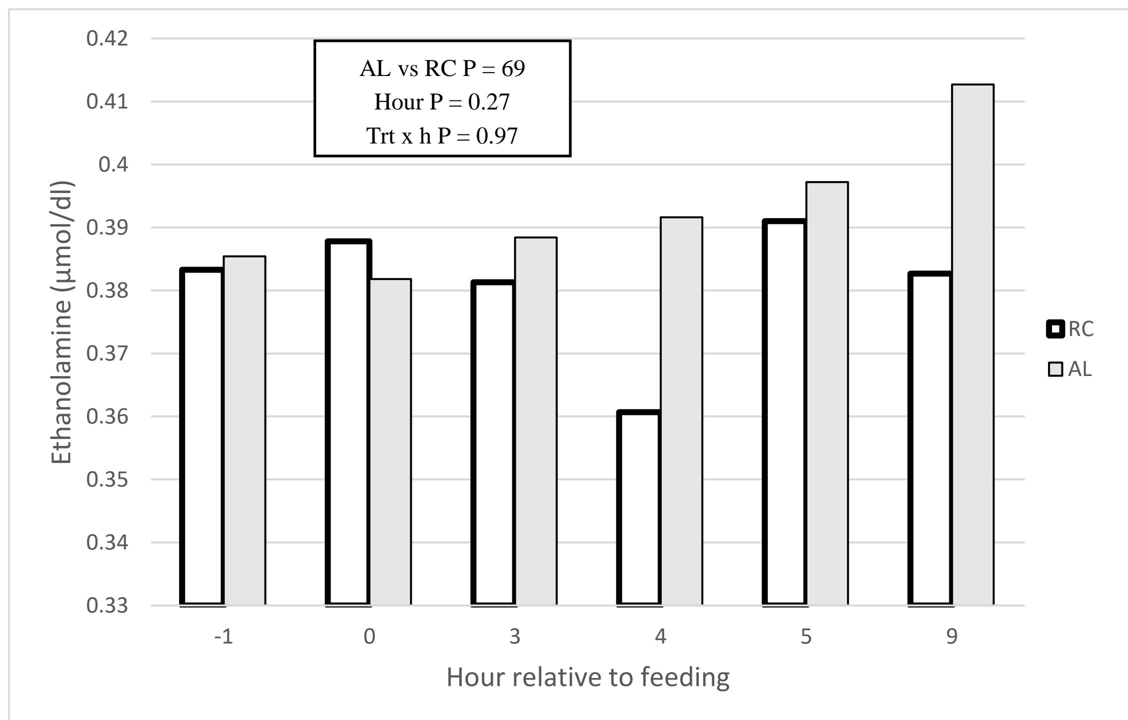

Figure 88 **Ethanolamine (PEA) concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

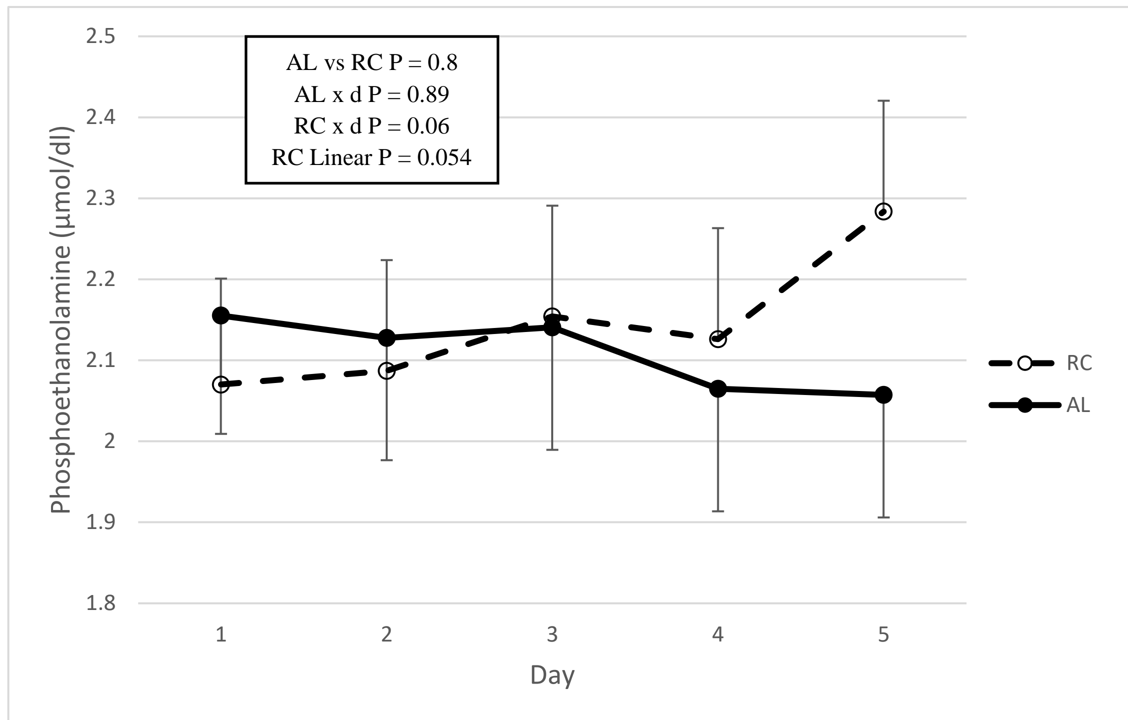

Figure 89 **Daily average plasma concentrations of Phosphoethanolamine (PEA) in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

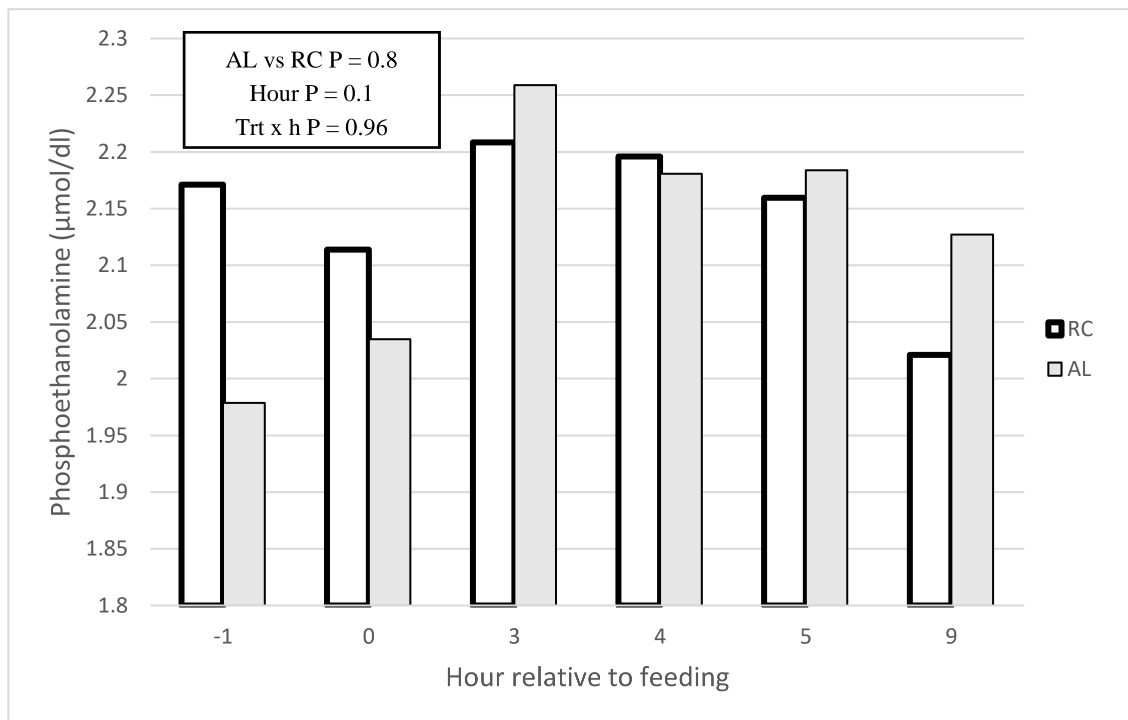

Figure 90 **Phosphoethanolamine (PEA) concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

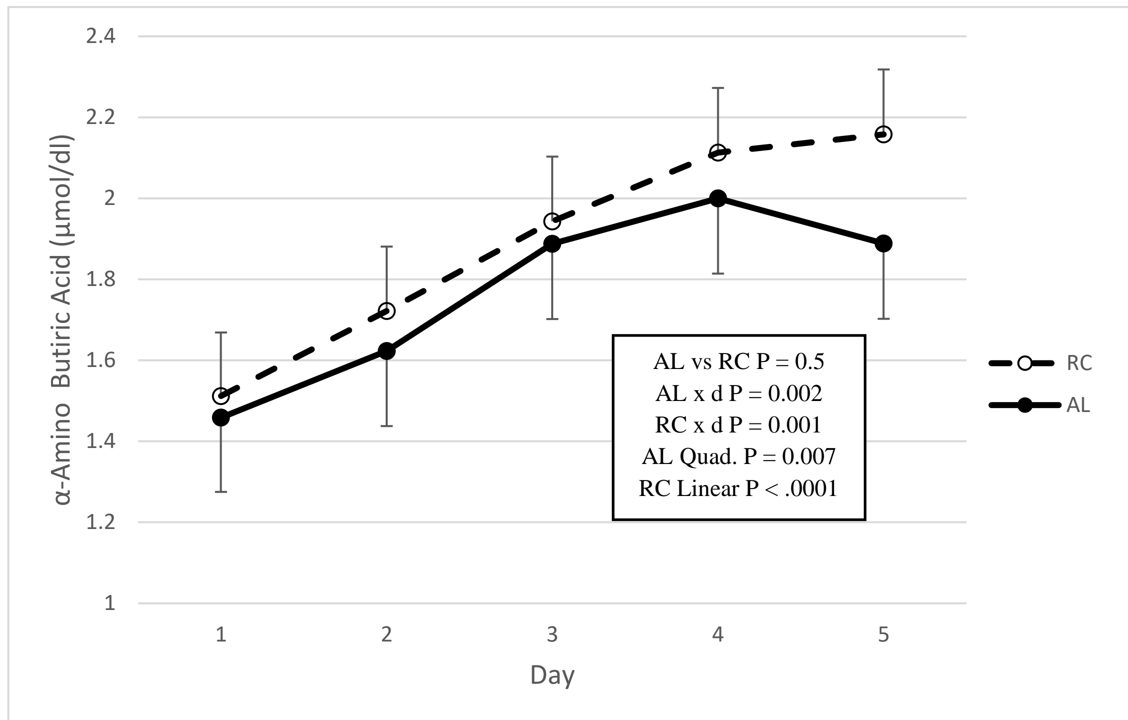

Figure 91 **Daily average plasma concentrations of α-Amino Butyric Acid in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

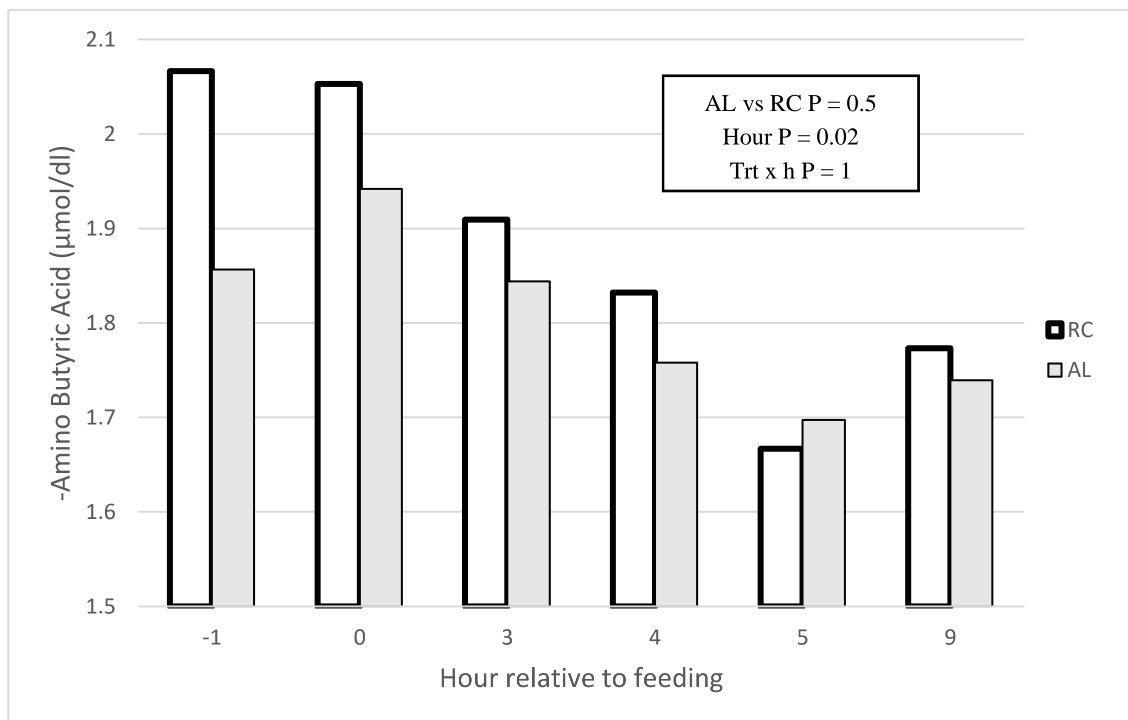

Figure 92 **α-Amino Butyric Acid concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

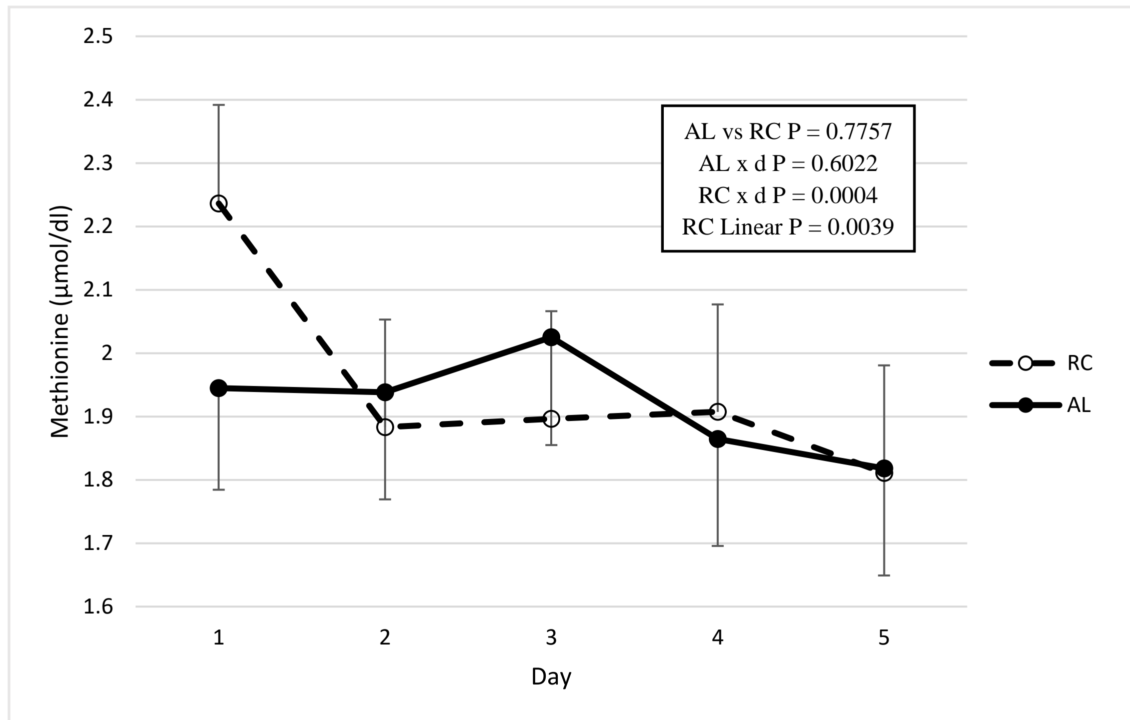

Figure 93 **Daily average plasma concentrations of Methionine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (**P **< 0.10) and * (**P **< 0.05).**

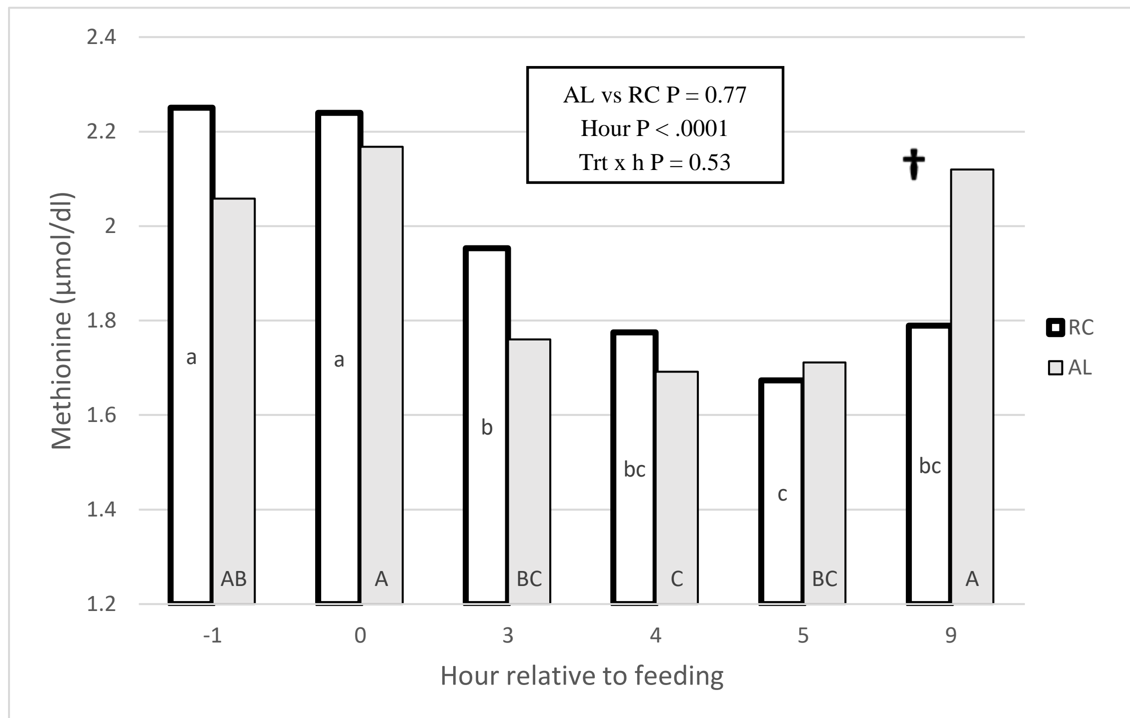

Figure 94 **Methionine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at **P**<0.05. Bars with same Upper case letter are not significant different at **P**<0.05. Differences between treatments (AL and RC) are marked with † (**P **< 0.10) and * (**P **< 0.05).**

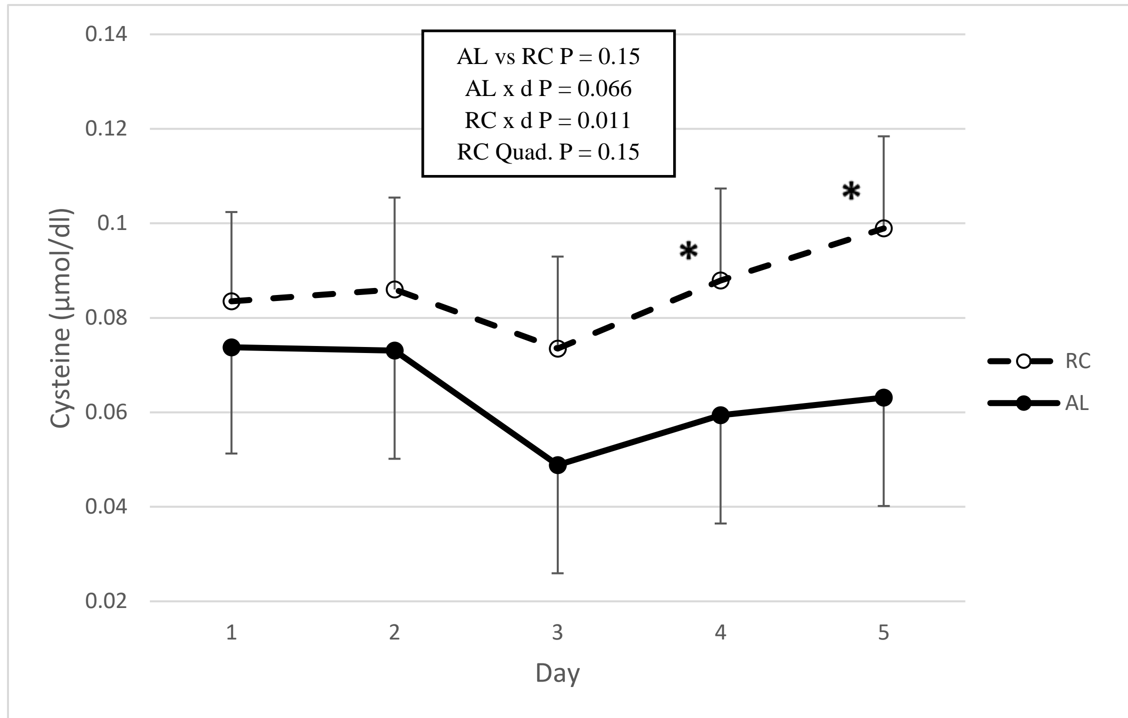

Figure 95 **Daily average plasma concentrations of Cysteine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

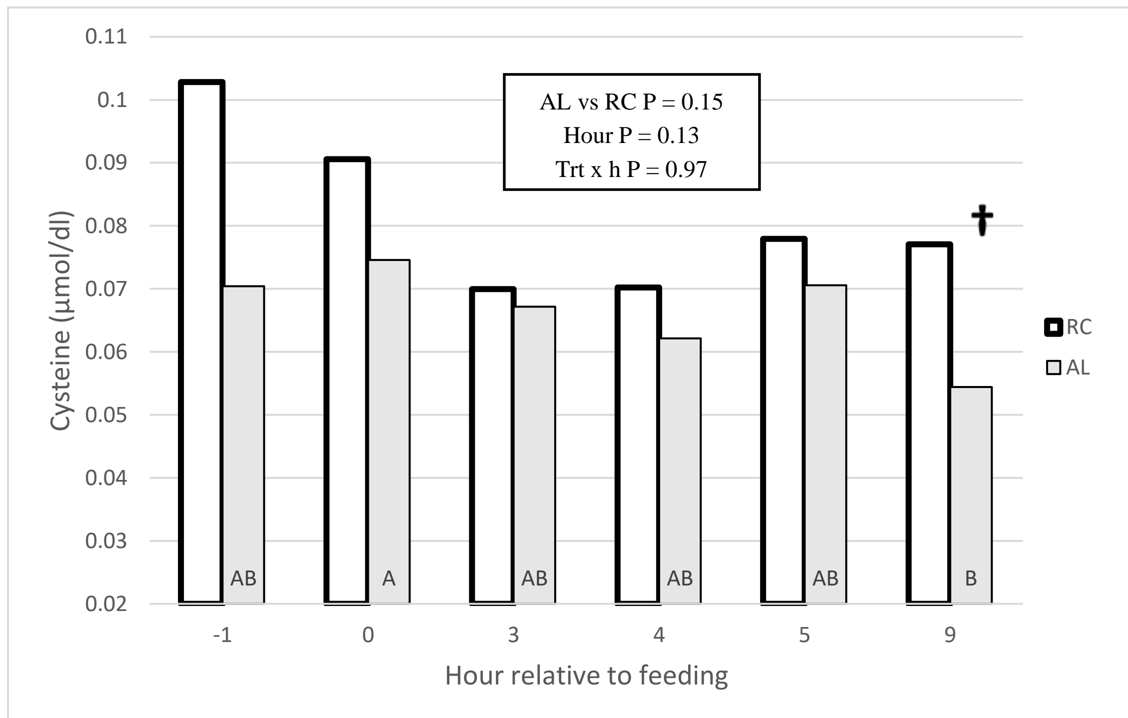

Figure 96 **Cysteine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

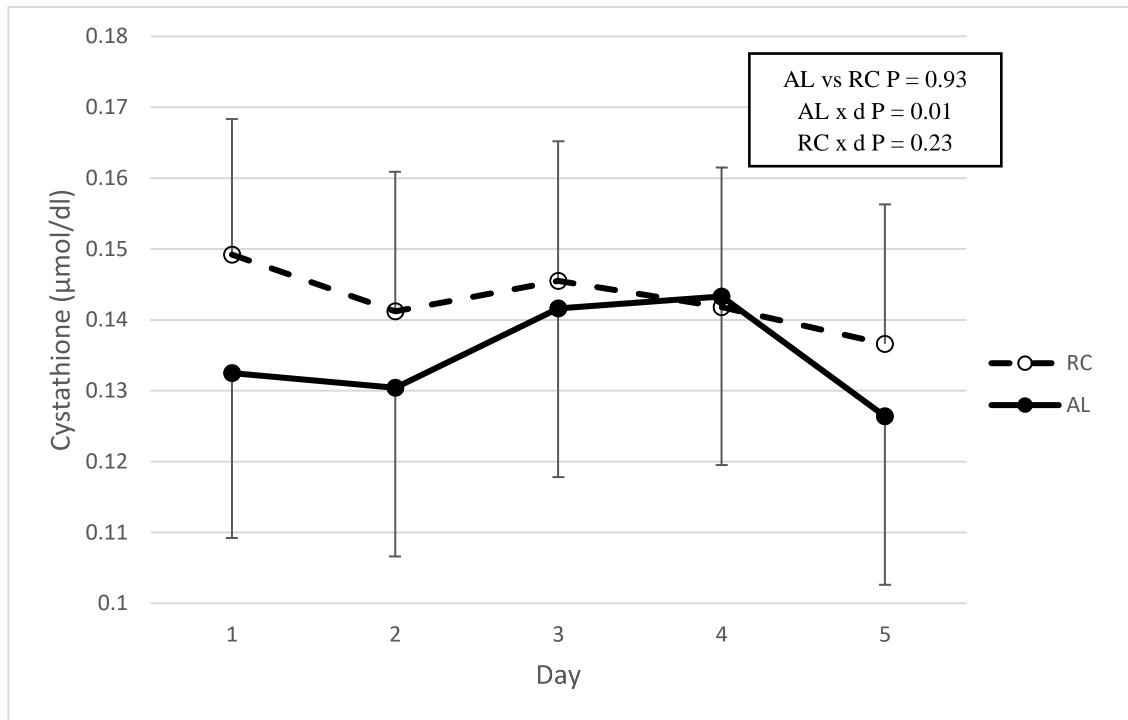

Figure 97 **Daily average plasma concentrations of Cystathionine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

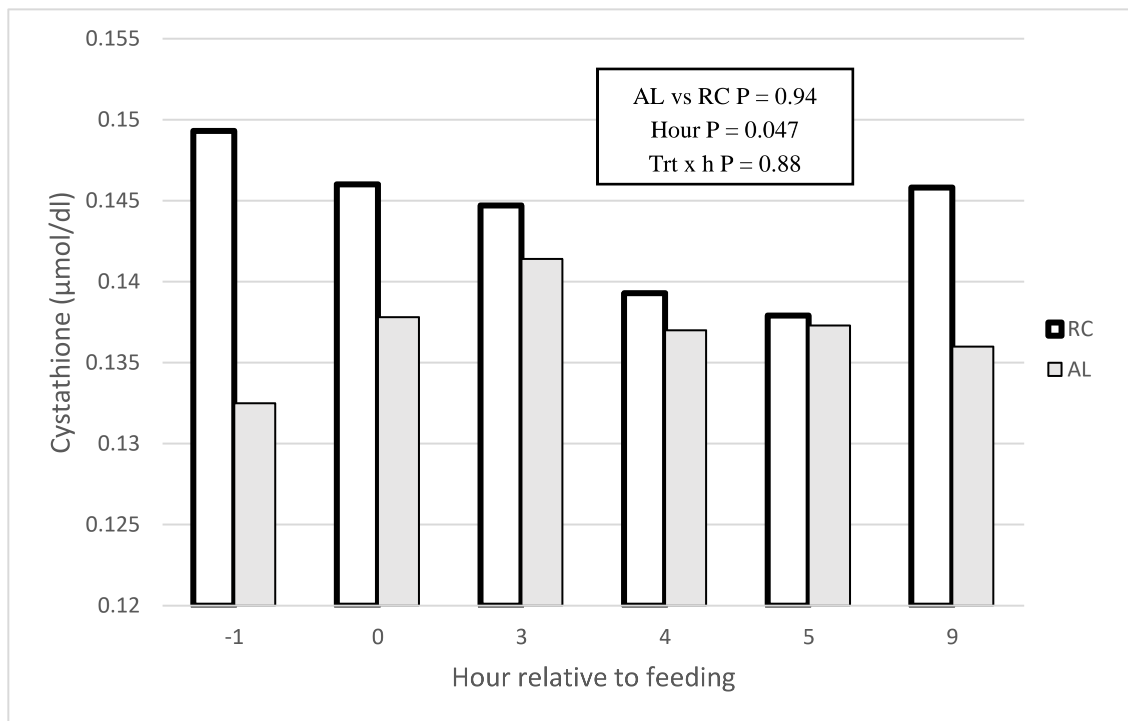

Figure 98 **Cystathionine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

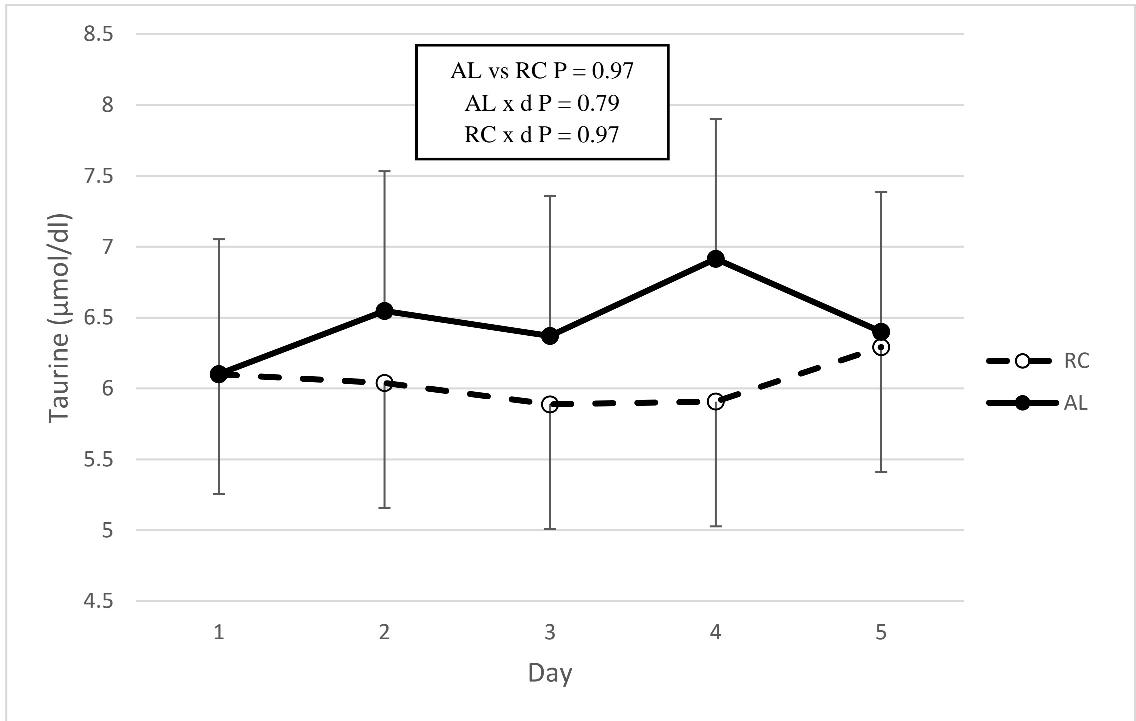

Figure 99 **Daily average plasma concentrations of Taurine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

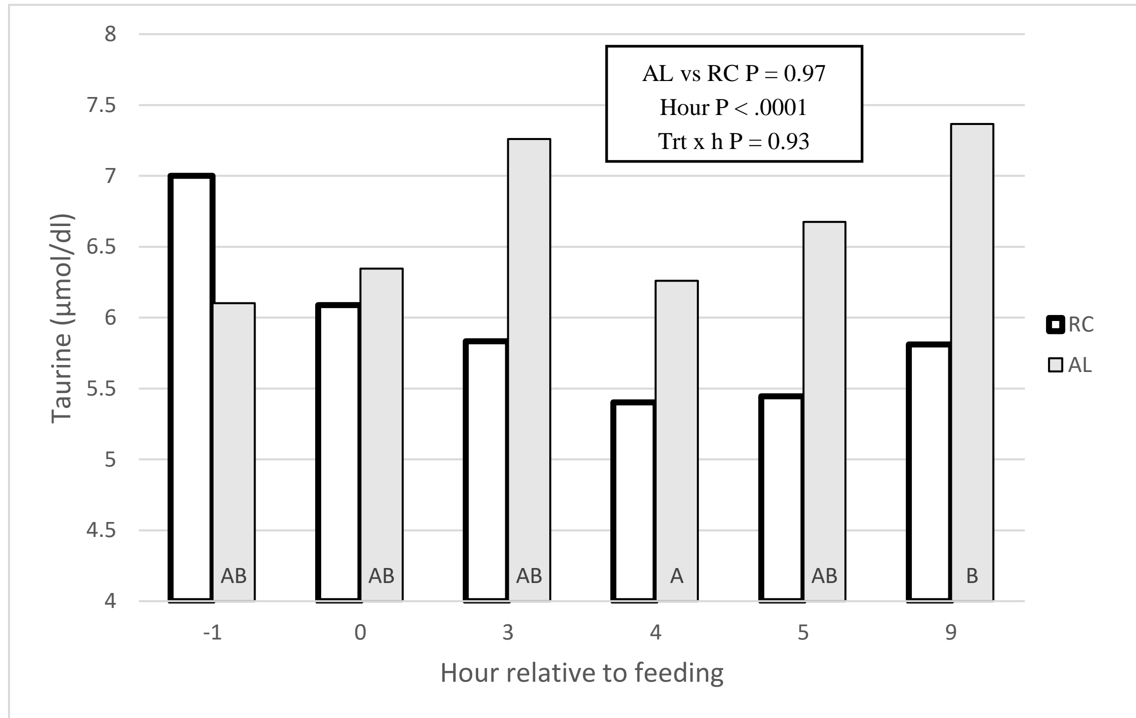

Figure 100 **Taurine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

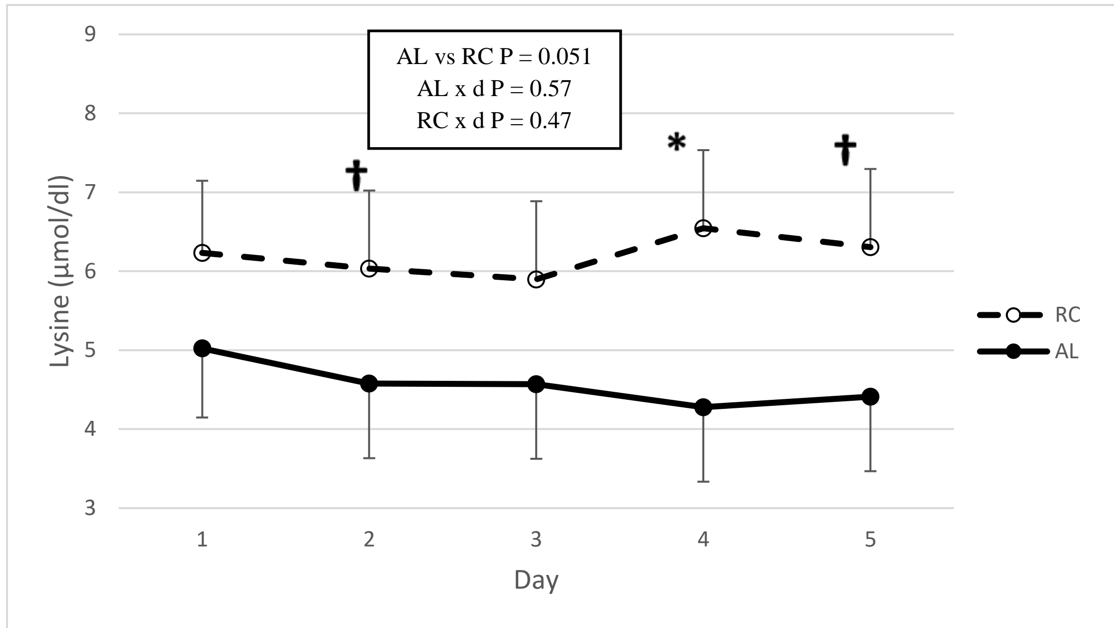

Figure 101 **Daily average plasma concentrations of Lysine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

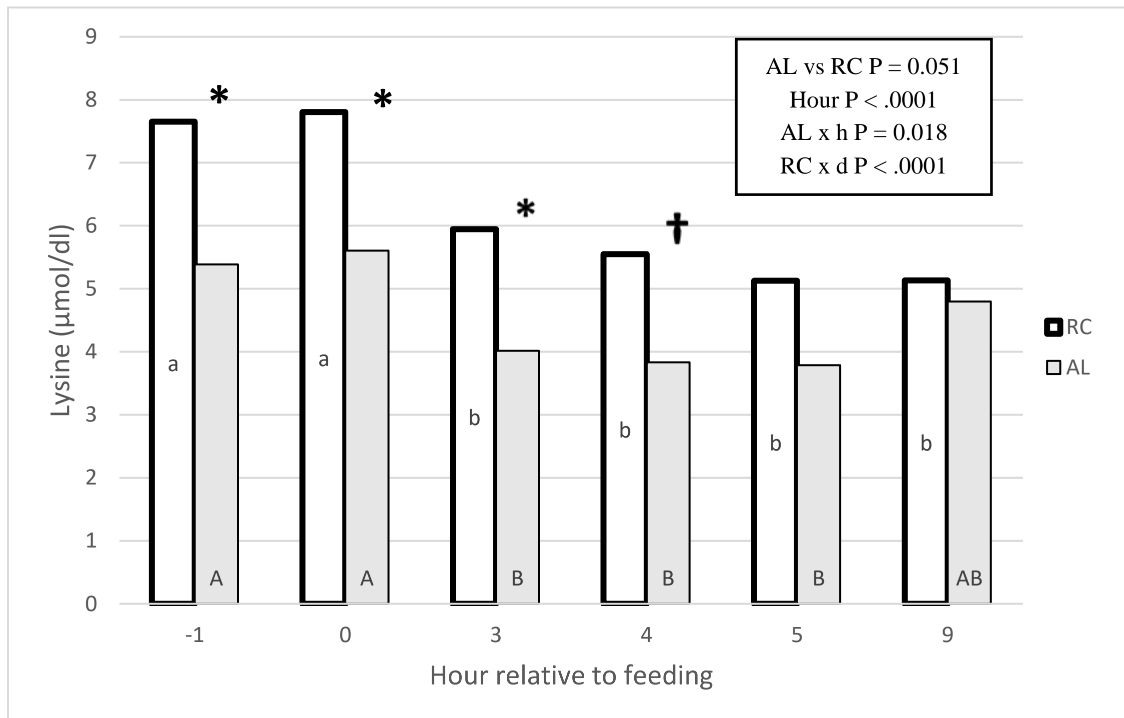

Figure 102 **Lysine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

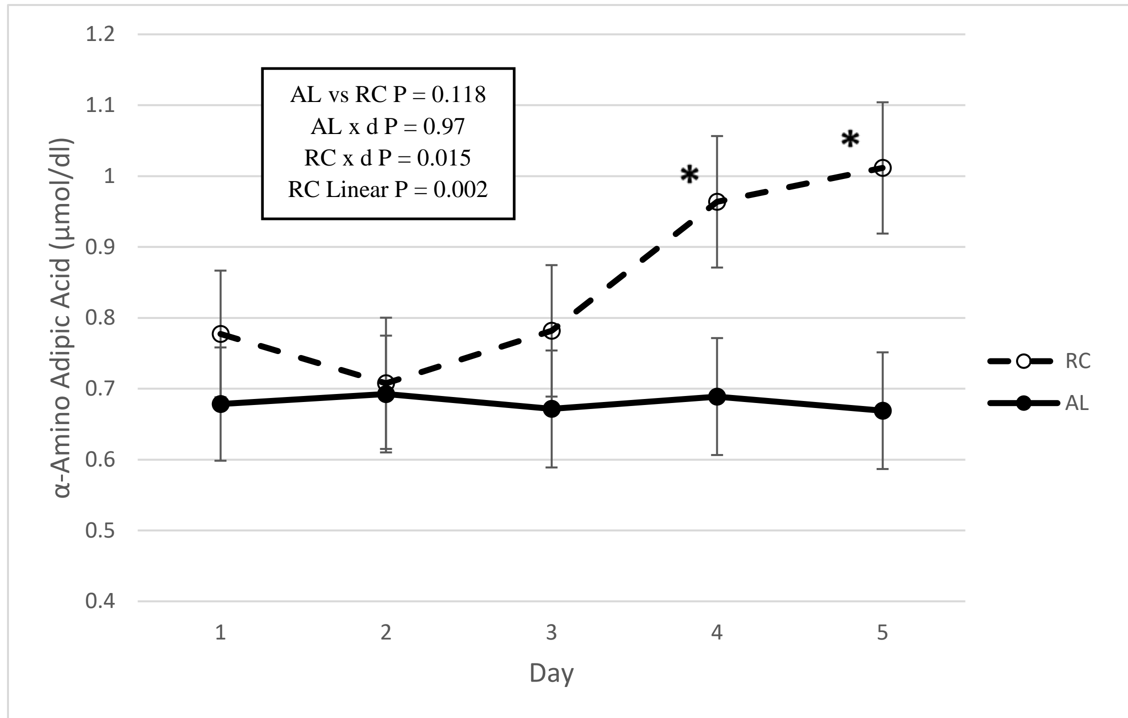

Figure 103 **Daily average plasma concentrations of α-Amino Adipic Acid in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

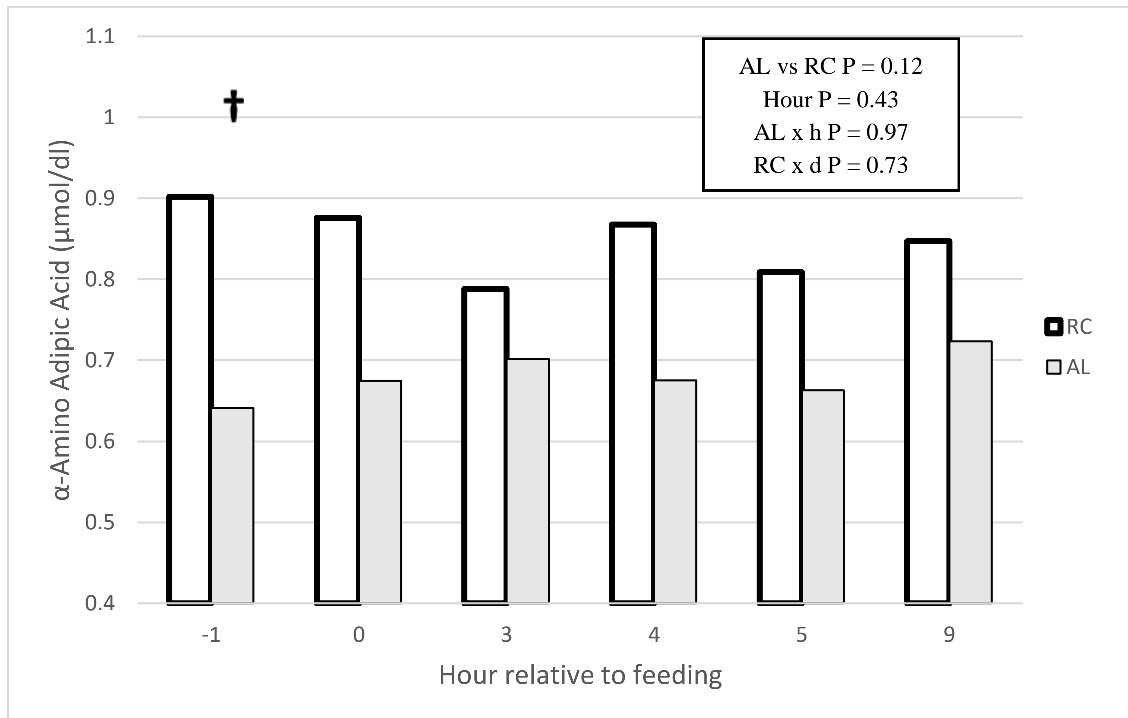

Figure 104 **α-Amino Adipic Acid concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

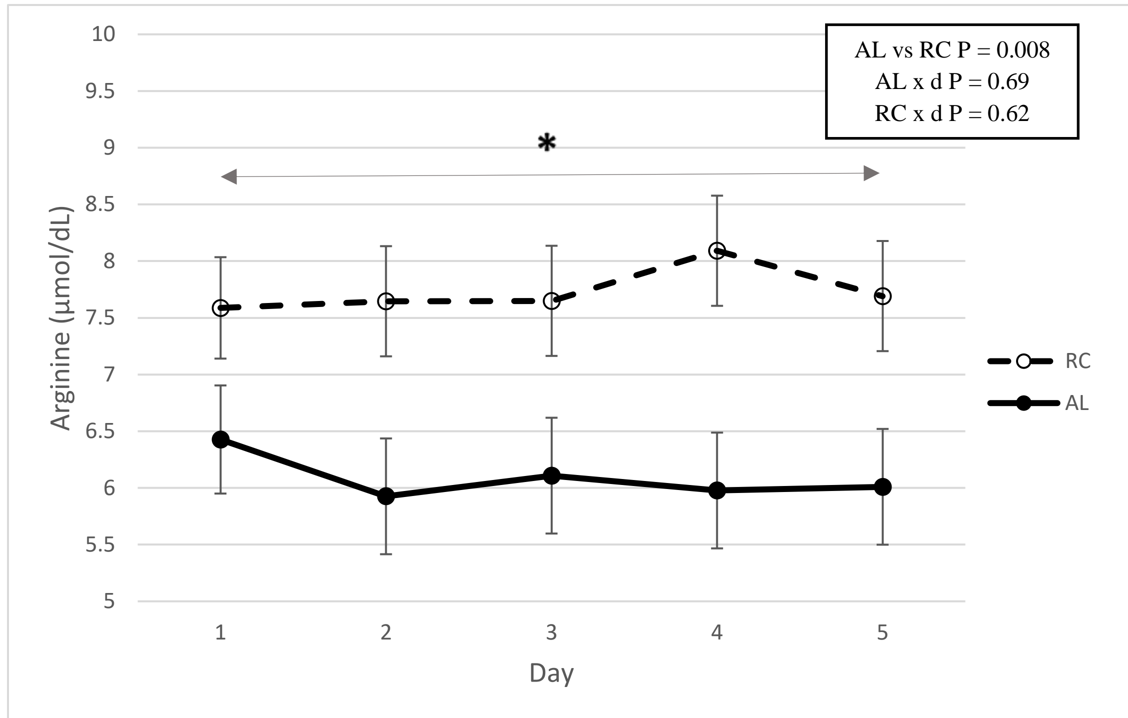

Figure 105 **Daily average plasma concentrations of Arginine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

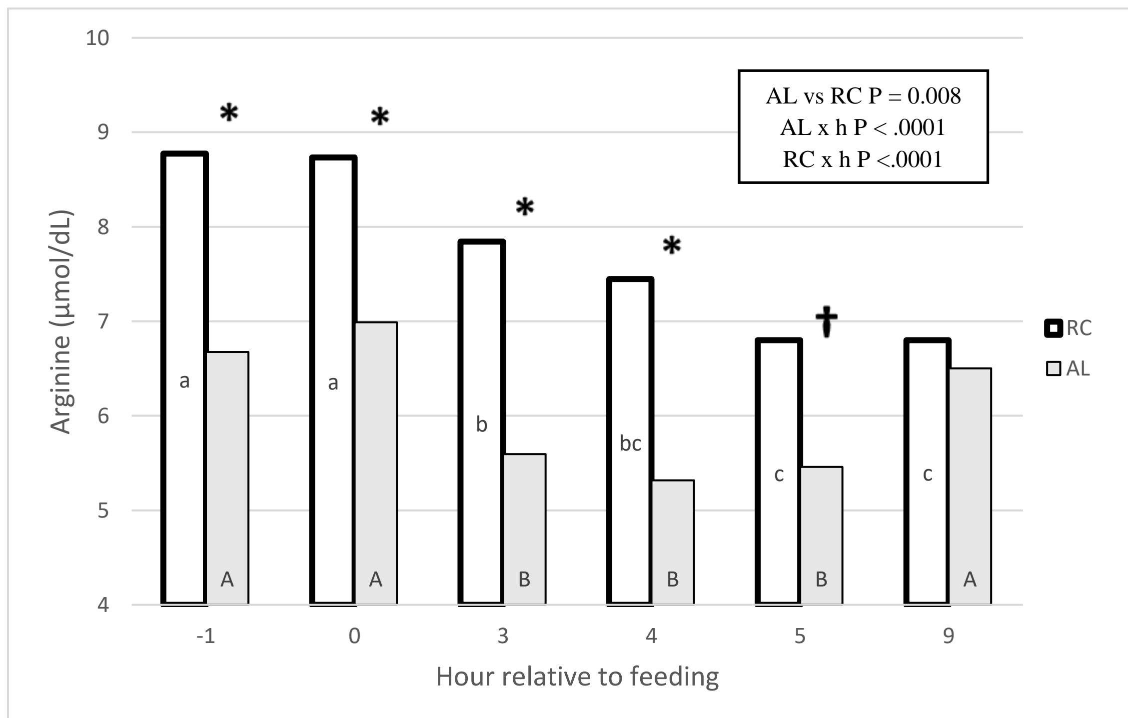

Figure 106 **Arginine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

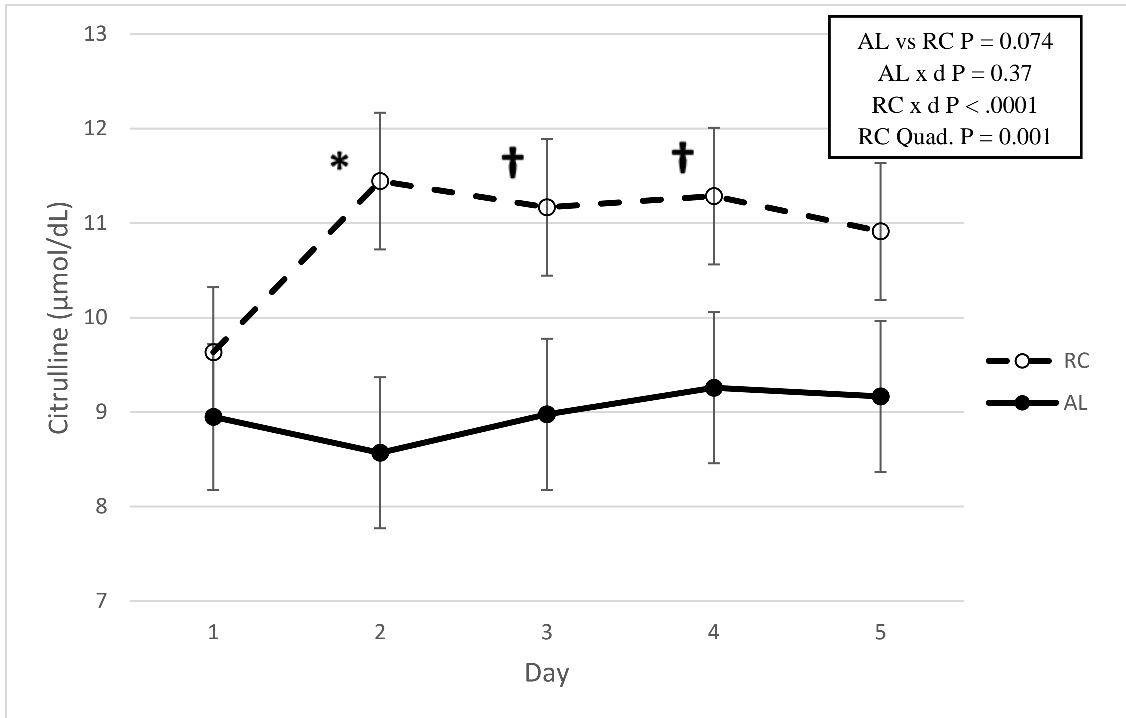

Figure 107 **Daily average plasma concentrations of Citrulline in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

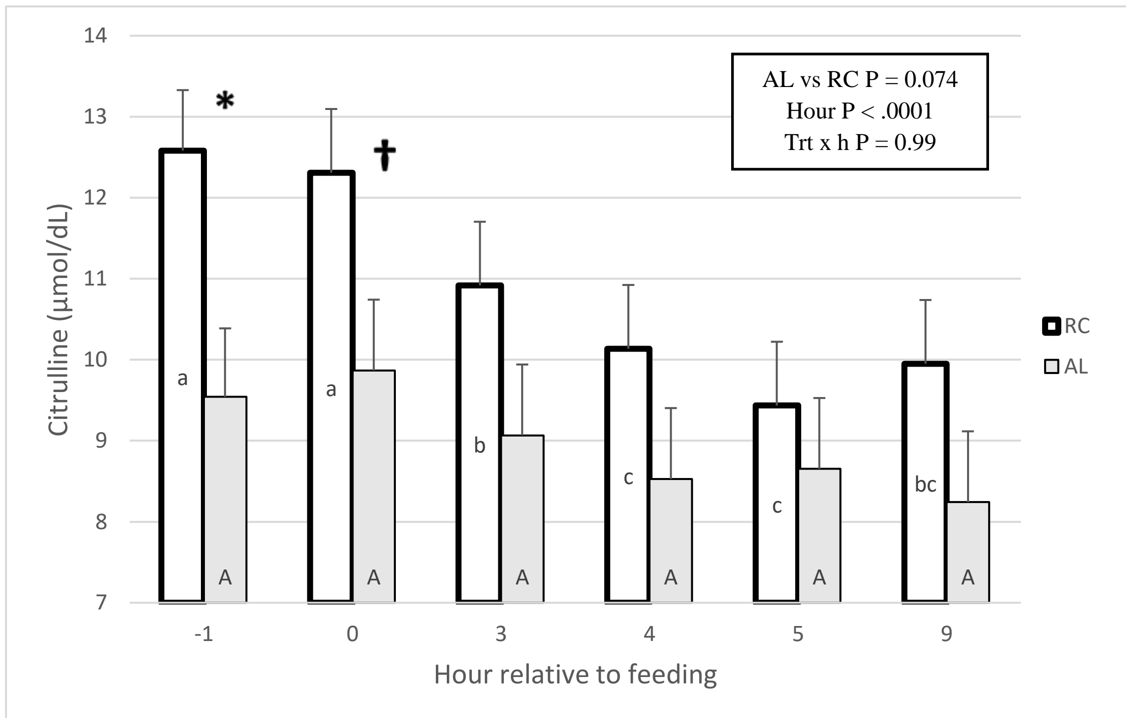

Figure 108 **Citrulline concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

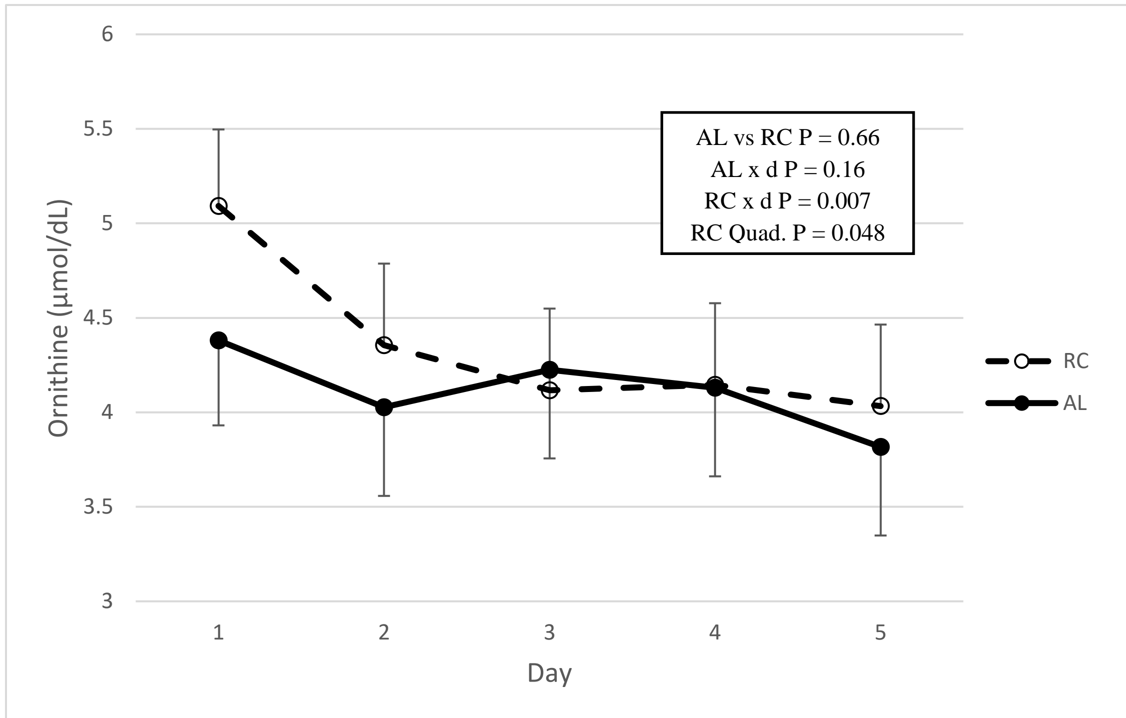

Figure 109 **Daily average plasma concentrations of Ornithine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

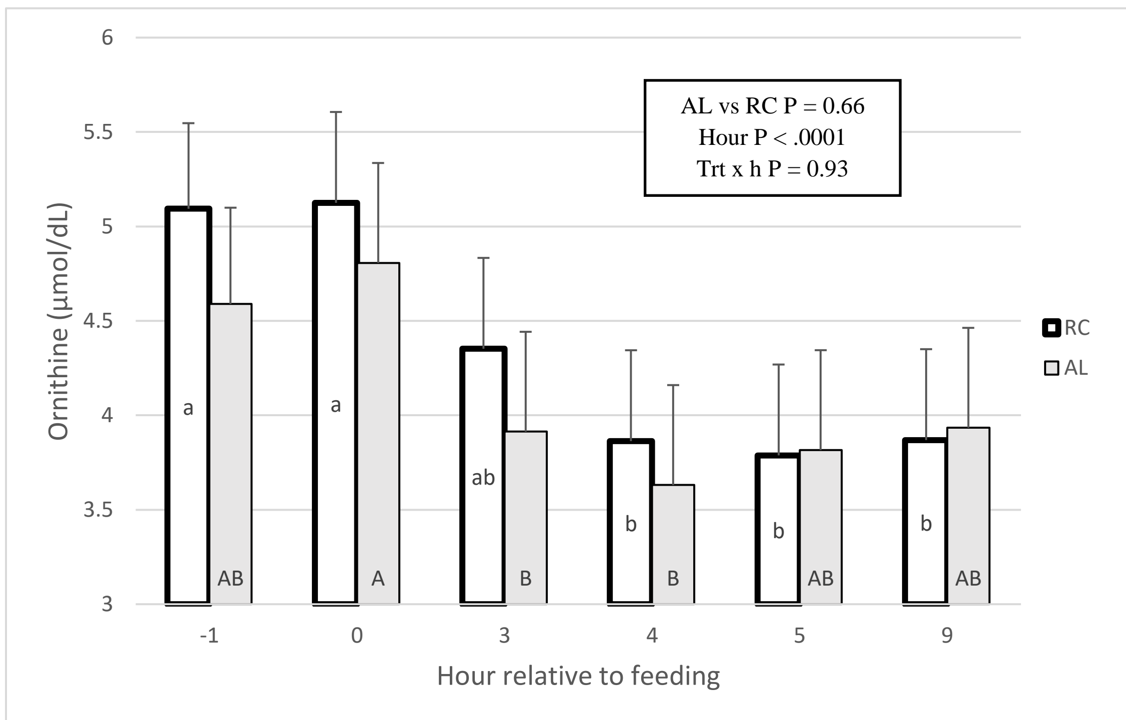

Figure 110 **Ornithine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

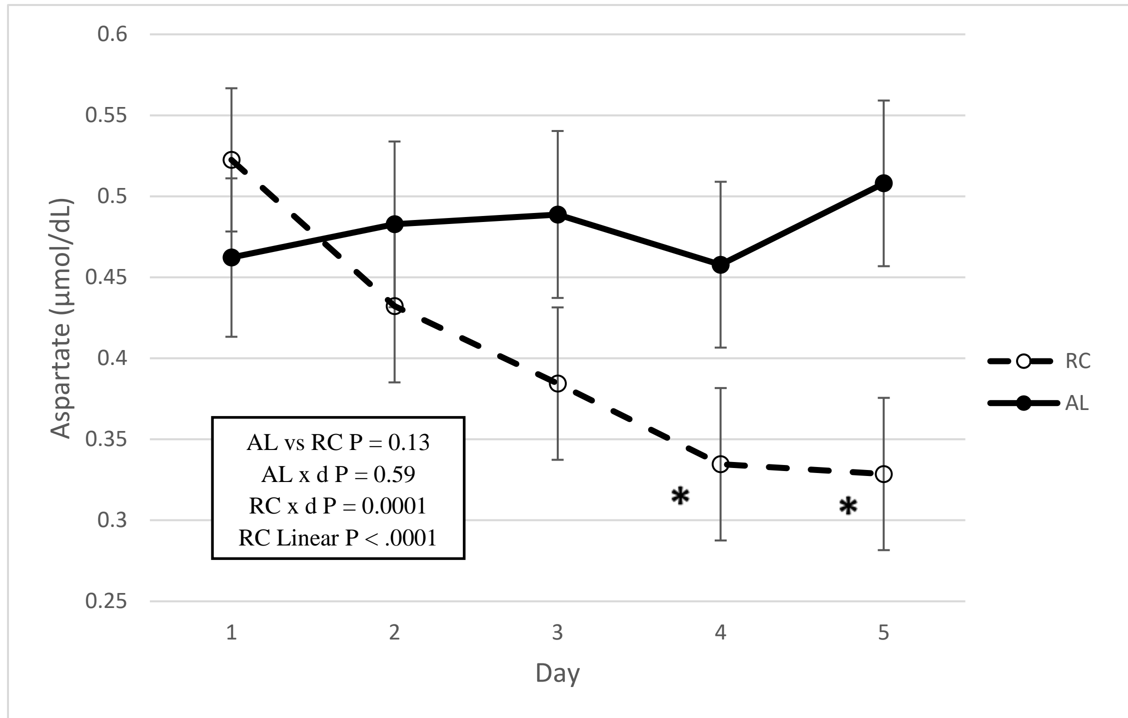

Figure 111 **Daily average plasma concentrations of Aspartate in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (**P **< 0.10) and * (**P **< 0.05).**

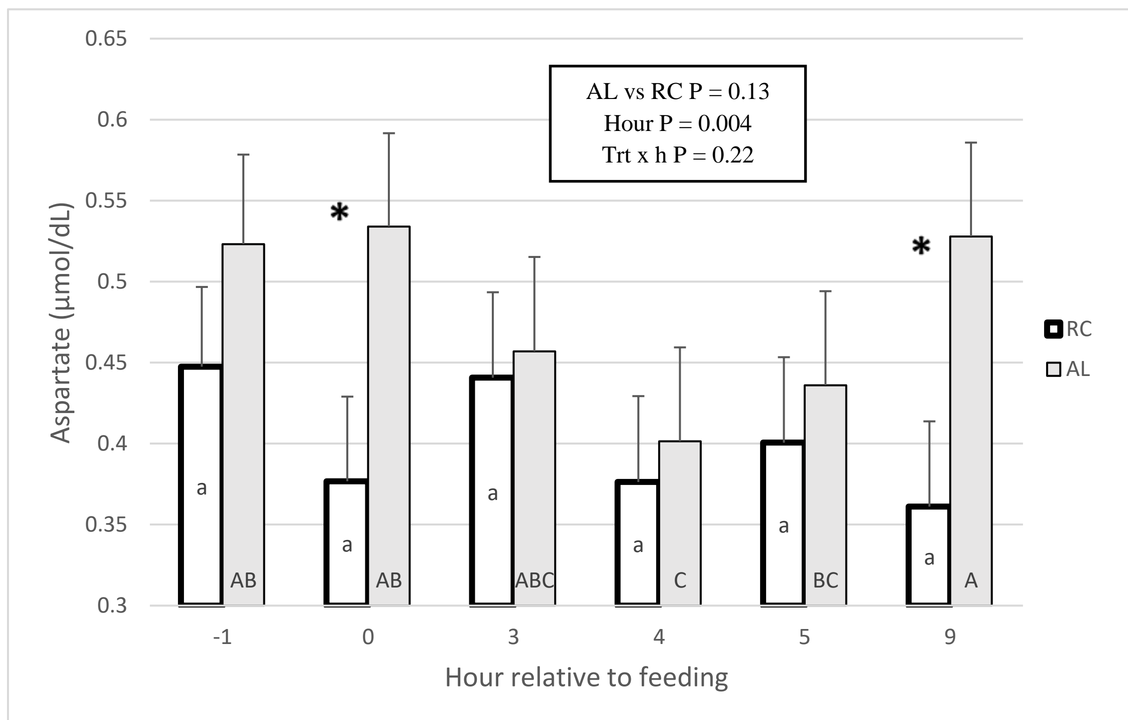

Figure 112 **Aspartate concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at **P**<0.05. Bars with same Upper case letter are not significant different at **P**<0.05. Differences between treatments (AL and RC) are marked with † (**P **< 0.10) and * (**P **< 0.05).**

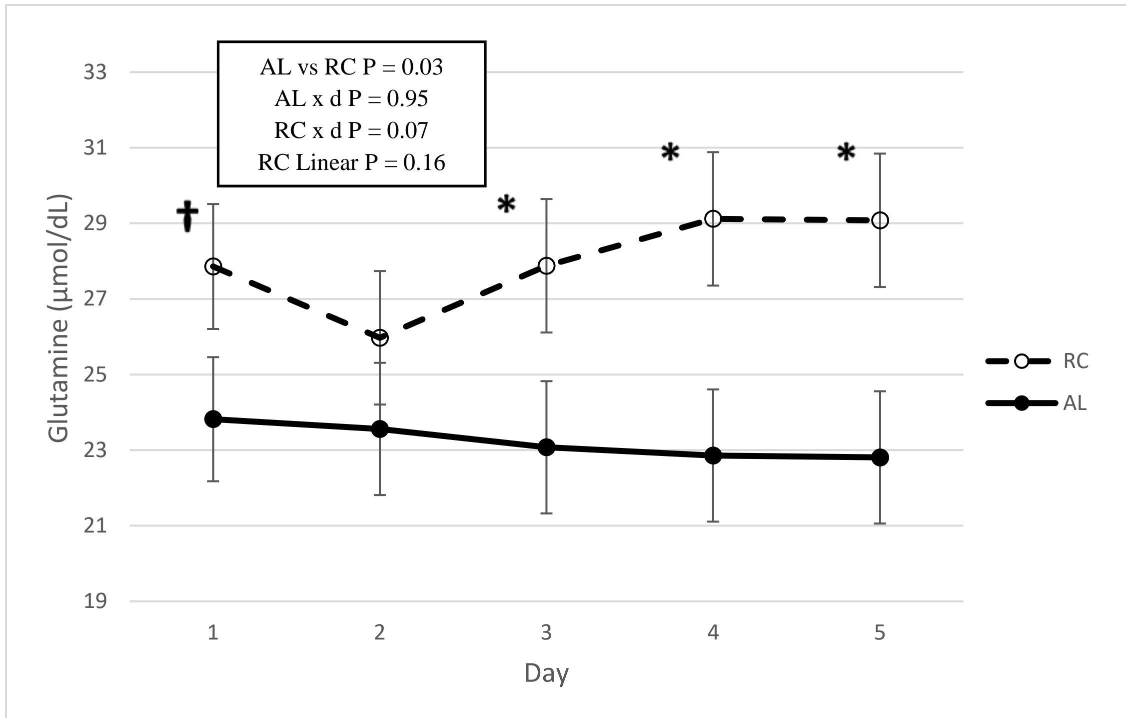

Figure 113 **Daily average plasma concentrations of Glutamine in RC (Feed Restriction Control group) and AL (Ad libitum feeding group). Values are given in Least Square Means (LSM) and ± standard error. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

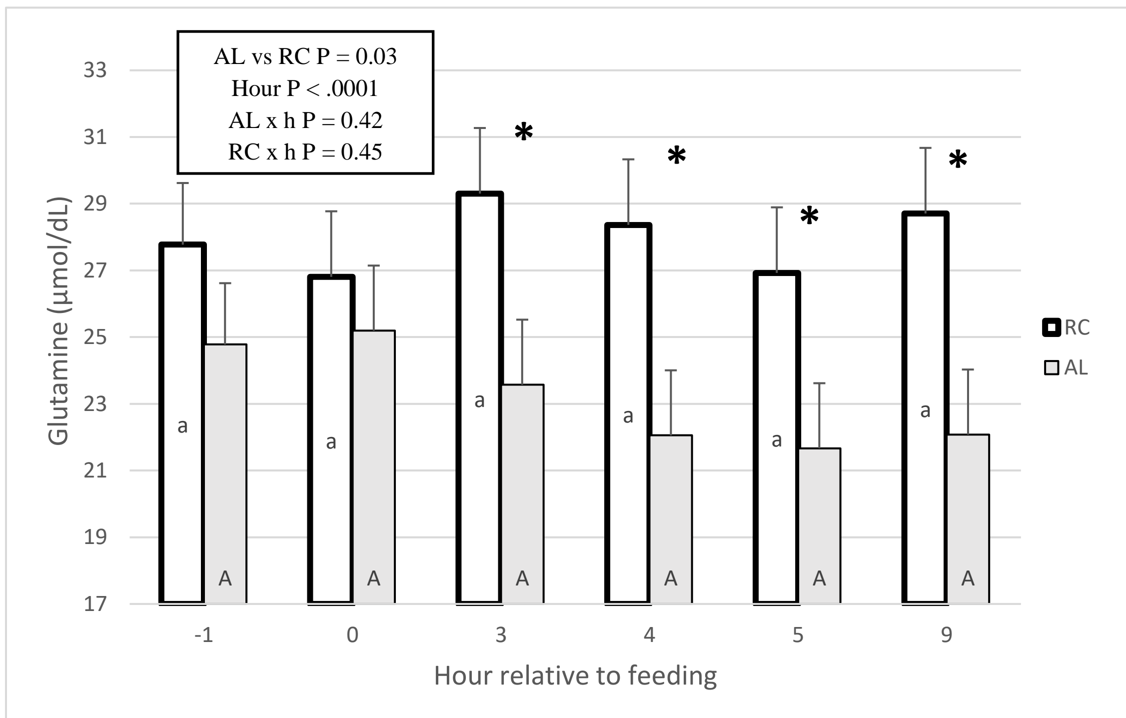

Figure 114 **Glutamine concentration by hour relative to feeding in RC (Feed Restricted control) and AL (Ad Libitum control). Average of the 5 days experimental period. Values are given in Least Square Means (LSM) and ± standard error. Bars with same lower case letter are not significant different at P<0.05. Bars with same Upper case letter are not significant different at P<0.05. Differences between treatments (AL and RC) are marked with † (P < 0.10) and * (P < 0.05).**

TABLES

Table 1 Composition of diet fed as Total Mixed Ration

Item Ingredient	% DM	Chemical composition	% DM
Corn silage	34.12	Dry matter	51.87
Alfalfa silage	5.50	Crude protein	16.47
Wheat straw	1.00	RUP	6.61
Cottonseed fuzzy	5.00	RDP	9.86
Corn grain	17.00	Soluble CP	5.17
Soybean hulls pellet	7.26	ADF	17.51
Molasses beet	5.15	NDF	28.90
Soybean meal 47.5%	5.94	peNDF	17.49
Soyplus	4.90	Lignin	2.89
Corn gluten feed dry	4.81	NFC	41.06
Wheat midds	3.00	Starch	28.59
Energy Booster 100	1.64	Sugar	6.534
ProvAAl Advantage®	1.50	Fat total	5.41
Limestone ground	1.22	NEl (Mcal/kg)	1.67
Sodium bicarbonate	0.75		
Biotin	0.39		
Calcium phosphate	0.38		
Salt white	0.20		
Min/Vit premix	0.17		
Magnesium Oxide	0.05		
Vitamin A premix	0.02		

Table 2 EAA balance during feed restriction (RC)

AA	Absorbed (g/d)	Required (g/d)	Balance (g/d)	% Req	% MP
MET	28	57.4	-29.4	48.70%	2.18%
LYS	87.9	160.7	-72.7	54.70%	6.86%
ARG	86.1	107.7	-21.6	79.90%	6.72%
THR	61.8	85.9	-24.1	72.00%	4.82%
LEU	100.3	214.6	-114.3	46.70%	7.83%
ILE	65.4	116	-50.6	56.40%	5.10%
VAL	72.5	126.6	-54.1	57.30%	5.66%
HIS	34.7	53.5	-18.8	64.90%	2.71%
PHE	64.7	119	-54.3	54.40%	5.04%
TRP	18.1	33.4	-15.3	54.20%	1.41%
TOTAL	**619.5**		**AVG**	**58.92%**	

Table 3 LS means per treatment

Variable	Treatments		SE	P-value								
Production variables	AL	RC	SE	ALvsRC[1]	Day	Trt*Day	Hour	Trt*Hour	Diet[2]	Diet*Day	Diet*Hour	ALvsRES[3]
Dry Matter Intake (kg)	27.5[a]	12.4[b]	1.91	**<.0001**	**0.042**	0.94	--	--	**<.0001**	**0.003**	--	**<.0001**
Body Weight (kg)	671[a]	634[b]	8.84	**0.001**	**<.0001**	**0.001**	--	--	**0.001**	**0.001**	--	**0.001**
Energy Balance (MJ/d)	-11.3[a]	-43.1[ab]	14.30	**0.031**	**<.0001**	0.47	--	--	**0.002**	0.1	--	**0.005**
Feed Efficiency (kg milk/kg DM)	2.35	2.28	0.12	0.81	**<.0001**	**0.056**	--	--	0.96	**0.056**	--	0.86
Feed Efficiency (kg ECM/kg DM)	1.93[a]	2.66[b]	0.14	**0.022**	**<.0001**	0.1	--	--	**0.011**	0.49	--	**0.012**
Milk weight (kg)	37.7[a]	30.5[b]	2.00	**0.002**	**<.0001**	**0.047**	--	--	**<.0001**	**0.001**	--	**0.057**
Energy Corrected Milk (kg)	36.2	33.3	3.91	0.078	**<.0001**	0.14	--	--	0.06	0.54	--	0.065
Milk protein												
%	3.09[a]	2.95[b]	0.06	**0.034**	**<.0001**	0.12	--	--	**0.011**	**0.032**	--	**0.003**
kg	1.15[a]	0.9[b]	0.07	**<.0001**	**<.0001**	**<.0001**	--	--	**<.0001**	**0.001**	--	**<.0001**
Milk Fat												
%	3.13[b]	4.30[ab]	0.37	**0.005**	**0.003**	0.34	--	--	**0.001**	**0.03**	--	**0.001**
kg	1.15	1.30	0.21	0.99	0.51	0.28	--	--	0.71	0.93	--	0.75
Lactose												
%	4.66	4.56	0.10	0.2	**<.0001**	0.31	--	--	0.39	0.64	--	0.34
kg.	1.75[a]	1.38[b]	0.07	**<.0001**	**<.0001**	**0.015**	--	--	**0.001**	**0.005**	--	**0.001**
Total Solids												
%	16.71	16.86	0.35	0.67	0.19	**0.052**	--	--	**0.049**	**0.022**	--	**0.049**
kg	6.1[a]	5.14[ab]	0.33	**0.007**	**<.0001**	0.097	--	--	**0.005**	0.09	--	**0.005**
Milk Urea Nitrogen (mg/dL)	10.48[a]	15.27[b]	1.34	**0.007**	**<.0001**	0.79	--	--	**0.04**	0.09	--	**0.013**
Energy-Related Metabolites and Hormones												
Non Esterified Fatty Acid (mEq/L)	0.062[a]	0.324[b]	0.09	**0.001**	**<.0001**	**<.0001**	**<.0001**	0.12	**<.0001**	**<.0001**	**0.003**	**0.001**
Insulin (μg/L)	0.64[a]	0.44[b]	0.13	**0.013**	**<.0001**	**<.0001**	**<.0001**	0.97	**0.015**	**<.0001**	0.071	**0.011**
Glucose (mg/dL)	64.4[a]	59.3[b\]	2.47	**0.041**	**<.0001**	**<.0001**	**<.0001**	0.89	0.26	**0.03**	0.75	0.083
Hydroxy-Butyrate (mmol/L)	0.62	0.59	0.15	0.46	**<.0001**	0.064	**<.0001**	0.99	0.99	**0.025**	0.14	0.56
Glucagon (pg/ml)	48.5	51.4	11.45	0.67	**0.002**	**0.005**	0.67	0.99	0.98	**0.001**	0.86	0.87
Leptin (ng/ml)	4.99	4.87	0.12	0.32	**<.0001**	**0.01**	--	--	0.061	0.12	--	0.06
Acute-Phase Response												
Total Protein (g/dL)	7.76	7.746	0.13	0.92	**<.0001**	0.36	--	--	0.52	0.71	--	0.53
Albumin (g/dL)	3.253[a]	3.108[b]	0.06	**0.053**	0.4	0.11	--	--	0.83	0.96	--	0.84
Globulin (g/dL)	4.563	4.542	0.10	0.83	**<.0001**	0.45	--	--	0.97	0.73	--	0.73
Haptoglobin (mg/ml)	0.92	0.82	0.21	0.25	0.53	0.14	--	--	0.62	**0.023**	--	0.5
Serum Amyloid A (ng/ml)	109	83	58.09	0.44	0.47	0.79	--	--	0.96	0.67	--	0.9
Liver Function variables												
Alkaline Phosphatase Total (U/L)	38.9	36.5	1.41	0.18	0.08	0.69	--	--	**0.042**	**0.028**	--	0.071
Aspartate Aminotransferase (U/L)	73.3	74.9	4.61	0.73	**<.0001**	0.31	--	--	0.72	0.69	--	0.99
Gamma-Glutamyl Transferase (U/L)	28.42	28.39	1.03	0.98	**<.0001**	**0.05**	--	--	0.099	0.21	--	0.15
Total Bilirubin (mg/dL)	0.167[b]	0.265[a]	0.05	**0.0123**	**<.0001**	0.11	--	--	**0.001**	**0.001**	--	**0.001**
Glutamate Dehydrogenase (U/L)	51	59	21.72	0.2	**<.0001**	0.94	--	--	0.48	0.94	--	0.5
Cholesterol Total (mg/dL)	190.9	195.3	5.91	0.47	**0.001**	0.46	--	--	0.11	0.53	--	0.16
Triglycerides (mg/dL)	8.04[a]	10.54[b]	1.04	**0.015**	**<.0001**	0.54	--	--	**0.02**	0.37	--	**0.029**
Protein Metabolism variables												
Aspartate (μmol/dl)	0.480	0.401	0.05	0.13	**<.0001**	**<.0001**	**0.004**	0.22	0.25	**0.002**	0.1	0.41
Alanine (μmol/dl)	19.7	18.5	1.70	0.23	**<.0001**	**<.0001**	**<.0001**	0.34	0.16	**0.001**	0.62	0.25

Table 3 (cont.) LS means per treatment

Serine (µmol/dl)	8.20	8.03	0.85	0.73	**<.0001**	**<.0001**	**<.0001**	0.99	0.23	**0.001**	0.69	0.33
Threonine (µmol/dl)	7.21	6.44	0.82	0.28	**<.0001**	**<.0001**	**<.0001**	0.96	0.4	**0.001**	0.49	0.34
Cysteine (µmol/dl)	0.064	0.086	0.03	0.15	**<.0001**	**<.0001**	0.13	0.97	**0.048**	**0.001**	0.63	**0.048**
Isoleucine (µmol/dl)	10.04	9.91	1.03	0.8	**<.0001**	**0.023**	**0.045**	0.99	0.85	0.11	0.39	0.8
Leucine (µmol/dl)	21.2	19.0	1.90	0.63	**<.0001**	**<.0001**	**<.0001**	0.99	0.24	**0.001**	0.68	0.19
Valine (µmol/dl)	26.4	24.4	4.02	0.64	**<.0001**	**<.0001**	0.28	**<.0001**	0.57	**0.001**	0.79	0.35
Tyrosine (µmol/dl)	5.59^{a}	4.04^{b}	0.49	**0.001**	**<.0001**	**<.0001**	**<.0001**	0.98	**0.001**	**<.0001**	0.42	**0.001**
Phenylalanine (µmol/dl)	5.47^{a}	4.18^{b}	0.56	**0.032**	**<.0001**	**<.0001**	**<.0001**	0.94	**0.041**	**<.0001**	0.75	0.1
Tryptophan (µmol/dl)	3.02	2.81	0.23	0.37	**<.0001**	**0.002**	**<.0001**	0.91	0.72	0.2	0.62	0.63
Histidine (µmol/dl)	4.94	4.85	0.33	0.17	**<.0001**	**<.0001**	**0.042**	0.99	0.17	**<.0001**	0.81	0.15
Asparagine (µmol/dl)	4.07	3.75	0.48	0.47	**<.0001**	**<.0001**	**<.0001**	0.35	0.36	**<.0001**	0.39	0.42
Proline (µmol/dl)	8.84	8.18	1.12	0.24	**<.0001**	**<.0001**	**<.0001**	0.91	0.082	**<.0001**	0.3	0.19
Ornithine (µmol/dl)	4.12	4.35	0.55	0.66	**<.0001**	**0.015**	**<.0001**	0.93	0.23	0.31	0.72	0.27
Citrulline (µmol/dl)	8.98	10.89	0.95	0.074	**<.0001**	**<.0001**	**<.0001**	0.99	0.16	**0.001**	0.15	0.077
Glycine (µmol/dl)	29.0^{a}	36.1^{b}	3.96	**0.056**	**<.0001**	**<.0001**	**<.0001**	0.98	0.21	0.57	0.14	0.1
Methionine (µmol/dl)	1.92	1.95	0.21	0.77	**<.0001**	**<.0001**	**<.0001**	0.56	0.89	**0.002**	0.3	0.88
Arginine (µmol/dl)	6.09^{a}	7.73^{b}	0.59	**0.008**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**0.022**	0.066	**0.036**	**0.003**
Lysine (µmol/dl)	4.57^{b}	6.20^{a}	1.15	**0.051**	**<.0001**	**0.038**	**<.0001**	**<.0001**	**0.043**	0.25	0.38	**0.001**
Glutamine (µmol/dL)	23.2^{a}	28.0^{b}	2.09	**0.03**	**<.0001**	**<.0001**	**<.0001**	0.097	0.089	0.3	0.27	**0.022**
Glutamate (µmol/dl)	4.32	4.03	0.25	0.095	0.35	**<.0001**	**<.0001**	**<.0001**	0.25	**0.004**	**0.003**	0.25
Urea (µmol/dl)	426	530	54.64	0.077	**<.0001**	**<.0001**	**<.0001**	0.99	0.3	**<.0001**	0.91	0.058
NH3 (µmol/dl)	9.53	10.18	0.72	0.35	**0.046**	0.24	**<.0001**	0.32	0.86	0.97	0.5	0.83
α- AAA (µmol/dl)	0.68^{b}	0.848^{ab}	0.10	0.12	**<.0001**	0.27	0.43	**<.0001**	0.87	0.12	0.91	0.4
3-Methylhistidine (µmol/dl)	0.197^{b}	0.244^{b}	0.06	0.56	**<.0001**	**<.0001**	**<.0001**	0.99	**0.027**	**<.0001**	0.94	**0.027**
1-Methylhistidine (µmol/dl)	0.963	0.935	0.07	0.57	**<.0001**	0.13	**0.006**	0.88	0.44	0.37	0.63	0.44
Carnosine (µmol/dl)	1.13	0.12	0.20	0.99	**<.0001**	**0.039**	**<.0001**	0.99	0.94	0.2	0.72	0.94
Sarcosine (µmol/dl)	0.64	0.48	0.17	0.96	**<.0001**	**<.0001**	0.11	0.83	0.29	**0.003**	0.75	0.44
Hydroxyproline (µmol/dl)	1.03^{ab}	1.13^{a}	0.17	0.22	0.1	0.66	**0.002**	0.75	0.61	0.78	0.98	0.93
Cystathionine (µmol/dl)	0.135	0.143	0.04	0.93	**<.0001**	**<.0001**	**0.047**	0.88	0.36	**0.003**	0.22	0.51
β-Alanine (µmol/dl)	0.247	0.240	0.06	0.98	**<.0001**	**<.0001**	0.099	0.96	0.99	0.68	0.87	0.99
Phosphoethanolamine (µmol/dl)	2.11	2.14	0.18	0.8	0.065	**0.001**	0.1	0.96	0.5	0.6	0.59	0.5
Ethanolamine (µmol/dl)	0.395	0.383	0.03	0.69	**0.033**	**0.028**	0.27	0.97	0.88	0.12	0.81	0.88
α-Amino Butyric Acid (µmol/dl)	1.77	1.89	0.22	0.5	**<.0001**	**<.0001**	**0.02**	0.99	0.64	**0.036**	0.97	0.64
Taurine (µmol/dl)	6.47	6.05	1.14	0.97	**<.0001**	**0.002**	**<.0001**	0.93	0.93	0.37	0.76	0.99
P-Serine (µmol/dl)	1.371	1.322	0.10	0.78	**<.0001**	**<.0001**	0.13	0.99	0.59	0.59	0.41	0.44

[1] Contrast statement between ad libitum (AL) and restricted control (RC) treatments

[2] Effect of feed condition among all the treatments: fed (AL) or restricted (RC, LYS, MSG, GLN, VAL, GLC)

[3] Contrast statement between ad libitum (AL) and the group of restricted treatments (RES; RC, LYS, MSG, GLN, VAL, GLC)

[a,b] LS means within a row with different superscripts differ (P < 0.05)

Table 4 LS means per diet and day

Variables		Days[1]					
Production variables		**1**	**2**	**3**	**4**	**5**	**SE**
Dry Matter Intake (kg)	**AL**	26.4[b]	28.1[ab]	29.5[a]	27.9[ab]	25.5[b]	1.34
	RES	12.9	13.0	12.9	12.9	12.6	0.95
Energy Balance (MJ/d)	**AL**	-12.7	-2.1	-14.3	-4.1	-15.6	15.04
	RES	-63.0[b]	-49.7[a]	-43.9[a]	-40.8[a]	-38.0[a]	3.69
Feed Efficiency (milk yield/ DM)	**AL**	2.24	2.35	2.28	2.32	2.40	0.25
	RES	2.754[a]	2.37[b]	2.185[bc]	2.116[c]	2.111[c]	0.07
Feed Efficiency (ECM/DM)	**AL**	2.05	1.83	1.83	1.81	1.92	0.29
	RES	2.908	2.623	2.500	2.468	2.404	0.08
Milk weight (lb.)	**AL**	35.8	38.3	38.6	38.2	35.7	1.74
	RES	35.8[a]	31.6[b]	29.1[c]	28.3[c]	27.2[c]	0.91
Energy Corrected Milk (lb.)	**AL**	37.1	35.5	37.6	35.6	34.0	3.14
	RES	37.9	34.6	32.8	32.6	30.5	1.77
Milk protein							
%	**AL**	3.05	3.11	3.12	3.08	2.99	0.06
	RES	3.05[a]	3.03[a]	2.91[b]	2.85[c]	2.80[c]	0.02
kg.	**AL**	1.09	1.19	1.20	1.18	1.07	0.05
	RES	1.08[a]	0.95[b]	0.84[c]	0.80[cd]	0.76[d]	0.02
Milk Fat							
%	**AL**	3.71	2.73	3.07	2.81	3.16	0.41
	RES	3.76[b]	4.17[a]	4.52[a]	4.52[a]	4.56[a]	0.14
kg.	**AL**	1.29	1.02	1.17	1.04	1.04	0.18
	RES	1.38	1.32	1.30	1.33	1.23	0.09
Lactose							
%	**AL**	4.653	4.679	4.645	4.623	4.655	0.06
	RES	4.667	4.662	4.599	4.551	4.537	0.02
kg.	**AL**	1.677	1.801	1.800	1.771	1.668	0.09
	RES	1.676[a]	1.479[b]	1.341[c]	1.290[c]	1.246[c]	0.03
Total Solids							
%	**AL**	7.82	7.47	7.59	7.43	7.58	0.17
	RES	7.726[b]	7.887[a]	7.932[a]	7.890[a]	7.809[ab]	0.05
kg.	**AL**	6.13	6.25	6.42	6.19	5.86	0.38
	RES	6.12[a]	5.49[b]	5.06[c]	4.94[c]	4.71[c]	0.19
Milk Urea Nitrogen (mg/dL)	**AL**	11.5	11.1	12.1	11.6	11.6	1.36
	RES	12.12[c]	15.36[a]	14.01[b]	13.49[bc]	13.66[abc]	0.58
Energy Corrected Milk (kg)	**AL**	37.1	35.5	37.6	35.6	34.0	3.14
	RES	37.9	34.6	32.8	32.6	30.5	1.77
Energy-Related Metabolites and Hormones							
Non Esterified Fatty Acid (mEq/L)	**AL**	0.111	0.097	0.096	0.099	0.118	0.07
	RES	0.119[a]	0.311[b]	0.391[c]	0.371[bc]	0.358[bc]	0.03
Insulin (µg/L)	**AL**	0.55	0.69	0.66	0.53	0.46	0.13
	RES	0.741[a]	0.367[b]	0.273[c]	0.289[bc]	0.297[c]	0.03
Glucose (mg/dL)	**AL**	64.7	65.2	63.0	63.3	64.7	3.09
	RES	65.36[a]	60.64[b]	59.05[c]	59.01[c]	59.76[bc]	0.80
Hydroxy-Butyrate (mmol/L)	**AL**	0.56	0.60	0.58	0.57	0.56	0.10
	RES	0.532[a]	0.557[a]	0.639[b]	0.719[c]	0.735[c]	0.03
Glucagon (pg/ml)	**AL**	37.5[c]	45.3[bc]	53.6[a]	55.1[ab]	47.6[abc]	6.91
	RES	51.6[a]	50.3[a]	48.6[a]	41.9[b]	45.0[ab]	2.24
Leptin (ng/ml)	**AL**	4.86	5.03	5.01	5.11	4.94	0.15
	RES	5.014	4.872	4.767	4.704	4.596	0.07
Acute-Phase Response							
Total Protein (g/dL)	**AL**	7.66	7.81	7.71	7.65	7.85	0.16
	RES	7.673	7.853	7.961	7.786	7.878	0.05
Albumin (g/dL)	**AL**	3.226	3.270	3.235	3.237	3.270	0.05
	RES	3.208	3.250	3.260	3.218	3.253	0.02
Globulin (g/dL)	**AL**	4.48	4.64	4.54	4.51	4.68	0.13
	RES	4.460	4.613	4.688	4.555	4.638	0.04
Haptoglobin (mg/mL)	**AL**	0.80[ab]	0.79[ab]	0.74[a]	0.90[ab]	1.34[b]	0.25
	RES	0.912	0.924	0.964	0.890	0.933	0.08

Table 4 (cont.) LS means per diet and day

Serum Amyloid A (ng/mL)	**AL**	86	68	100	140	166	56.87
	RES	109	112	120	113	140	18.32
Liver Function Variables							
Alkaline Phosphatase Total (U/L)	**AL**	38.6	37.8	38.1	38.5	39.8	1.55
	RES	37.12^{bc}	38.06^{c}	36.80^{bc}	35.55^{ab}	34.77^{a}	0.85
Aspartate Aminotransferase (U/L)	**AL**	79.0	77.1	73.1	70.4	69.1	5.01
	RES	76.4	77.0	76.6	69.7	66.4	1.64
Gamma-Glutamyl Transferase (U/L)	**AL**	28.28	29.55	28.88	26.55	28.55	0.96
	RES	28.47	29.93	30.39	29.36	29.42	0.31
Total Bilirubin (mg/dL)	**AL**	0.181	0.176	0.142	0.176	0.176	0.05
	RES	0.169^{c}	0.286^{b}	0.349^{a}	0.311^{ab}	0.286^{ab}	0.02
Glutamate Dehydrogenase (U/L)	**AL**	56.9	52.9	50.3	50.1	47.3	17.79
	RES	72.5	61.0	47.8	37.5	29.6	5.84
Cholesterol Total (mg/dL)	**AL**	189.4	192.8	190.5	189.2	188.8	6.24
	RES	191.5	195.7	200.6	199.9	203.1	2.05
Triglycerides (mg/dL)	**AL**	7.6	7.0	7.4	7.4	8.0	1.27
	RES	7.75	10.33	10.83	10.46	10.29	0.42
Protein Metabolism Variables							
Aspartate (µmol/dl)	**AL**	0.468	0.500	0.471	0.518	0.504	0.05
	RES	0.535^{a}	0.479^{b}	0.448^{c}	0.412^{c}	0.381^{d}	0.02
Alanine (µmol/dl)	**AL**	21.3	20.3	20.2	18.9	18.2	1.31
	RES	22.87^{a}	18.03^{b}	17.51^{b}	17.86^{b}	17.42^{b}	0.40
Serine (µmol/dl)	**AL**	8.19	8.22	8.36	8.16	7.17	0.60
	RES	9.02^{a}	7.32^{b}	6.88^{c}	7.23^{bc}	7.09^{bc}	0.19
Threonine (µmol/dl)	**AL**	7.36	7.25	7.42	7.13	6.51	0.62
	RES	8.27^{a}	6.35^{b}	6.14^{b}	6.52^{b}	6.22^{b}	0.19
Cysteine (µmol/dl)	**AL**	0.074	0.073	0.049	0.059	0.063	0.02
	RES	0.0862^{b}	0.0959^{b}	0.0870^{b}	0.1106^{a}	0.1199^{a}	0.01
Isoleucine (µmol/dl)	**AL**	10.83	10.47	10.54	9.77	8.93	0.94
	RES	11.25^{a}	10.16^{bc}	10.26c	10.93^{ab}	10.12^{c}	0.49
Leucine (µmol/dl)	**AL**	21.6	21.2	22.1	20.9	18.6	1.83
	RES	23.0^{a}	19.0^{bc}	18.5^{c}	19.7^{b}	18.1^{c}	1.14
Valine(µmol/dl)	**AL**	27.9^{ab}	26.8^{ab}	27.8^{a}	26.2^{ab}	23.4^{b}	2.30
	RES	29.8^{a}	25.00^{ab}	24.5^{c}	25.8^{b}	24.1^{c}	1.38
Tyrosine (µmol/dl)	**AL**	5.77	5.79	5.94	5.48	4.95	0.41
	RES	6.66^{a}	4.52^{b}	3.67^{cd}	3.61^{c}	3.35^{d}	0.12
Phenylalanine (µmol/dl)	**AL**	5.37	5.60	5.89	5.53	5.34	0.40
	RES	5.37^{c}	4.45^{ab}	4.4263^{a}	4.69^{b}	4.60^{ab}	0.12
Tryptophan (µmol/dl)	**AL**	3.18	2.99	3.02	2.99	2.93	0.20
	RES	3.461	3.233	3.058	3.009	2.824	0.06
Histidine (µmol/dl)	**AL**	5.43^{a}	5.48^{a}	5.46^{a}	5.26^{a}	4.75^{b}	0.32
	RES	6.00^{a}	4.83^{b}	4.38^{c}	4.56^{bc}	4.49^{c}	0.10
Asparagine (µmol/dl)	**AL**	4.05	4.06	4.22	4.11	3.69	0.34
	RES	5.04^{a}	3.73^{b}	3.35^{c}	3.62^{b}	3.31c	0.10
Proline (µmol/dl)	**AL**	9.16	9.02	9.37	9.15	8.31	0.75
	RES	9.99^{a}	7.61^{b}	6.98^{c}	7.47^{b}	7.24^{bc}	0.24
Ornithine (µmol/dl)	**AL**	4.43	4.11	4.28	4.15	3.78	0.41
	RES	5.23	4.85	4.46	4.40	4.09	0.13
Citrulline (µmol/dl)	**AL**	9.39	9.12	9.46	9.69	9.56	0.74
	RES	9.51^{c}	10.96^{ab}	10.61^{ab}	10.87^{a}	10.30^{bc}	0.24
Glycine (µmol/dl)	**AL**	31.2	30.1	29.8	30.9	29.3	2.99
	RES	31.48	30.44	31.89	33.95	33.64	0.94
Methionine (µmol/dl)	**AL**	1.95	1.94	2.04	1.84	1.80	0.15
	RES	2.3^{a}	1.9^{b}	1.9^{b}	1.9^{b}	1.8^{b}	1.96
Arginine (µmol/dl)	**AL**	6.63	6.20	6.38	6.25	6.28	0.50
	RES	7.09^{b}	7.28^{b}	7.56^{b}	8.12^{a}	7.39^{b}	0.16
Lysine (µmol/dl)	**AL**	5.58	5.16	5.11	4.81	4.90	0.49
	RES	6.17	6.04	6.27	6.65	6.02	0.18
Glutamine (µmol/dl)	**AL**	24.9	24.6	25.2	24.9	24.5	1.84
	RES	28.88	26.53	26.43	27.37	26.71	0.77

Table 4 (cont.) LS means per diet and day

Glutamic Acid (µmol/dl)	**AL**	4.05	4.31	4.35	4.28	4.44	0.22
	RES	4.27[a]	4.15[ab]	4.03[ab]	4.01[b]	4.00[b]	0.14
Urea (µmol/dl)	**AL**	471	464	470	477	473	39.78
	RES	472[c]	600[a]	533[b]	493[c]	473[c]	13.10
NH_3 (µmol/dl)	**AL**	9.73	9.67	9.59	9.35	9.38	0.59
	RES	9.78	9.88	9.76	9.78	9.70	0.20
α- AAA (µmol/dl)	**AL**	0.694	0.703	0.682	0.699	0.680	0.07
	RES	0.601[c]	0.643[c]	0.709[b]	0.775[a]	0.758[ab]	0.03
3-Methylhistidine (µmol/dl)	**AL**	0.162[b]	0.192[ab]	0.189[ab]	0.198[ab]	0.243[a]	0.05
	RES	0.198[d]	0.258[d]	0.336[b]	0.3683[a]	0.371[a]	0.02
1-Methylhistidine (µmol/dl)	**AL**	0.893	0.938	0.978	0.978	1.005	0.06
	RES	0.946	0.982	1.053	1.076	1.040	0.02
Carnosine (µmol/dl)	**AL**	1.13	1.13	1.12	1.15	1.13	0.16
	RES	1.252	1.181	1.102	1.082	1.066	0.05
Sarcosine (µmol/dl)	**AL**	0.54	0.54	0.57	0.57	0.53	0.16
	RES	0.490[c]	0.589[b]	0.650[a]	0.697[a]	0.714[a]	0.06
Hydroxyproline (µmol/dl)	**AL**	0.97	0.87	0.92	0.90	0.87	0.20
	RES	0.961	0.899	0.926	0.954	0.900	0.06
Cystathionine (µmol/dl)	**AL**	0.135[ab]	0.133[b]	0.144[a]	0.146[a]	0.129[b]	0.02
	RES	0.1511[a]	0.1319[b]	0.1296[b]	0.1399[b]	0.1325[b]	0.01
β-Alanine (µmol/dl)	**AL**	0.251	0.255	0.261	0.255	0.236	0.05
	RES	0.262	0.259	0.240	0.247	0.222	0.02
Phosphoethanolamine (µmol/dl)	**AL**	2.17	2.15	2.16	2.09	2.08	0.15
	RES	2.188	2.154	2.244	2.280	2.252	0.05
Ethanolamine (µmol/dl)	**AL**	0.366	0.386	0.396	0.421	0.408	0.03
	RES	0.4053[a]	0.3947[ab]	0.4[a]	0.3867[ab]	0.3723[b]	0.01
α-Amino Butyric Acid (µmol/dl)	**AL**	1.46[c]	1.62[bc]	1.89[a]	2.00[ab]	1.89[abc]	0.19
	RES	1.559[d]	1.734[c]	1.826[b]	2.078[a]	2.013[a]	0.06
Taurine (µmol/dl)	**AL**	6.2	6.7	6.5	7.0	6.5	1.05
	RES	5.61	6.15	6.50	7.11	6.85	0.43
P-Serine (µmol/dl)	**AL**	1.442	1.402	1.364	1.325	1.268	0.08
	RES	1.368	1.322	1.294	1.285	1.264	0.03

[1] LS means per day and per feed condition (AL or RES). Data from treatments where values were significantly modified by the infusion, were removed from the calculations

[a,b,c,d] LS means within a row with different superscripts differ ($P < 0.05$)

Table 5 P-values from the contrast statements to test linear and quadratic trends of daily averages

Variable	**Treatments**					
	AL		**RC**		**RES**[1]	
Production Variables	Linear	Quad	Linear	Quad	Linear	Quad
Dry Matter Intake	0.34	**<.0001**	0.95	0.89	0.40	0.34
Energy Balance (MJ/d)	0.78	0.19	**0.053**	0.94	**0.001**	0.077
Feed Efficiency (kg milk/kg DM)	0.68	0.87	**0.001**	0.45	**<0.0001**	**<0.0001**
Feed Efficiency (kg ECM/kg DM)	0.79	0.48	**0.016**	0.97	**<.0001**	**0.043**
Milk weight (kg.)	0.91	0.063	**0.001**	0.91	**<0.0001**	**<0.0001**
Energy Corrected Milk (lb.)	0.47	0.67	**0.049**	0.53	**<0.0001**	0.18
Milk protein						
%	0.60	**0.015**	**<0.0001**	0.47	**<0.0001**	0.82
kg	0.71	**0.002**	**<0.0001**	0.59	**<0.0001**	**<0.0001**
Milk Fat						
%	0.42	0.16	0.098	0.21	**0.000**	**0.026**
kg	0.42	0.60	0.78	0.43	0.55	0.71
Lactose						
%	0.57	0.60	**<0.0001**	0.74	**<0.0001**	0.79
kg	0.82	0.076	**<0.0001**	0.91	**<0.0001**	**<0.0001**
Total Solids						
%	0.28	0.16	0.11	0.10	0.30	**0.001**
kg	0.60	0.23	**0.001**	0.65	**<0.0001**	**0.003**
Milk Urea Nitrogen (mg/dL)	0.65	0.56	0.62	**0.004**	0.52	**0.001**
Energy Corrected Milk (kg.)	0.47	0.67	**0.049**	0.53	**<0.0001**	0.18
Energy-Related Metabolites and Hormones						
Non Esterified Fatty Acid (mEq/L)	0.97	0.53	**0.002**	**<.0001**	**<.0001**	**<.0001**
Insulin (µg/L)	0.31	0.068	**<.0001**	**<.0001**	**<.0001**	**<.0001**
Glucose (mg/dL)	0.89	0.55	**0.001**	**0.001**	0.083	**0.041**
Hydroxy-Butyrate (mmol/L)	0.74	0.56	**0.042**	0.66	**<.0001**	0.77
Glucagon (pg/ml)	0.074	**0.011**	**0.055**	0.86	**0.001**	0.45
Leptin (ng/ml)	0.61	0.24	0.57	0.48	**<.0001**	0.54
Acute-Phase Response						
Total Protein (g/dL)	0.82	0.68	0.71	0.33	**0.035**	**0.008**
Albumin (g/dL)	0.88	0.90	0.28	**0.032**	0.37	0.36
Globulin (g/dL)	0.6742	0.5657	0.6287	0.7812	**0.009**	**0.0053**
Haptoglobin (mg/ml)	0.2106	**0.0273**	0.2107	0.9186	0.834	0.6488
Serum Amyloid A (ng/ml)	0.3842	0.2898	0.8534	0.9883	0.23	0.7095
Liver Function Variables						
Alkaline Phosphatase Total (U/L)	0.71	0.39	**0.018**	0.65	**0.002**	0.067
Aspartate Aminotransferase (U/L)	0.16	0.93	0.25	0.28	**<.0001**	**0.009**
Gamma-Glutamyl Transferase (U/L)	0.43	0.97	0.76	0.84	0.21	**0.001**
Total Bilirubin (mg/dL)	0.81	0.50	0.15	0.12	**<.0001**	**<.0001**
Glutamate Dehydrogenase (U/L)	0.79	0.98	**0.042**	0.41	**<.0001**	0.62
Cholesterol Total (mg/dL)	0.64	0.91	0.21	0.19	**<.0001**	0.18
Triglycerides (mg/dL)	0.68	0.74	0.17	**0.001**	**0.001**	**0.001**
Protein Metabolism parameters						
Aspartate (µmol/dl)	0.62	0.85	**<.0001**	0.11	**<.0001**	0.31
Alanine (µmol/dl)	**0.051**	0.77	**0.015**	**<.0001**	**<.0001**	**<.0001**
Serine (µmol/dl)	0.41	0.056	0.055	**0.001**	**<.0001**	**<.0001**
Threonine (µmol/dl)	0.25	0.27	**0.015**	**<.0001**	**<.0001**	**<.0001**
Cysteine (µmol/dl)	0.43	0.22	0.40	0.15	**<.0001**	**0.011**
Isoleucine (µmol/dl)	**0.044**	0.33	**0.027**	0.22	0.060	0.091
Leucine (µmol/dl)	0.37	0.090	**0.001**	**0.001**	**<.0001**	**<.0001**
Valine(µmol/dl)	0.27	0.20	**0.037**	**0.001**	**0.03**	**<.0001**
Tyrosine (µmol/dl)	0.099	0.093	**<.0001**	**<.0001**	**<.0001**	**<.0001**
Phenylalanine (µmol/dl)	0.74	**0.045**	**0.001**	**<.0001**	**<.0001**	**<.0001**
Tryptophan (µmol/dl)	0.25	0.62	**0.001**	**0.012**	**<.0001**	0.13
Histidine (µmol/dl)	0.06	**0.043**	**0.001**	**<.0001**	**<.0001**	**<.0001**
Asparagine (µmol/dl)	0.67	0.16	**<.0001**	**0.001**	**<.0001**	**<.0001**
Proline (µmol/dl)	0.62	0.20	**0.001**	**0.001**	**<.0001**	**<.0001**
Ornithine (µmol/dl)	0.36	0.81	**0.018**	**0.048**	**<.0001**	0.053

Table 5 (cont.) P-values from the contrast statements to test linear and quadratic trends of daily averages

Citrulline (μmol/dl)	0.49	0.73	0.091	**0.001**	**0.001**	**<.0001**
Glycine (μmol/dl)	0.91	0.99	0.27	0.90	**0.040**	**0.022**
Methionine (μmol/dl)	0.32	0.36	**0.004**	0.097	**<.0001**	**<.0001**
Arginine (μmol/dl)	0.40	0.49	0.42	0.62	**0.001**	**<.0001**
Lysine (μmol/dl)	0.54	0.68	0.79	0.74	0.07	**0.001**
Glutamine (μmol/dl)	0.49	0.83	0.16	0.33	0.55	**<.0001**
Glutamate (μmol/dl)	0.06	0.52	0.059	**0.015**	0.10	0.062
Urea (μmol/dl)	0.97	0.84	0.51	**<.0001**	0.12	**<.0001**
NH3 (μmol/dl)	0.26	0.99	0.47	0.11	0.36	0.53
α- AAA (μmol/dl)	0.92	0.88	**0.002**	0.070	**<.0001**	**0.032**
3-Methylhistidine (μmol/dl)	**0.003**	0.46	**<.0001**	0.20	**<.0001**	**<.0001**
1-Methylhistidine (μmol/dl)	**0.033**	0.42	0.49	**0.019**	**<.0001**	**<.0001**
Carnosine (μmol/dl)	0.86	0.99	0.090	0.29	**<.0001**	**0.006**
Sarcosine (μmol/dl)	0.94	0.53	**0.028**	0.97	**<.0001**	**0.003**
Hydroxyproline (μmol/dl)	0.60	0.83	0.85	0.26	0.51	0.84
Cystathionine (μmol/dl)	0.98	0.21	0.31	0.93	**0.003**	**0.001**
β-Alanine (μmol/dl)	0.61	0.42	0.41	0.14	**0.001**	0.46
Phosphoethanolamine (μmol/dl)	0.35	0.86	**0.054**	0.44	**0.010**	0.66
Ethanolamine (μmol/dl)	0.12	0.41	0.59	0.070	**0.007**	0.25
α-Amino Butyric Acid (μmol/dl)	**0.011**	**0.007**	**<.0001**	0.091	**<.0001**	**0.005**
Taurine (μmol/dl)	0.58	0.50	0.87	0.49	**<.0001**	**0.030**
P-Serine (μmol/dl)	**0.001**	0.72	0.17	0.68	**0.004**	**0.049**

[1] RES: group of treatments under feed restriction (RC, MSG, GLN, VAL, LYS, GLC)

Table 6 LS means per diet and hour relative to feeding

Variable	Hours relative to feeding/infusion[1]						
Energy-Related Metabolism Variables	-1	0	3	4	5	14	SE
Non Esterified Fatty Acid (mEq/L)	0.479^{c}	0.519^{c}	0.121^{a}	0.112^{a}	0.123^{a}	0.313^{b}	0.022
Insulin (µg/L)	0.309^{c}	0.287^{c}	0.490^{ab}	0.524^{a}	0.548^{a}	0.384^{b}	0.043
Glucose (mg/dL)	61.3^{bc}	62.0^{b}	59.6^{bc}	59.0^{c}	61.0^{b}	66.1^{a}	1.308
Hydroxy-Butyrate (mmol/L)	0.423^{a}	0.419^{a}	0.797^{c}	0.818^{c}	0.813^{c}	0.517^{b}	0.041
Glucagon (pg/ml)	44.9	46.8	46.8	47.6	48.7	50.4	2.493
Protein Metabolism Variables							
Aspartate (µmol/dl)	0.429^{a}	0.470^{b}	0.468^{ab}	0.446^{ab}	0.437^{ab}	0.471^{ab}	0.019
Alanine (µmol/dl)	19.72^{ab}	19.11^{b}	17.77^{c}	17.45^{c}	17.45^{c}	21.29^{a}	0.448
Serine (µmol/dl)	8.47^{a}	8.18^{a}	7.21^{b}	6.98^{b}	6.87^{b}	8.45^{a}	0.244
Threonine (µmol/dl)	7.60^{a}	7.44^{a}	6.42^{b}	6.03^{bc}	5.84^{c}	7.13^{a}	0.205
Cysteine (µmol/dl)	0.1008	0.0957	0.0898	0.0934	0.0997	0.0891	0.007
Isoleucine (µmol/dl)	11.05^{a}	10.90^{ab}	10.18^{ab}	9.99^{b}	9.98^{b}	10.77^{ab}	0.545
Leucine (µmol/dl)	20.9^{b}	20.5^{b}	18.3^{c}	17.9^{c}	18.1^{c}	22.7^{a}	1.269
Valine(µmol/dl)	27.6	27.3	30.2	31.2	31.0	28.6	1.019
Tyrosine (µmol/dl)	5.19^{a}	4.86^{a}	4.20^{b}	4.03^{b}	4.04^{b}	5.05^{a}	0.148
Phenylalanine (µmol/dl)	5.10^{b}	4.97^{b}	4.44^{c}	4.34^{c}	4.45^{c}	5.59^{a}	0.128
Tryptophan (µmol/dl)	3.320^{a}	3.247^{a}	2.888^{b}	2.890^{b}	2.965^{b}	3.310^{a}	0.064
Histidine (µmol/dl)	5.15^{a}	5.08^{ab}	4.96^{ab}	4.79^{ab}	4.68^{b}	4.93^{ab}	0.123
Asparagine (µmol/dl)	4.28^{a}	4.06^{a}	3.59^{b}	3.45^{b}	3.33^{b}	4.27^{a}	0.114
Proline (µmol/dl)	8.67^{b}	8.30^{bc}	7.64^{cd}	7.27^{d}	7.15^{d}	9.41^{a}	0.263
Ornithine (µmol/dl)	5.02^{ab}	5.03^{a}	4.52^{bc}	4.19^{d}	4.06^{d}	4.33^{cd}	0.219
Citrulline (µmol/dl)	11.57^{a}	11.37^{a}	10.37^{b}	9.66^{c}	9.04^{d}	9.65^{bcd}	0.253
Glycine (µmol/dl)	36.3^{a}	35.7^{a}	29.3^{b}	27.2^{c}	26.9^{c}	34.8^{a}	1.065
Methionine (µmol/dl)	2.214^{a}	2.172^{a}	1.913^{b}	1.790^{c}	1.715^{c}	2.025^{b}	0.048
Arginine (µmol/dl)	8.04^{a}	7.88^{ab}	7.62^{b}	7.16^{c}	6.73^{d}	7.16^{cd}	0.136
Lysine (µmol/dl)	7.39^{a}	7.41^{a}	8.80^{ab}	8.47^{bc}	7.80^{c}	6.08^{c}	0.331
Glutamine (µmol/dl)	26.28^{ab}	25.49^{b}	28.20^{a}	27.09^{ab}	25.99^{b}	26.65^{ab}	0.790
Glutamate (µmol/dl)	3.84^{c}	3.68^{d}	4.45^{a}	4.45^{a}	4.40^{a}	4.12^{b}	0.123
Urea (µmol/dl)	513^{bc}	529^{bc}	576^{a}	560^{ab}	526^{c}	373^{d}	14.996
NH3 (µmol/dl)	10.37^{c}	9.98^{b}	9.60^{ab}	9.23^{ab}	9.21^{a}	9.98^{bc}	0.200
α- AAA (µmol/dl)	0.730	0.717	0.768	0.799	0.812	0.731	0.030
3-Methylhistidine (µmol/dl)	0.298^{ab}	0.292^{ab}	0.288^{b}	0.278^{b}	0.275^{b}	0.313^{a}	0.015
1-Methylhistidine (µmol/dl)	1.034^{a}	1.024^{ab}	1.006^{ab}	0.990^{ab}	0.969^{b}	1.042^{a}	0.020
Carnosine (µmol/dl)	1.240^{a}	1.198^{a}	1.075^{b}	1.060^{b}	1.072^{b}	1.170^{a}	0.051
Sarcosine (µmol/dl)	0.594	0.603	0.625	0.606	0.616	0.660	0.040
Hydroxyproline (µmol/dl)	0.998^{a}	1.002^{a}	0.879^{ab}	0.918^{ab}	0.869^{b}	0.927^{ab}	0.050
Cystathionine (µmol/dl)	0.140^{ab}	0.145^{a}	0.137^{ab}	0.134^{ab}	0.131^{b}	0.140^{ab}	0.010
β-Alanine (µmol/dl)	0.259	0.252	0.254	0.238	0.237	0.241	0.022
Phosphoethanolamine (µmol/dl)	2.195	2.153	2.254	2.256	2.256	2.143	0.047
Ethanolamine (µmol/dl)	0.4000	0.3980	0.3948	0.3829	0.3925	0.3856	0.010
α-Amino Butyric Acid (µmol/dl)	1.899^{a}	1.926^{a}	1.833^{a}	1.788^{a}	1.702^{b}	1.843^{ab}	0.074
Taurine (µmol/dl)	6.53^{ab}	6.21^{a}	6.58^{a}	6.06^{a}	6.04^{a}	7.23^{b}	0.378
P-Serine (µmol/dl)	1.337	1.315	1.330	1.303	1.314	1.293	0.026

[1] LS means per hour relative to feeding/beginning of daily infusion and per feed condition (AL or RES). Data from treatments where values were significantly modified by the infusion, were removed from the calculations. Average of the 5 treatment days.

[a,b,c,d] LS means within a row with different superscripts differ ($P < 0.05$)

Table 7 P-values of the slice statement from the significant interactions between Trt and Day effects

Variables	Treatments	
Production Variables	**RC**	**AL**
Milk yield (kg)	**0.008**	0.39
Feed Efficiency (milk yield/DM)	**0.007**	0.94
Feed Efficiency (ECM/DM)	0.15	0.96
Protein %	**<.0001**	0.17
Protein yield (kg)	**0.001**	0.43
Lactose Yield (kg)	**<.0001**	0.44
Total Solids %	**0.028**	0.10
Total Solids Yield (kg)	**0.002**	0.68
Energy/Liver metabolism Variables		
NEFA	**<.0001**	0.14
BHBA	0.16	0.80
Insulin	**<.0001**	0.38
Glucose	**<.0001**	0.79
Glucagon	0.128	**0.02**
Leptin	0.88	0.30
Albumin	0.16	0.97
Haptoglobin	0.43	**0.027**
Gamma-Glutamyl Transferase	0.39	0.061
Total Bilirubin	**0.040**	0.46
Protein Metabolism Variables		
Arginine	0.62	0.69
Citrulline	**<.0001**	0.38
Ornithine	**0.008**	0.16
Aspartate	**0.001**	0.59
Glutamine	0.070	0.95
Glutamate	**0.005**	0.21
Valine	**<.0001**	**0.049**
Leucine	**<.0001**	0.093
Isoleucine	**0.038**	0.14
Tyrosine	**<.0001**	0.16
Tryptophan	**<.0001**	0.53
Phenylalanine	**<.0001**	0.25
Glycine	**0.005**	0.33
Serine	**<.0001**	0.074
Methionine	**0.001**	0.60
Cysteine	**0.011**	0.066
Histidine	**<.0001**	**0.035**
Lysine	0.48	0.57
Threonine	**<.0001**	0.20
Asparagine	**<.0001**	0.32
Proline	**<.0001**	0.13
Alanine	**<.0001**	0.34
Urea	**<.0001**	0.88
NH3	**0.020**	0.73
Carnosine	0.27	0.92
3-Methylhistidine	**<.0001**	**<.0001**
1-Methylhistidine	0.23	0.088
P-Serine	0.90	**0.004**
Sarcosine	**0.002**	0.53
Cystathionine	0.23	**0.014**
β-Alanine	0.38	0.80
Phosphoethanolamine	0.060	0.89
Ethanolamine	**0.030**	0.47
α-ABA	**0.001**	**0.002**
Taurine	0.97	0.79

Table 8 P-values of the slice statement from the significant interactions between Trt and Hour effects

Variable	Trt*Hour	Treatment RC	AL
NEFA	0.124	**<.0001**	0.092
Arginine	**<.0001**	**<.0001**	**<.0001**
Glutamine	0.097	0.45	0.43
Glutamate	**<.0001**	**0.002**	0.17
Valine	**<.0001**	0.75	0.67
Lysine	**<.0001**	**<.0001**	**0.018**
α- AAA	**<.0001**	0.73	0.97

Table 9 Pearson correlation coefficients for milk yield (kg/d), milk components, DIM and BW

Variables	**Milk Production**	**Milk Fat %**	**Protein %**	**Lactose %**	**Total Solids %**	**Milk Urea Nitrogen**	**Days in milk**	**Body Weight**
Milk Production	1	-0.309	-0.351	0.271	-0.257	--	-0.465	0.253
Milk Fat %	-0.309	1	0.220	-0.327	0.882	0.445	0.339	-0.118
Protein %	-0.351	0.220	1	-0.563	0.187	--	0.214	0.141
Lactose %	0.271	-0.327	-0.563	1	--	-0.031	--	-0.363
Total Solids %	-0.257	0.882	0.187	--	1	0.430	0.353	-0.291
Milk Urea Nitrogen	--	0.445	--	--	0.430	1	--	-0.209
Days in milk	-0.465	0.339	0.214	--	0.353	--	1	--
Body Weight	0.253	-0.118	0.141	-0.363	-0.291	-0.209	--	1

Table 10 Pearson correlation coefficients for plasma amino acids concentrations (µmol/dL)

Variables	Day	Urea	Asp	Thr	Ser	Glu	Gln	Gly	Ala	Val	Cys	Met	Ile	Leu	Tyr	Phe	Trp	NH3	Lys	His	Arg	Asn	Pro	Cit	Orn
Day	**1**	--	-0.21	-0.24	-0.29	--	--	0.08	-0.28	--	0.10	-0.23	-0.07	-0.21	-0.55	-0.15	-0.27	--	--	-0.34	0.08	-0.36	-0.29	0.11	-0.26
Urea	--	**1**	-0.27	0.13	-0.22	-0.15	0.23	-0.29	--	0.09	0.18	0.10	0.29	0.11	-0.09	-0.50	0.18	-0.17	0.11	0.23	0.23	--	--	0.59	0.21
Aspartate	-0.21	-0.27	**1**	--	0.08	0.58	-0.13	0.16	0.08	--	--	0.08	--	--	0.22	0.34	--	0.41	-0.16	--	-0.07	0.07	--	-0.27	--
Threonine	-0.24	0.13	--	**1**	0.67	--	0.38	0.28	0.61	0.23	--	0.82	0.64	0.69	0.73	0.40	0.71	--	0.07	0.71	0.47	0.80	0.76	0.48	0.63
Serine	-0.29	-0.22	0.08	0.67	**1**	-0.10	0.32	0.55	0.57	--	--	0.61	0.16	0.33	0.64	0.43	0.41	0.12	--	0.60	0.34	0.79	0.71	0.17	0.46
Glutamate	--	-0.15	0.58	--	-0.10	**1**	0.16	-0.14	-0.08	0.12	--	--	--	-0.08	--	0.21	--	--	-0.13	--	--	-0.08	-0.17	-0.31	--
Glutamine	--	0.23	-0.13	0.38	0.32	0.16	**1**	0.17	0.20	--	0.08	0.37	0.25	0.15	0.22	--	0.25	-0.32	0.09	0.56	0.48	0.44	0.37	0.26	0.50
Glycine	0.08	-0.29	0.16	0.28	0.55	-0.14	0.17	**1**	0.14	-0.07	0.13	0.23	--	0.07	0.15	0.27	0.16	0.29	--	0.32	0.26	0.41	0.31	0.15	0.29
Alanine	-0.28	--	0.08	0.61	0.57	-0.08	0.20	0.14	**1**	--	-0.23	0.56	0.26	0.43	0.55	0.32	0.49	0.22	--	0.67	0.25	0.61	0.79	0.20	0.40
Valine	--	0.09	--	0.23	--	0.12	--	-0.07	--	**1**	0.19	0.27	0.49	0.44	0.20	0.15	0.32	0.10	-0.08	0.12	0.09	0.15	0.08	0.17	0.17
Cysteine	0.10	0.18	--	--	--	--	0.08	0.13	-0.23	0.19	**1**	--	0.18	0.18	--	--	--	--	--	0.09	0.12	--	--	0.19	0.07
Methionine	-0.23	0.10	0.08	0.82	0.61	--	0.37	0.23	0.56	0.27	--	**1**	0.61	0.62	0.72	0.44	0.64	0.08	0.08	0.62	0.54	0.74	0.64	0.36	0.66
Isoleucine	-0.07	0.29	--	0.64	0.16	--	0.25	--	0.26	0.49	0.18	0.61	**1**	0.88	0.48	0.27	0.57	--	0.13	0.42	0.43	0.47	0.43	0.55	0.47
Leucine	-0.21	0.11	--	0.69	0.33	-0.08	0.15	0.07	0.43	0.44	0.18	0.62	0.88	**1**	0.66	0.43	0.62	--	--	0.53	0.25	0.60	0.63	0.45	0.44
Tyrosine	-0.55	-0.09	0.22	0.73	0.64	--	0.22	0.15	0.55	0.20	--	0.72	0.48	0.66	**1**	0.61	0.58	0.12	--	0.64	0.24	0.81	0.69	0.15	0.52
Phenylalanine	-0.15	-0.50	0.34	0.40	0.43	0.21	--	0.27	0.32	0.15	--	0.44	0.27	0.43	0.61	**1**	0.43	0.22	--	0.22	0.17	0.44	0.35	-0.22	0.23
Tryptophan	-0.27	0.18	--	0.71	0.41	--	0.25	0.16	0.49	0.32	--	0.64	0.57	0.62	0.58	0.43	**1**	0.09	0.08	0.60	0.33	0.58	0.57	0.33	0.51
NH_3	--	-0.17	0.41	0.03	0.12	--	-0.32	0.29	0.22	0.10	--	0.08	--	--	0.12	0.22	0.09	**1**	--	0.10	--	0.12	0.17	--	--
Lysine	--	0.11	-0.16	--	--	-0.13	0.09	--	--	-0.08	--	0.08	0.13	--	--	--	0.08	--	**1**	--	0.55	0.09	--	--	0.36
Histidine	-0.34	0.23	--	0.71	0.60	--	0.56	0.32	0.67	0.12	0.09	0.62	0.42	0.53	0.64	0.22	0.60	0.10	--	**1**	0.32	0.78	0.81	0.36	0.61
Arginine	0.08	0.23	-0.07	0.47	0.34	--	0.48	0.26	0.25	0.09	0.12	0.54	0.43	0.25	0.24	0.17	0.33	--	0.55	0.32	**1**	0.44	0.28	0.48	0.70
Asparagine	-0.36	--	0.07	0.80	0.79	-0.08	0.44	0.41	0.61	0.15	--	0.74	0.47	0.60	0.81	0.44	0.58	0.12	0.09	0.78	0.44	**1**	0.80	0.36	0.60
Proline	-0.29	--	--	0.76	0.71	-0.17	0.37	0.31	0.79	0.08	--	0.64	0.43	0.63	0.69	0.35	0.57	0.17	--	0.81	0.28	0.80	**1**	0.36	0.50
Citrulline	0.11	0.59	-0.27	0.48	0.17	-0.31	0.26	0.15	0.20	0.17	0.19	0.36	0.55	0.45	0.15	-0.22	0.33	--	--	0.36	0.48	0.36	0.36	**1**	0.37
Ornithine	-0.26	0.21	--	0.63	0.46	--	0.50	0.29	0.40	0.17	0.07	0.66	0.47	0.44	0.52	0.23	0.51	--	0.36	0.61	0.70	0.60	0.50	0.37	**1**