

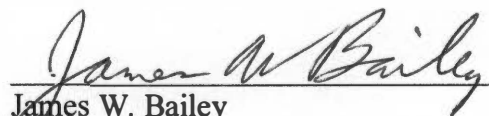
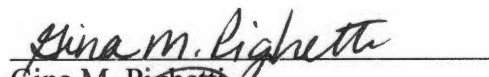
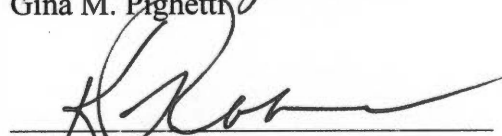
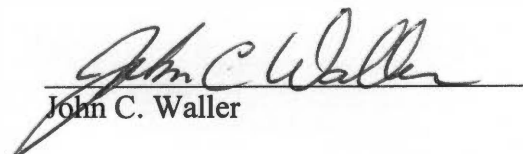
To the Graduate Council:

I am submitting herewith a dissertation written by James Levi Klotz entitled "Effects of Weaning Transition and Ionophore on Metabolic Profile and Portal and Hepatic Nutrient Flux During Pre- and Post-Weaning Growth in Dairy Calves." I have examined the final paper copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

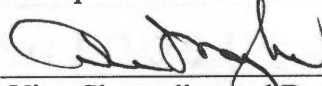


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Accepted for the Council:



Vice Chancellor and Dean of
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**EFFECTS OF WEANING TRANSITION AND IONOPHORE ON
METABOLIC PROFILE AND PORTAL AND HEPATIC NUTRIENT
FLUX DURING PRE- AND POST-WEANING GROWTH IN DAIRY
CALVES**

A Dissertation
Presented for the
Doctor of Philosophy
Degree

The University of Tennessee, Knoxville

James Levi Klotz
May 2004

ACKNOWLEDGMENTS

The author wishes to express his sincere gratitude to the numerous individuals who in various ways have directly or indirectly contributed to the completion of this dissertation:

To the Department of Animal Science at The University of Tennessee, Knoxville, for the opportunity to pursue a Ph.D.

To Dr. Richard N. Heitmann, major professor, mentor, and a friend without whom this work and my sanity would not have been possible. It was your idiom "...improvise, adapt, and overcome" that helped me persevere through the variety obstacles I encountered during the completion of this work.

To Dr. James W. Bailey, Dr. Gina M. Pighetti, Dr. Kelly R. Robbins, and Dr. John C. Waller, for your time, patience, and participation as members of my graduate committee. Your contributions and commitment to a level of excellence has made for a quality education.

To Dr. Chris Richards, your surgical consulting, lending of equipment, and patience in teaching research techniques has made me a better scientist.

To Ann Reed, for your statistical expertise in analyzing datasets that required magical abilities.

To Dr. John Hodges of the Knoxville Experiment Station, thank you for all of the calves and allowing me access to the facilities and resources for a,.....slightly longer than expected time. Also, to Dr. Mark Campbell, Roger Long, and Charlie Young, your patience with my requests and ability to get the job done was invaluable.

To Henry Dowlen of the Dairy Experiment Station, for providing the many of the calves used in this project and your patience with my research requests. Also, to Phillip Lunn, Hugh Moorehead, and Terry Austin for your contributions in the completion of this research, your ability to get the job done was invaluable.

To Dr. Fred Hopkins, for acting as our veterinarian of record and all your help in keeping the calves healthy and me happy. Also, Dr. Matt Welborn, Dr. Bob Henry, and Dr. Robert Reed for helping me find a home for the experimental units.

To my fellow graduate students, thank you for your friendship and support. A special thanks to Tracey Livingston, Jan Pulliam, Rob Gillis, Magdalena Rambeaud, Brent Pugh, and Allison Corrigan for assisting in the surgeries and regulating the entropy.

To Linda Miller, Nancy Rohrbach, and TanTan Sun, without these individuals I would still be in the laboratory. Also, to Eddie Jarboe, for patiently taking care of my numerous supply orders and laboratory questions and making Brehm feel like a home, especially when my work forced it to become one.

To all my “special problems” undergraduates, your daily assistance with the seven-days-a-week animal care and laboratory work was a real time and lifesaver and you also taught me a little about being the boss.

To my family, your lifelong support and confidence in me has made these many years of college possible.

Lastly, but not leastly I would like to thank my wife Ursula, without you I would have never made it this far and for that I am eternally indebted.

“It is not the critic who counts, not the man who points out how the strong man stumbles or where the doer of deeds could have had them done better. The credit belongs to the man who is actually in the arena, whose face is marred by dust and sweat and blood, who strives valiantly, who errs and comes short again and again because there is no effort without error and shortcomings, who knows the great devotion, who spends himself in a worthy cause, who at best knows in the end the high achievement of triumph and who at worst, if he fails while daring greatly, knows his place shall never be with those timid and cold souls who know neither victory nor defeat.”

- Theodore Roosevelt

ABSTRACT

Dairy calf weaning is associated with increases in ketone levels that exceed measured rates of utilization in adults and present a potential energy loss that may be mitigated by ionophores. Therefore, effects of weaning and ionophore supplementation on nutrient metabolism and growth in dairy calves pre- and post-weaning were examined in two experiments. The first experiment consisted of 24 Jersey bull calves blocked in groups of two according to birth date and weight and randomly assigned to receive either a commercial pelleted starter (CON), or the same diet containing lasalocid (TRT; 83 mg/kg DM) to examine effects of weaning transition on weight (BW), gain (ADG), and blood concentrations of glucose, acetoacetate (ACAC), β -hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA), volatile fatty acids (VFA), lactate, pyruvate, insulin, and glucagon over 16 wk. The second experiment assessed the effects of weaning and lasalocid supplemented starter (listed above) on BW, ADG, net portal-drained viscera, hepatic, and total splanchnic fluxes of the same metabolites listed above for Experiment 1, but were measured on d 35, 56, 84, and 112 in nineteen Jersey bull calves that were equipped with catheters in portal, hepatic, and mesenteric veins and artery at wk 3 - 4. In Experiments 1 and 2 all calves were fed milk replacer from d 3 - 34. On d 35, calves began receiving either CON or TRT calf starter *ad libitum* plus their milk replacer ration until weaning on d 49. From d 49-112 calves received only CON or TRT *ad libitum*. Post-weaning intake, ADG, and feed:gain did not differ between CON and TRT in either Experiment 1 and 2. Total VFA and ketones significantly increased and NEFA and glucose significantly decreased following introduction of solid feed in both experiments. Both experiments demonstrated significant effects of ionophore supplementation on

ACAC, BHBA, and VFA during the early post-weaning period. In both experiments significant changes in metabolic profile of transition calves were demonstrated peri-weaning and ionophore appears to moderate alimentary output at a post-weaning period where ketone concentrations have potential to exceed whole animal capacity for utilization.

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PART I

INTRODUCTION

The existence and categorical success of mature ruminant animals is largely dependent on the end-products of microbial fermentation of complex carbohydrates and post-ruminal digestion of microbial protein. However, prior to weaning the animal is dependent on a liquid diet of milk and an essentially monogastric digestive system with which to process ingesta. The first two months of a young ruminants life require several major adaptations. First, the animal must adjust to extra-uterine life and establish a functional digestive system capable of obtaining required nutrients from a feedstuff instead of maternal blood supply. The second adaptation is weaning to a solid feed. This transformation of a preruminant to a functional ruminant is one of the greatest modifications of digestive physiology that the animal will ever experience. It is not surprising that this period is one of the more perilous in the ruminant animals life.

Economic loss due to weaning related stress can be substantial for all species of production animals. Dairy calf mortality in the first three months of life can range between 7 – 14% of all live births (James et al., 1984; Hartman et al., 1974; Oxender et al., 1973; Speicher and Hepp, 1973). In addition to mortality, production losses can be due to a post-weaning lag in growth rate, increased susceptibility to disease, delayed genetic progress, reduced replacements for voluntary culling, and increased purchase of replacement calves (James et al., 1984). Furthermore, rapid rates of daily gain (0.6 – 0.7 kg/d, depending on breed size) are required to allow dairy calves to reach sufficient weight (~ 340 kg) for breeding at 14–15 months of age.

The average cost of raising a replacement heifer has been reported to be \$1000 to \$1300 per animal (Cady and Smith, 1996). One management strategy currently in practice to reduce costs of replacement heifers is to wean the calf as early as possible.

This is an attempt to minimize mortality losses and reduce feed and labor costs associated with milk feeding. Through daily encouragement of early dry feed intake to stimulate ruminal development calves can be weaned as early as 3 – 4 weeks of age without negatively affecting subsequent rates of gain (Anderson et al., 1987a; Quigley et al., 1991a; Winter, 1985).

Encouraging the calf to make the required metabolic, nutritional, and behavioral changes to become a functional ruminant and expecting rapid rates of gain immediately thereafter represents an extreme challenge to both the animal and the producer. Given that feed costs per unit of height or weight increase are lowest during the first 6 months of life (Davis and Drackley, 1998) and the dairy industry's increasing emphasis on accelerated growth schemes, a clear understanding of the metabolic and physiologic challenges the calf faces during this dietary transition is critical to efficient nutritional management of replacement heifers.

Currently, optimal management of the transition from neonatal mono-gastric digestion to ruminant fermentative digestion is still largely undefined due to a dearth of information concerning the underlying mechanisms controlling use and assimilation of nutrients in visceral and peripheral tissues. This includes the period where the animals declining dependence on milk-derived nutrients and the increasing dependence on fermentative end products do not equally offset each other. This may also require the adaptation of peripheral tissue nutrient utilization systems to a change in source and type of nutrients supplied. The necessity for nutrient production and utilization to act in concert is exemplified by the rapid transient nature that ruminal volatile fatty acids

(VFA) and circulating ketone concentrations increase in conjunction with weaning dry feed intake (Quigley et al., 1991a).

Considerable advancements were made during the 1960's in understanding enzyme activity changes relative to weaning and changes in energy metabolite concentrations in blood of young ruminants. However, there are few, if any, reports regarding changes in net nutrient flux across splanchnic tissues during the transition at weaning. Circulating concentrations of a metabolite are a function of the rate of appearance and (or) disappearance of that metabolite (Heitmann, 1989). Because the available data consists primarily of replicated single site blood samples, they only represent the metabolite of interest at a specific location and time and thus, limit the scope of interpretation. Because of increasing emphasis on early weaning and potential for the rate of post-weaning growth to influence production as an adult, more in depth studies that address the physiological changes occurring in digestive tissues surrounding weaning should be undertaken. While all species of young ruminants generally undergo these adaptations, the dairy calf is unique in the accelerated rate at which it is expected to digestively mature. Thus, it is the physiological predicament of the dairy replacement calf and the homeorhesis of weaning that is the focus of the following discussion and experiments.

PART II

LITERATURE REVIEW

DEVELOPMENT OF THE FOREGUT

Development Versus Diet

Critical to performance of a calf during and after weaning is the ability to progress from obtaining energy via post-ruminal digestion of milk/milk replacer to obtaining energy from the end products of rumen microbial fermentation of carbohydrates. Following parturition, the onset of ruminal development is primarily dependent on the diet. However, post-weaning diets cannot adequately provide the nutrients required to support maintenance and growth if rumen development is inadequate. At birth the rumen has a volume of less than one liter and comprises only one third of the neonate's total stomach capacity, while the rumen of a mature animal comprises approximately two thirds of the total stomach capacity and a volume of about 150 liters (Church, 1975). The absorptive tissue lining the luminal surface of the neonatal rumen is smooth, contains minimal papillation, and does not possess the ability produce ketones, which is a hallmark characteristic of a functionally mature rumen (Baldwin and Jesse, 1992; Giesecke et al., 1979).

The adult rumen epithelium is very similar in cell type to skin. A keratinized stratified squamous epithelium, it consists of four cell strata: the *stratum corneum*, *stratum granulosum*, *stratum spinosum*, and *stratum basale* (Steven and Marshall, 1970). Like skin, rumen epithelial cells are keratinocytes that provide a protective barrier, but differ from skin as the rumen lining is also an absorptive epithelium. Cells making up the *stratum basale* are thought to be responsible for the majority of metabolic activity attributed to mature form of this tissue, as demonstrated by their extensive rate of

ketogenesis relative to cells isolated from the other layers (Baldwin and Jesse, 1991; 1992). The number of metabolically active cells per gram of rumen epithelium decreases with age (Lavker et al., 1969). This results from the increasing proportion of metabolically inactive cells that comprise the *granulosa* and *corneal* cell strata increasing with age relative to the number of metabolically active cells in *basal* in *spinous* strata (Lane et al., 2000). The newborn epithelium is non-keratinized and contains a high concentration of glycogen (Henrikson, 1970; Wardrop, 1961). However, seven days after birth the epithelium begins to resemble the keratinizing epithelium of an adult and retains only traces of glycogen, which are completely lost by day 35 (Henrikson, 1970). The complete disappearance of cellular glycogen is concomitant with the appearance of a prominent intercellular glycocalyx and basal infoldings which are indicative of a developing transport function in the tissue (Henrikson, 1970).

Functional development of rumen epithelium, particularly papillary growth appears to coincide with the initiation of dry feed intake (Warner et al., 1956; Tamate et al., 1962). However, the development in physical size of the organ is not *pari passu* with papillary growth (Brownlee, 1956). Blaxter et al. (1952) noted that calves fed milk and hay had a marked increase in the capacity of the rumen, reticulum, and omasum without an increase in the empty weight of the respective organs. Tamate et al. (1962) introduced indigestible sponges into the rumen of milk fed calves and observed an increase in overall rumen capacity and musculature, but not papillary development. Thus, there is a requirement for bulk, but growth, development and differentiation of the rumen mucosa appear to be dependent on resulting chemical stimuli and end products of rumen fermentation. Specifically, production and subsequent absorption of volatile fatty acids

(VFA) have been associated with the onset of rumination as early as seven days following birth (Tamate et al., 1962). Two weeks after parturition the oxidation of both butyrate and glucose to carbon dioxide by the rumen epithelial cells has been shown to occur at similar rates *in vitro* (Baldwin and Jesse, 1992). However, as noted above, the capacity of rumen cells to produce ketones from butyrate is very low prior to weaning and increases rapidly post-weaning (Baldwin and Jesse, 1992; Giesecke et al., 1979). The post-weaning increase in ketogenic potential coincides with a decline in glucose oxidation and an increased preference for butyrate as an oxidative substrate (Baldwin and Jesse, 1992; Bush, 1988).

Establishment of ruminal fermentation and successive production of VFA stimulates neonatal rumen development. Many changes in the ruminal environment are necessary for increases in surface area and metabolic activity of the rumen to coincide with dry feed intake. Increased bacterial numbers, ruminal synthesis and concentration of VFA, and decreased ruminal pH have all been related to feed consumption (Anderson et al., 1987a; Anderson et al., 1987b). Two to three weeks following weaning, forestomach development and concentrations of ruminal metabolites typically reach levels associated with those found in mature ruminants (Wardrop, 1961; Fahey and Berger, 1993). Through ruminal infusion of VFA, an increased absorptive capacity and metabolic activity of the epithelium including the production of ketones, primarily from butyrate has been observed (Hird and Weidemann, 1964; Sutton et al., 1963a; Sutton et al., 1963b; Walker and Simmonds, 1962). Other studies have shown that infusion of VFA into the rumen of calves promoted increases in papillary size and greater blood flow to epithelial tissue (Tamate et al., 1962; Sander et al., 1959; Flatt et al., 1959). Conversely, the degree

of subepithelial vesiculation in the rumen, a necessary component of metabolite absorption from the rumen to the portal blood, has not been shown to be a result of age of the calf or dietary management (Lotz et al., 1997).

These increases in absorption and metabolic activity result from the large increases in papillae numbers and size, which expand the luminal surface area of the rumen available for VFA absorption (Warner et al., 1956; Tamate et al., 1962). Butyrate is thought to be the primary luminal stimulator of rumen epithelial development *in vivo* (Sakata and Tamate, 1976, 1978) and also has, through dietary treatments, been implicated in influencing the size, thickness of the corneal layer, and metabolism of the rumen mucosa in adult ruminants (Harmon et al. 1991). The rapid increase in ruminal VFA peri-weaning results in significant increases in cell mitotic indices of sheep *in vivo* (Sakata and Tamate, 1978) with butyrate being the most efficacious. In contrast, cultured rumen epithelial cells treated with butyrate demonstrate decreased cell DNA synthesis (Neogady et al. 1989) and decreased cell proliferation (Baldwin, 1999). This disparity in actions of butyrate *in vivo* and *in vitro* is unclear and is still an area of research that is being actively investigated. In light of the conflicting reports, it is unlikely that butyrate is solely responsible for stimulation of development, but may require the complement of some other product of rumen fermentation for complete development to occur (Lane and Jesse, 1997).

Glucose and Propionate Metabolism

Prior to weaning, the calf is dependent on dietary carbohydrates for its supply of glucose (Lyford and Huber, 1993). Following weaning, all glucose must be derived from

non-carbohydrate compounds via hepatic gluconeogenesis, or post-ruminal glucose absorption. From three to eight weeks of age rumen development must be sufficiently advanced to provide adequate VFA to satisfy energy requirements of the young animal. By about eight weeks of age capacity for glucose uptake and oxidation by rumen epithelium becomes limited (Baldwin and Jesse, 1992; Bush, 1988; Giesecke et al., 1979).

It has been estimated that as much as 60 to 80% of an adult ruminant's daily energy requirement is derived from VFA absorbed from the rumen (Ørskov, 1980). As the young animal becomes increasingly reliant on VFA as a primary energy source, propionate stands out as the only glucogenic VFA. Propionate undergoes varying degrees of metabolism to lactate and pyruvate by the rumen epithelium during absorption and numerous laboratories have suggested that the amount of propionate converted to lactate by the rumen epithelium is between 30 - 50% (Pennington and Sutherland, 1956; Leng et al., 1967; Stevens, 1970; Bergman and Wolff, 1971). However, subsequent investigations concluded that an average of 2 to 3% propionate was converted to lactate by rumen epithelium and was of little quantitative significance to the animal (Weekes and Webster 1975; Weekes 1972; Weigand et al., 1972). This disparity in propionate metabolism is likely the result of species (sheep have a higher propionyl-CoA synthetase activity than cattle and may be partially the cause of the increased epithelial metabolism) and experimental diet differences. The purpose of epithelial metabolism of propionate to lactate relative to the conversion of butyrate to acetoacetate (ACAC) and β -hydroxybutyrate (BHBA) may serve to satisfy mitochondrial and cytosolic redox states of the cell (Baldwin and Jesse, 1996; Weekes and Webster, 1975).

Regardless of the extent of propionate conversion by the rumen epithelium, there is little propionate found in arterial blood because of effective hepatic removal of it from the portal vein (Bergman et al., 1966). In a normally fed animal the liver removes up to 80-100% of the propionate from the portal blood (Bergman and Wolff, 1971). In addition to the extensive utilization of propionate for gluconeogenesis, amino acids have shown increased potential as gluconeogenic substrates as the demand for glucose increases (Wolff et al., 1972; Overton et al., 1999). Nocek et al. (1980) reported higher propionyl-CoA synthetase (E.C. 6.2.1.17) activity in the rumen epithelium of calves fed ground hay compared to those fed chopped hay at eight weeks of age. Also, the amount of propionate converted to lactate by the rumen epithelium is affected by concentrations of butyrate, which has been shown *in vitro* to inhibit the metabolism of propionate, as a result of the inhibition of the activating enzyme propionyl-CoA synthetase (Ash and Baird, 1973). Thus, the metabolic fate of propionate in ruminal and hepatic tissue is directly related not only to the animal's stage of development, but also to diet type.

Butyrate and Ketone Metabolism

In addition to supporting increased absorption of VFA, the papillae appear to be the major site of metabolism and are one of the few mammalian ketogenic organs (Leighton et al., 1983). In fed and non-pregnant ruminants, the rumen epithelium has been shown to be the most active ketogenic organ (Heitmann et al., 1987; Huntington and Reynolds, 1987). Typically in the adult ruminant, as much as 90% of the ruminally absorbed butyrate is metabolized by the rumen epithelium (Bergman, 1975; Bergman, 1990) and approximately 75% to 80% of the absorbed butyrate is converted to BHBA

and ACAC by the rumen prior to release into portal circulation (Heitmann, et al., 1987; Ramsey and Davis, 1965; Stevens, 1970). It has been demonstrated that mature rumen papillae contain enzymes necessary for ketone production, including β -hydroxy, β -methylglutaryl-CoA (HMG-CoA) synthase (E.C. 4.1.3.5), HMG-CoA lyase (E.C. 4.1.3.4), acetoacetyl-CoA thiolase (E.C. 2.3.1.9), and BHBA dehydrogenase (E.C. 1.1.1.30; Baird et al., 1970; Bush and Milligan, 1971; Koundakjian and Snoswell, 1970). In contrast, the undeveloped papillae of a preruminant lacks sufficient activity of key enzymes demonstrated by the inability to produce notable amount of ketones from butyrate prior to 6 weeks of age (Baldwin and Jesse, 1992; Giesecke et al., 1979). Through the developmental processes papillae making up the maturing rumen mucosa achieve high activities of these enzymes. Recent findings by Lane et al. (2000) using isolated rumen epithelial cells from lambs consuming milk replacer demonstrated that consumption of solid feed and resultant VFA production were not required to stimulate development of rumen epithelial ketogenic potential. This strongly supports findings of Giesecke et al. (1979) and Bush (1988) who used rumen epithelial slices taken from preweanling lambs and calves (respectively) to demonstrate that rumen ketogenesis is largely increased with age, particularly between 4 and 10 weeks, even when stimulation by rumen fermentation products is prevented. These three *in vitro* studies indicate that consumption of solid feed and production of VFA are not required to stimulate development of epithelial ketogenesis. This suggests an ontogenic response rather than a diet induced event and that certain aspects of rumen metabolic development may occur independently from morphological development.

β -Hydroxybutyrate is the main circulating ketone body released into portal circulation in fed ruminants (Katz and Bergman, 1969a; Heitmann et al., 1987) and ACAC produced by rumen epithelium is quantitatively removed from circulation by conversion to BHBA by the liver (Heitmann et al., 1986). This is the result of rumen epithelial BHBA dehydrogenase being located in the mitochondria (Leighton et al., 1983), but the hepatic isozyme of BHBA dehydrogenase resides primarily in the cytosol (Watson and Lindsay, 1972; Koundakjian and Snoswell, 1970). However, during fasting, changes in the circulating insulin-to-glucagon ratio, and release of non-esterified fatty acids (NEFA) from adipose will result in hepatic metabolism of NEFA to ketone bodies (Robinson and Williamson, 1980) causing a shift from alimentary to hepatic ketogenesis. Peripheral utilization of the ketones ACAC and BHBA may be for oxidation or lipogenesis and because the presence of ketones serves to conserve other body fuels (in particular glucose, alanine, and glycerol) they are associated with metabolic regulation (Robinson and Williamson, 1980). The portal-drained viscera (PDV) and hepatic tissues contribute to nutrient partitioning by regulating the structure and quantity of absorbed nutrients available (a cost of service function) to the peripheral tissues and by regulating the amounts of insulin and glucagon released to peripheral tissues (Reynolds and Maltby, 1994). Ketones produced by the rumen epithelium and liver can be used as an energy source by most tissues. The initial step in the oxidation of BHBA is dehydrogenation to ACAC by β -hydroxybutyrate dehydrogenase (E.C. 1.1.1.30). Acetoacetate must then be converted to acetoacetyl-CoA by succinyl-CoA transferase (E.C. 2.8.3.5), cleaved by acetoacetyl-CoA thiolase (E.C. 2.3.1.9) to yield two acetyl-CoA moieties, which are subsequently oxidized in the TCA cycle (Robinson and Williamson, 1980). Alternatively,

ketones may be used for lipid synthesis through acetyl-CoA and malonyl-CoA formation (Kronfeld, 1970).

A prerequisite for the neo-ruminant to utilize ketones is the presence of the necessary enzymes in peripheral tissues in sufficient concentrations to accommodate a sudden increase in circulating ketone concentrations. Varnam et al. (1978a) evaluated succinyl-CoA transferase (E.C. 2.8.3.5) activities in renal, cardiac, and cerebral tissue from fed and fasted sheep. Following a four-day fast, succinyl-CoA transferase activity in all three tissues was significantly lower than the respective activity measured in fed sheep. Because succinyl-CoA transferase is required for metabolism of acetoacetate, a lack of activity or significant decrease will reduce the capacity of the tissue to oxidize ketones. In a review of lipid and carbohydrate metabolism during ruminant post-natal development, Leat (1970) points out that as the calf makes the transition from milk to a solid feed diet between 3 to 8 weeks of age the metabolic predominance of absorbed glucose is decreasing, but plasma VFA levels can be insufficient because ruminal development has not progressed sufficiently to provide enough VFA to satisfy energy requirements. This incomplete development may also be the case for ketone utilization components in peripheral tissues. This places the animal in a precarious balance between fed and fasted states and a mild stress is enough to deplete liver glycogen and increase circulating concentrations of NEFA.

In vivo measures of circulating metabolites have demonstrated that a strong positive relationship exists between increasing calf starter dry matter intake (DMI) and blood BHBA and ACAC concentrations (Quigley et al., 1991a; Quigley et al., 1992; Quigley, 1996). In the early 1990's a series of studies monitored blood metabolite

concentrations in young calves. These studies evaluated the effects of grain feeding and weaning age (4 or 8 weeks) on concentrations of blood metabolites (Quigley et al., 1991a; Quigley et al., 1991b), the relationship of time of feeding and sampling on concentrations of ruminal and blood metabolites (Quigley and Bernard, 1992), and the inclusion of lasalocid on ruminal fermentation and blood metabolites in young calves (Quigley et al., 1992). Each experiment reported that concentrations of blood ketones were positively correlated with increased grain intake in young calves. Normal levels of NEFA and glucose measured in the above studies by Quigley and co-workers suggests that the elevated ketones were not the result of hepatic ketogenesis. Unexpectedly, average postweaning increases of total ketone concentrations greater than 1 mmole/L and, occasionally greater than 2 mmole/L were observed (Quigley et al., 1991a). Generally, in adults, blood ketone concentrations of 1 mmole/L are indicative of subclinical ketosis (Bergman, 1984) and concentrations above 2 mmole/L result in clinical symptoms. However, it was noted by Quigley et al. (1991a) that no negative effects or symptoms were observed that could be related to the observed hyperketonemia. It was reported by Bergman (1971) for ACAC and also by Leng (1965) for BHBA that rate of ketone turnover and oxidation to carbon dioxide by peripheral tissues is proportional to the circulating concentrations up to approximately 2 mmole/L. Beyond this concentration, slight increases in ketone production resulted in considerable increases in blood concentration. This phenomenon is a potential explanation for the rapid increase in circulating ketone concentrations found in the weanling calf.

Unfortunately, the majority of data concerning the peri-weanling calf including the Quigley studies discussed above were obtained via peripheral, single site sampling

(e.g., jugular venipuncture) of metabolites that are solely (e.g., propionate), or partially (e.g., amino acids) dependent on dietary intake, rumen fermentation, or absorption and are confounded by PDV and hepatic metabolism. In addition, the liver extracts amino acids from the portal blood of fed sheep at rates similar to their addition by the PDV (Heitmann and Bergman, 1980). Currently, the only publications concerning net nutrient flux in preruminant calves have been published by Ortigues et al. (1994, 1995, 1996). However, they were using veal calves, thus only concerned with events pre-weaning and not the effect of weaning. Consequently, changes in interorgan metabolism that may represent nutritional or physiological stress may be occurring peri-weaning that are not reflected by single site sampling in the current literature and is an area of research that should be pursued in greater detail.

HEPATIC ADAPTATIONS TO FOREGUT DEVELOPMENT

Development Versus Adaptation

The concentration of any circulating metabolite is a function of relative rates of production and utilization. Production may be endogenous (biosynthesis) or exogenous (dietary). Hepatic output is a function of the type and quantity of nutrients delivered mainly (~90 %; Reynolds et al., 1995) via the portal vein. Because all portal derived exogenous nutrients must pass through it, the liver has a central role in determining the amount and variety of nutrients available to peripheral tissues. In developing animals, the peri-weaning digestive adaptation from a preruminant to functional ruminant causes a significant change in the portal blood profile. Consequently, this change in dietary

nutrient pattern and supply causes substantial alterations in hepatic function and output. Most research directed at elucidating hepatic mechanisms involved with this period of transition have attempted to determine whether the preruminant liver is always equipped to accommodate the digestive adaptations of the foregut or whether the liver undergoes a maturation process of its own in response to ruminal development.

Hepatic Enzymatics

The most notable of changes in principle metabolic processes during ruminal development is the shift from a glycolytic to glucogenic liver. Table 2.1 is a representative compilation of enzymes and changes in activities relative to weaning. As microbial fermentation increases, less carbohydrate is available for post-ruminal digestion and the dietary supply of glucose diminishes. Like the rumen epithelium, these changes in glucose metabolism were reviewed in detail by Leat (1970). There is a basic reduction in enzyme capacity for hepatic glucose oxidation via glycolytic and hexose monophosphate pathways (Bartley et al., 1966) caused by decreased activities of hepatic glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49), 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44) and fructose 1,6-bisphosphate aldolase (E.C. 4.1.2.13), glyceraldehyde 3-phosphate dehydrogenase (E.C. 1.2.1.12; Goetsch, 1966; Leat, 1970). Hepatic tissue must now support a larger portion of the animals glucose requirements. Concurrent with decreased importance of glycolytic pathways is the rapid increase in activity of hepatic gluconeogenic enzymes (Table 2.1) with ruminal development, with glucose 6-phosphatase (E.C. 3.1.3.9) activity shown to double during this period (Bartley et al.,

Table 2.1. Changes in hepatic enzyme activity with the shift in metabolic priorities associated with ruminal development.

Enzyme	Species	Activity ¹		Source
		Pre-ruminant	Ruminant	
<i>Pentose Phosphate Pathway</i>				
Glucose-6-phosphate dehydrogenase	Bovine	+	-	Goetsch, 1966
6-Phosphogluconate dehydrogenase	Bovine	+	-	Howarth et al., 1968
<i>Glycolysis</i>				
Hexokinase	Ovine	+	-	Leat, 1970
Fructose-1,6-bisphosphate aldolase	Bovine	+	-	Howarth et al., 1968
Glyceraldehyde 3-phosphate dehydrogenase	Bovine	+	-	Howarth et al., 1968
Pyruvate kinase	Ovine	NC	NC	Edwards et al., 1975
<i>Tricarboxylic Acid Cycle</i>				
Citrate Synthase	Ovine	NC	NC	Edwards et al., 1975
Malate dehydrogenase	Ovine	NC	NC	Edwards et al., 1975
<i>Gluconeogenesis</i>				
Glucose-6-phosphatase	Bovine	-	+	Bartley et al., 1966
	Ovine	-	+	Ballard and Oliver, 1965
Fructose-1,6-bisphosphatase	Ovine	-	+	Ballard and Oliver, 1965
Pyruvate carboxylase	Ovine	+	-	Warnes et al., 1977
Lactate dehydrogenase	Ovine	+	-	Edwards et al., 1975
<i>Glycogen Metabolism</i>				
Glycogen synthase	Ovine	+	-	Ballard and Oliver, 1965
Glycogen phosphorylase	Ovine	+	-	Ballard and Oliver, 1965
	Bovine	-	+	Bartley et al., 1966
<i>Ketone Metabolism</i>				
β-hydroxybutyrate dehydrogenase	Ovine	-	+	Varnam et al., 1978b
Acetyl-CoA acetyltransferase	Ovine	-	+	Varnam et al., 1978b
Acetyl-CoA synthetase	Ovine	NC	NC	Leat, 1970

¹Changes in enzyme activity relative to before or after weaning: (+) = increased enzyme activity, (-) = reduced enzyme activity, (NC) = no change in enzyme activity.

1966). At this point in the animal's post-weaning development, propionate can be taken up from the portal blood by hepatic tissues and activated to propionyl-CoA, carboxylated to methylmalonyl-CoA, and enter the tri-carboxylic acid (TCA) cycle as succinyl-CoA (Bergman, 1990). The three carbons originally from propionate can now be converted through the gluconeogenic pathway to be released in the hepatic vein as glucose or remain in the liver stored as glycogen.

Concomitant with changes in enzyme activity associated with decreased glucose oxidation is the fact that basal rates of hepatic gluconeogenesis from propionate are similar in newborn, weaned, and adult sheep (Ballard and Oliver, 1965) and in cattle (Young et al., 1965; Donkin and Armentano, 1995). Hepatic glucose production is thought to be the primary means of lactate clearance in the preruminant (Edwards et al., 1975). The capacity for hepatic metabolism of L-lactate or pyruvate to glucose is two to three-fold greater in preweanling than adult sheep (Ballard and Oliver, 1965; Savan et al., 1986). More recently, using hepatocyte monolayers taken from preruminating and ruminating calves, Donkin and Armentano (1995) demonstrated that gluconeogenesis from lactate was approximately 10-fold greater in preruminating than ruminating calves. Ortigues et al. (1996) determined the maximum contribution of lactate to hepatic glucose production was 20% in preruminant calves fitted with chronic indwelling catheters in portal, hepatic, and mesenteric vein and artery. Van der Walt et al. (1983) also attributed 20% of hepatic uptake of lactate being released as glucose in adult sheep. This apparent contradiction by lack of difference using preruminant and ruminant *in vivo* models is probably attributable to a species difference, because cattle and sheep show different rates of propionate conversion to lactate by rumen mucosa (reviewed by Bergman, 1990).

While preruminants can convert either lactate or propionate to glucose, the inhibition of gluconeogenesis from lactate in ruminants is probably a result of increased availability of propionate (Donkin and Armentano, 1994). Thus, the shift in hepatic gluconeogenic metabolism may be a response to development of the functional rumen generating a more continuous supply of glucogenic substrate in the form of propionate in contrast to preruminant prandial patterns of nutrient delivery.

Insulin and Glucagon

Differences in preruminant and ruminant portal nutrient delivery to the liver are also evident in hormonal regulation of liver function. As ruminal development progresses there is a decreased sensitivity of hepatic gluconeogenesis in response to acute aberrations in insulin and glucagon concentrations. In hepatocytes isolated from preruminants, glucagon effectively increases hepatic gluconeogenesis from propionate and lactate, but had no effect on mature ruminant hepatocytes (Donkin and Armentano, 1995). Donkin and Armentano (1995) also demonstrated decreased gluconeogenesis and increased net de novo glycogenesis from propionate in the presence of insulin only in preruminant hepatocytes. Activities of enzymes (Table 2.1) glycogen synthetase (E.C. 2.4.1.11) and phosphorylase (E.C. 2.4.1.1) associated glycogen formation and degradation were elevated, but appeared to decrease to adult levels by 2-3 months (Ballard and Oliver, 1965). Hepatic glycogen reserves of the preruminant typically equal or exceed adult levels (Ballard and Oliver, 1965). However, a short 16 hour fast results in consumption of almost all liver glycogen, while similar fasts had little effect on hepatic glycogen in adults (Boda et al., 1962). Much more sensitive to insulin, the young

preruminant is similar to non-ruminants (Leat, 1970), but concurrent with ruminal development is decreased sensitivity to glucagon and a complete loss of insulin sensitivity (Donkin and Armentano, 1995). This is probably also a result of the liver becoming a steady producer of glucose and cessation of post-prandial spikes in circulating concentrations of glucose.

Hepatic Ketogenesis

Unlike the adult where the foregut is the primary producer of ketones, the preruminant foregut produces negligible amounts of ketones due to the absence of microbial fermentation, the liver being the primary site of ketogenesis. The immature fetal lamb liver is incapable of acetoacetate synthesis, but hepatic tissue of term fetus and the 15 week old lamb are capable of both synthesis of acetoacetate and conversion to β -hydroxybutyrate (Varnam et al., 1970). Following birth, the preruminant liver has the ability to synthesize acetoacetate from acetyl-CoA (Varnam et al., 1978b) and from butyrate (Hird and Weidemann, 1964). In developing sheep, hepatic activities (Table 2.1) of β -hydroxybutyrate dehydrogenase (1.1.1.30) and acetyl-CoA transferase (E.C. 2.3.1.9) increased with increasing β -hydroxybutyrate concentrations, but were not as high as activities measured in fed adults when the ruminal epithelium was the primary ketone producer (Varnam et al., 1978a). This is a likely indication of the adult hepatic conversion of portal-derived acetoacetate to β -hydroxybutyrate by cytosolic β -hydroxybutyrate dehydrogenase (Koundakjian and Snoswell, 1970) that results in a net hepatic output of β -hydroxybutyrate and uptake of acetoacetate (Heitmann et al., 1987).

In the event of fasting, the unfed preruminant is capable of hepatic ketogenesis from the time of birth, but does not assume the role of secondary ketone producer until ruminal development is complete.

While the change in diet that occurs peri-weaning causes a morphological and metabolic transformation in the foregut, changes in hepatic physiology appear to be secondary and more of an adjustment to a change in portal supplied nutrients brought on by a prolific ruminal fermentation.

LASALOCID SUPPLEMENTATION

Basic Chemical and Biologic Properties

Lasalocid (Figure 2.1) belongs to a class of compounds identified as carboxylic polyether ionophore antibiotics (commonly referred to as ionophores) and was isolated from *Streptomyces lasaliensis* (Westley et al., 1974). Ionophores as a group have a carbon backbone and oxygen atoms located throughout the molecule. The backbone can assume conformations that allow the oxygen molecules to congregate in the center of a

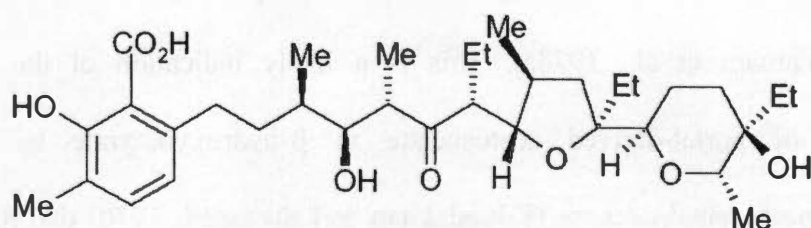


Figure 2.1. Chemical structure of lasalocid, acyclic form

ring or cavity. Through hydrogen bonding between the ether, hydroxyl, and carboxylic oxygen and the cation, the ionophore can effectively trap a cation (McGuffey et al., 2001). It is this conformation and binding that gives most ionophores the biological ability to alter movements of cations such as sodium, potassium, and calcium across cell membranes (Chalupa, 1980). Ionophores have both polar and nonpolar regions that enhance cation entrapment and interaction with cell membranes (McGuffey et al., 2001). When bound with a cation the ionophore-cation complex is able to form a stable zwitterion that is lipophilic and can readily solubilize in the phospholipid bilayer making up cell membranes (Pressman, 1976). Because ionophores have this ability to attach to membranes of bacteria and protozoa (Chow et al., 1994), they facilitate the movement of cations across the membrane or concentration gradient.

Cation gradients and relative affinities drive the resultant primary and secondary cation-proton exchanges. These exchanges occur rapidly and possibly at rates exceeding 1000 events per second (Pressman, 1976). These actions culminate in disruption of normal physiological concentration gradients (Russell and Strobel, 1989). Ultimately, the bacteria (typically gram-positive) are forced to utilize energy consuming transport systems such as proton and sodium ATPase pumps to dissipate the accumulating intracellular H^+ and Na^+ . This generates an energetic preoccupation with active transport that reduces energy reserves and diminishes the means for cell division. For ruminal microbes this typically leaves the affected organism unable to sustain a rate of cell division necessary to adequately maintain metabolic significance (Russell and Strobel, 1989).

Lasalocid has affinities for both monovalent and divalent cations and when immersed in a membrane, exchanges them for one or two protons, respectively (Pressman, 1976). Specifically, lasalocid has affinities for monovalent cations, Na^+ and K^+ with a greater affinity for K^+ (Painter and Pressman, 1985; McGuffey et al., 2001). Lasalocid also has the ability to form dimer complexes (Westley, 1978) that creates a larger chelation cavity and causes a significant affinity for divalent cations (e.g., Ca^{2+}) and K^+ over Na^+ .

As mentioned above, lasalocid and ionophores in general have a predominant effect on gram-positive bacteria. The resistance of the majority of gram-negative bacteria to ionophorous perturbations is thought to be a consequence of the outer membrane that is impermeable to large molecules (Russell and Strobel, 1989). The cell envelope of gram-negative bacteria consists of a multilayered complex of two distinct membranes that are divided by a rigid peptidoglycan layer (Bergen and Bates, 1984). This broad selection of bacteria by the ruminal presence of an ionophore modulates the resident populations and is the predominant cause of all effects associated with supplementation.

Effects of Lasalocid Supplementation

Supplementing animal feed with ionophores results in a large number of biological responses, a number of which are advantageous to production efficiency. Ionophores have been fed to reduce the occurrence of bloat and coccidiosis and to improve feed efficiency and growth rate (Goodrich et al., 1984). Spears (1990) demonstrated that lasalocid-supplemented cattle had 2.0% increase in apparent

digestibility that was significant over controls. Lasalocid has been reported to have similar effects on ADG and feed:gain as monensin (Bergen and Bates, 1984).

The most widely recognized response associated with the supplementation of ionophores is the modification of ruminal microbial populations and subsequent shift in VFA production ratios. Due to the microbial selectivity of the ionophore, shifts in rumen fermentation patterns occur (Nagaraja et al., 1987) causing a decrease in gram-positive bacteria that are commonly producers of acetate, butyrate, H_2 , and ammonia. This response, in turn, allows increased production of the gluconeogenic precursor propionate (Schelling, 1984). Chalupa (1977) determined the efficiencies of fermenting hexoses to acetate, propionate, and butyrate, and demonstrated that production of propionate was more energy efficient than production of either butyrate or acetate. Increased ruminal propionate production increases energy efficiency for retention by decreasing fermentative energy lost as eructated methane. Ionophores do not inhibit methanogenesis *per se*, but divert metabolic H_2 and formate away from methane production to propionate production (Bergen and Bates, 1984). Moreover, propionate appears to be more efficiently used by tissues than acetate (Ørskov et al., 1979) and may either be oxidized or used for gluconeogenesis depending on the prevailing metabolic demand.

Because it is possible to manipulate the proportions of VFA produced in ruminal fluid, it should be possible to manipulate the proportions of energy substrates released in the portal blood from the rumen epithelium. Using monensin-supplemented steers consuming a high concentrate diet, Harmon and Avery (1987) reported a decrease in net portal flux of butyrate and no significant increase in propionate release into the portal vein. Murdock and Wallenius (1980) suggested that increasing proportions of ruminal

butyrate might increase the rate of ruminal development, as butyrate is especially mitogenic to ruminal epithelium (Tamate et al., 1962; Sander et al., 1959). However, feeding high grain rations to increase concentrations of ruminal butyrate may serve to further increase production of blood ketones from ruminally produced butyrate and contribute to elevation of ketone concentrations at weaning.

Krehbiel et al. (1992) demonstrated that increased ruminal butyrate resulted in an increased portal-drained visceral flux of butyrate, which was associated with an increased portal-drained visceral flux of ACAC, but not BHBA. In contrast, monensin reduced blood ketone concentrations in lactating dairy cows (Sauer et al., 1989), which appears to result from a shift towards higher ruminal production of propionate (Bull et al., 1979). Research examining lasalocid demonstrated decreased ruminal butyrate and blood β -hydroxybutyrate in weaned calves (Quigley et al., 1992) and appeared to decrease alimentary ketogenesis in mature ewes (Heitmann et al., 1994) and wethers (Housewright et al., 1994).

Ionophores are implicated in myriad of effects that contribute to a greater overall ruminant efficiency. As such, supplementing weanling calf diets with ionophores may be an effective method to decrease circulating levels of ketones or increase circulating levels of glucose-sparing propionate. This change in ruminally derived nutrients may minimize any potential exacerbation of the post-weaning lag in growth reported by James et al. (1984).

PART III

EFFECTS OF WEANING AND AN IONOPHORE ON SELECTED BLOOD METABOLITES AND GROWTH IN DAIRY CALVES

ABSTRACT

Dairy calf weaning is associated with elevated ketone levels in excess of measured rates of utilization in adults and excess concentrations excreted in urine present a potential energy loss. Lasalocid is frequently supplemented as an anticoccidial in calf starters, but in adults is also known to alter molar ratios of ruminal VFA. Jersey bull calves ($n = 24$) were blocked in groups of two according to birth date and weight and randomly assigned to receive either a commercial pelleted starter (CON), or the same diet containing lasalocid (TRT; 83 mg/kg DM) to examine effects of weaning transition on weight (BW), dry matter intake (DMI), gain (ADG), and blood glucose, β -hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA), volatile fatty acids (VFA), insulin, and glucagon (GLN) concentrations over 16 wk. From d 3 - 34 all calves were fed milk replacer twice daily, d 35 - 48 received replacer and CON or TRT, and d 49 - 112 received *ad libitum* CON or TRT. Body weights and metabolite concentrations from jugular samples were measured and recorded weekly. Postweaning intake (2.35 vs. 2.34 kg/d; ± 0.07), ADG (0.78 vs. 0.75 kg/d; ± 0.03), and feed : gain (3.10 vs. 3.19; ± 0.11) did not differ between CON and TRT. Glucose and NEFA concentrations did not differ between CON and TRT, but declined with age. Insulin and GLN concentrations did not differ between CON and TRT, but GLN increased with weaning. Total VFA significantly increased following introduction of solid feed at d 35, but there was a one-wk lag period with TRT. Acetate and butyrate concentrations were greater in CON than TRT during wk 7 ($P < 0.05$). Propionate concentrations and acetate : propionate ratios did not differ between CON and TRT. Blood BHBA concentrations were greater in CON than TRT (P

< 0.05) during wk 8 and 9 (1.0, 1.1 vs. 0.7, 0.8 mmol/L; $\pm$ 0.1). Consumption of starter with lasalocid delayed peak acetate and butyrate and lowered peak BHBA concentrations, but did not appear to significantly influence post-weaning growth or efficiency.

INTRODUCTION

Rumen development in the young calf originates from dry feed intake, establishment of ruminal fermentation, and resultant volatile fatty acid (VFA) production (Tamate et al., 1962; Sutton et al., 1963a). Butyrate is the most mitogenic of the three major ruminal VFA (Sakata and Tamate, 1976; 1978) and once absorbed, butyrate is extensively metabolized by the rumen epithelium to acetoacetate (ACAC) and β -hydroxybutyrate (BHBA) prior to release into portal circulation (Bergman, 1990). At weaning there is a large increase in ACAC and BHBA production by the rumen epithelium (Baldwin and Jesse, 1992). Quigley et al. (1991a) reported post-weaning ketone concentrations that exceeded levels typically used as an indication of subclinical ketosis (Bergman, 1984). Utilization of ketones by peripheral tissues is first order kinetics, i.e., a concentration dependent process. Once blood ketone concentrations reach 2.0 mM, however, the system appears to achieve a maximum velocity ($V_{\max}$; Bergman, 1971; Leng, 1965) and a small increase in production beyond this level generates large increases in circulating concentrations. The elevated ketone levels measured in the post-weaning calf approximate this concentration limit and could be a potential loss of energy at a developmental stage when energy is at a premium.

Supplementing animal feed with ionophores results in varied biological responses, many of which are advantageous to production efficiency (Bergen and Bates, 1984; Spears, 1990). Increases in production efficiency of cattle results from modification of rumen fermentation and selection against protozoa that cause coccidiosis (McGuffey et al., 2001). The most widely recognized response associated with the supplementation of ionophores is the modification of ruminal VFA production. In studies using mature ruminants, supplementation generally results in greater ruminal propionate production at the expense of acetate and butyrate productions (Russell and Strobel, 1989).

Quigley et al. (1992) investigated the effects of lasalocid on pre- and post-weaning calves and found that lasalocid administered directly into the rumen via cannula significantly reduced circulating concentrations of ACAC and BHBA during the postweaning period. This is not a common route for ingestion of feed and is not a method available to producers, who must incorporate additives into feeds. Also, the experimental period ended when the calves reached 12 weeks of age, which coincided with the experiment's highest measured concentrations of BHBA. Thus, the age at which circulating concentrations decrease to adult levels was not apparent. The objectives of this experiment, therefore, were to examine the effects of weaning on selected metabolites in dairy calves from birth to 16 wks of age and to determine the post-weaning effect of lasalocid supplementation through a commercially available calf starter on blood metabolites, growth, intake and efficiency.

MATERIALS AND METHODS

Animals and Management

Calves and housing. All procedures were conducted under protocols approved by the University of Tennessee Animal Care and Use Committee (protocol # 1004). Twenty-four Jersey bull calves were obtained from the University of Tennessee Dairy Experiment Station (Lewisburg, TN) where they were maintained for the duration of the experiment. Calves received adequate colostrum intake and were castrated before 3 d of age. Calves were housed in individual calf hutches with individual pens providing outdoor access for the duration of the experiment.

Diets and feeding. Calves had *ad libitum* access to fresh water and trace-mineralized salt (Champions Choice; Cargill Inc., Minneapolis, MN) for the duration of the study. Starting at 3 d of age, all calves received 227 g commercial milk replacer brought to an approximate volume of 2 L with H₂O (12% solids; w/v; Instant Nursing Formula NT Medicated, Land O'Lakes Animal Milk Products Co., Fort Dodge, IA) twice daily at 0800 and 1630 hours from nipple bottles. At 21 d of age the milk replacer concentration was increased to 284 g per 2 L H₂O (15% solids; w/v). At 5 weeks of age (d 35) a commercially prepared high fiber calf starter (Calf Primer-I and Calf Primer-I-B; Tennessee Farmers Cooperative, Laverne, TN) was offered for *ad libitum* consumption. At this time, the calves were still receiving milk replacer twice daily, but was reduced to 227 g/2 L. Starting at week six (d 42), only the AM replacer ration was offered and at week seven (d 49) the calves were weaned. Calves often acquired scours at least once during their time on the study. Calves with scours were treated with a commercial oral

electrolyte solution (Revitilyte, Vets Plus, Inc., Knapp, WI) between the AM and PM feedings (1200).

Calf starter diets were commercially prepared and pelleted without (CON) or with lasalocid (TRT) supplement (83 mg/kg DM). Calves were sequentially assigned by birth weight and birth date to either CON (n = 12) or TRT (n = 12) starter in blocks of two. Starter was fed in a single ration at 0800. When a calf consumed an entire previous days ration, the ration was increased by 105% of previous days consumption. Feed rations and refusals were recorded daily.

Measurements

Blood sampling. Weekly blood samples were taken from the jugular vein for 16 weeks at 0800 and 0900. This was always within one hour post-prandial to the AM ration. Two 10-mL samples were collected in 16 × 75 Vacutainer tubes (Becton, Dickinson and Co., Franklin Lakes, NJ) containing K₃EDTA (15% solution, 0.117 mL). The samples were then packaged in an insulated shipping container with cold packs and shipped overnight to our laboratory in Knoxville for next day metabolite analysis.

Body weights and feed samples. Prior to feeding, calves were weighed weekly for the entirety of the study. Additional body weight measures were also recorded at birth, weaning, and termination. All diets in this study were commercially prepared and were obtained from the same lot number. Feeds (milk replacer and calf starters) were sampled monthly and composited for later proximate analysis.

Chemical Analyses

Blood samples. Whole blood samples were analyzed for concentrations of glucose, L-(+)-lactate, pyruvate, ACAC, and BHBA. Spectrophotometric analyses for glucose, ACAC, and BHBA were performed the same day samples were received. Deproteinized lactate and pyruvate samples were centrifuged ($1600 \times g$ at 4°C) and supernatants were harvested and frozen (-20°C) for analysis within two weeks. Plasma was harvested from the remaining blood sample following centrifugation at $1600 \times g$ at 4°C then frozen (-20°C) and stored for later analysis of NEFA, VFA, glucagon, and insulin. Analyses for BHBA and ACAC (Appendices 4 and 5) were done using enzymatic assays of Williamson and Mellanby (1974) and Mellanby and Williamson (1974), respectively. L-(+)-lactate and pyruvate (Appendices 7 and 8) were determined using procedures from the Sigma kits for lactate and pyruvate (#726-UV and #826-UV, Sigma Chemical Co., St. Louis, MO). Analysis of NEFA (Appendix 9) utilized the WAKO NEFA-C kit (WAKO Chemical USA, Dallas, TX). Blood glucose (Appendix 6) was determined using the Sigma glucose kit (#510; Sigma Chemical Co., St. Louis, MO). Plasma glucagon and insulin were determined using the double antibody radioimmunoassay and the Coat-A-Count[®] insulin kits and protocols from Diagnostics Products Corporation (Los Angeles, CA). For hormone analyses, intra-assay errors greater than 10% were not accepted and samples were rerun. Plasma VFA (Appendix 11) were analyzed by the procedure described by Reynolds et al. (1986) and modified by Quigley et al. (1991b) for capillary column, and Benson et al., (2002) with a Hewlett Packard gas chromatograph (5890A), auto-sampler (7637A), integrator (3393A), and a

J&W Scientific 530 μm HP-FFAP capillary column (Agilent Technologies Inc., Palo Alto, CA).

Feed samples. Composite dietary samples of CON and TRT starter were ground through a 1-mm screen in a Wiley Mill (Author H. Thomas Co., Philadelphia, PA) and analyzed (Table 3.1) with composite milk replacer sample for DM, N (model FP-2000 nitrogen analyzer, LECO, St. Joseph, MI), gross energy (model 1241 adiabatic calorimeter, Parr Instruments, Moline, IL) ether extract (AOAC, 1999), NDF, ADF (ANKOM Technology, 2003a; 2003b; ANKOM 200 series fiber analyzer, ANKOM Technology Corp. Macedon, NY), and minerals (Unicam Solaar 969, atomic absorption spectrometer, Cambridge, UK).

Statistical Analyses

Data for weekly BW and blood variables were analyzed statistically as a randomized complete block design for the effect of age (week) and dietary treatment (starter with or without lasalocid) using the mixed models procedures of SAS (version 8.2; SAS Inst., Inc., Cary, NC). As the experimental units, calves ($n = 24$) did not start the project simultaneously, thus calves were blocked on birth date and birth weight in groups of two. The statistical model used was:

$$Y_{ijk} = \mu + \beta_i + D_j + \beta \cdot D_{ij} + W_k + W \cdot D_{jk} + \beta \cdot D \cdot W_{ijk}$$

where:

Table 3.1. Measured chemical composition of feeds¹

Component ²	Milk Replacer	Control	Treatment ³
DM, %	93.31	89.89	90.71
Crude Protein, %	23.00	19.81	19.38
Ether extract, %	17.51	3.17	2.92
NDF, %	0.17	34.45	33.97
ADF, %	0.03	19.03	19.13
Ash, %	11.93	8.36	8.44
Calcium, g/kg	7.17	11.58	10.55
Phosphorus, g/kg	9.81	8.27	7.99
Magnesium, g/kg	1.58	3.53	3.58
Potassium, g/kg	27.66	13.81	13.19
Gross Energy, Mcal/kg	4.91	4.37	4.33

¹Milk replacer was Instant Nursing Formula (Land O'Lakes, Fort Dodge, IA) and calf starters were Calf Primer I = control and Calf Primer I-B = treatment (Tennessee Farmers Cooperative, Laverne, TN).

²Composition on a dry matter basis.

³Treatment calf starter formulated to contained lasalocid supplement at 83 mg/kg DM.

Y_{ijk} = dependent variables,

μ = overall mean,

β_i = effect of block i ,

D_j = effect of diet j ,

$\beta * D_{ij}$ = effect of diet by block (error term for diet),

W_k = effect of week k (age),

$W * D_{jk}$ = effect of week by diet interaction,

$\beta * W * D_{ijk}$ = error term for week and week by diet (residual).

Because the age of a calf across the 16 sampling weeks is a treatment whose order was fixed by definition, a repeated measures model over time was included as a treatment design, with diet as between-subject and week as within-subject factors. Since the dietary treatment did not commence until wk 5, the sampling period of wk 1 – 4 was analyzed separately to ensure that any observed effects were the result of treatment effects and not pre-existing conditions. There were no differences detected in blood variables measured prior to initiation of dietary treatment at wk 5, so results presented include analysis across all 16 weeks.

Similar to factors described above, data for birth (d 0), weaning (d 49), and termination (112 d) weights; pre-weaning, post-weaning, and overall ADG; post-weaning feed efficiency, and DMI were analyzed as a randomized complete block design for effect of dietary treatment. Class variables were diet and block, but there was no week effect or repeated measures treatment design included in the analysis of these data. Consequently, the error term for analysis of these variables was block $\times$ diet.

Death of one calf between 8th and 9th sampling periods would have created an incomplete block and therefore this block was removed from the study. Also, two separate instances where samples were damaged during shipping resulted in additional missing observations and results presented are least square means. Thus, blood variables, weekly BW, and post-weaning measures of ADG, feed efficiency, and termination weights were analyzed using 22 calves. Measures of birth weight, weaning weight, and ADG from birth to weaning were analyzed using all 24 calves.

Due to heterogeneous variance causing abnormal distribution, blood variables propionate, butyrate, and insulin were rank transformed using rank procedure of SAS to improve normality. This resulted in data for these variables being ranked by block and ANOVA performed on ranks for least square mean separation. Least square mean separation was done using least significant difference features in SAS. Probabilities of $P < 0.05$ are discussed as significant throughout unless otherwise noted.

RESULTS

Overall, calves remained healthy throughout the study. Calves typically scoured at some time during the pre-weaning portion of the study and the average duration was 3.5 d (range 1-5 d). Two calves developed pink-eye within their second week in the experiment and were treated accordingly (Gentocin spray and LA-200). One calf died of unknown causes at 8 wk of age. No other health problems were noted following weaning and due to nearly identical weaning weights it is unlikely that any negative health effects from the pre-weaning period compromised the post-weaning treatment comparisons.

Analysis of chemical composition of milk replacer and calf starter supplemented with (treatment; TRT) or without (control; CON) lasalocid is in Table 3.1. All components for all feeds matched the ranges specified by the manufacturer. There were no apparent differences between analyses of CON and TRT calf starters.

Body Weights, Dry Matter Intake, and Efficiency of Gain

All calves had similar birth and weaning BW (Table 3.2) and wk 1 to 4 BW (Figure 3.1). This verifies that no pre-existing differences between calves influenced the measurements taken after dietary treatment was initiated at wk 5. Average daily gain calculated from birth to weaning does include the two-wk adaptation period where calves were receiving milk replacer and CON or TRT calf starter, but did not differ by dietary assignment. Final (16-wk) BW, ADG from weaning to 16-wk, ADG from birth to 16-wk, post-weaning DMI and feed:gain were not significantly different between CON and TRT calf starters (Table 3.2). Weekly body weight measurements progressed with similar trends between CON and TRT calves (Figure 3.1). There was a significant wk effect ($P < 0.01$) and a tendency ($P = 0.10$) for diet effect from wk 5 to 16. Prior to initiation of dietary treatment BW measures overlapped. Initiation of dry feed caused an apparent change in the slope between wk 5 and 7 and the lines diverged slightly and TRT remained slightly lower and progressed parallel with CON to wk-16. Body weight measures for CON became significantly greater than TRT ($P < 0.05$) during wk 14 and 15 (Figure 3.1).

Table 3.2. Means for body weight, dry matter intake, gain, and efficiency of gain for calves fed calf starter with or without lasalocid pre- and post-weaning

Variable ¹	Control	Treatment	SE	$P <^2$
Body weight, kg				
Birth (d 0)	25.06	25.89	0.84	0.31
Wean (d 49)	35.30	34.77	1.27	0.67
Final (d 112)	83.90	81.47	2.71	0.39
ADG, kg/d				
Birth to final (d 0-112)	0.53	0.50	0.02	0.13
Birth to wean (d 0-49)	0.21	0.18	0.02	0.22
Wean to final (d 49-112)	0.78	0.75	0.03	0.47
DMI, kg/d ³	2.35	2.34	0.07	0.92
Feed:Gain ³	3.10	3.19	0.11	0.44

¹Final BW, ADG birth to final, ADG wean to final, DMI, and feed:gain measurements are $n = 11$ per dietary treatment. All other observations are $n = 12$.

²Probability of greater F for the effect of dietary treatment on variable; not significant at $P > 0.10$.

³Calculated from post-weaning period data, thus represents period from wean to finish.

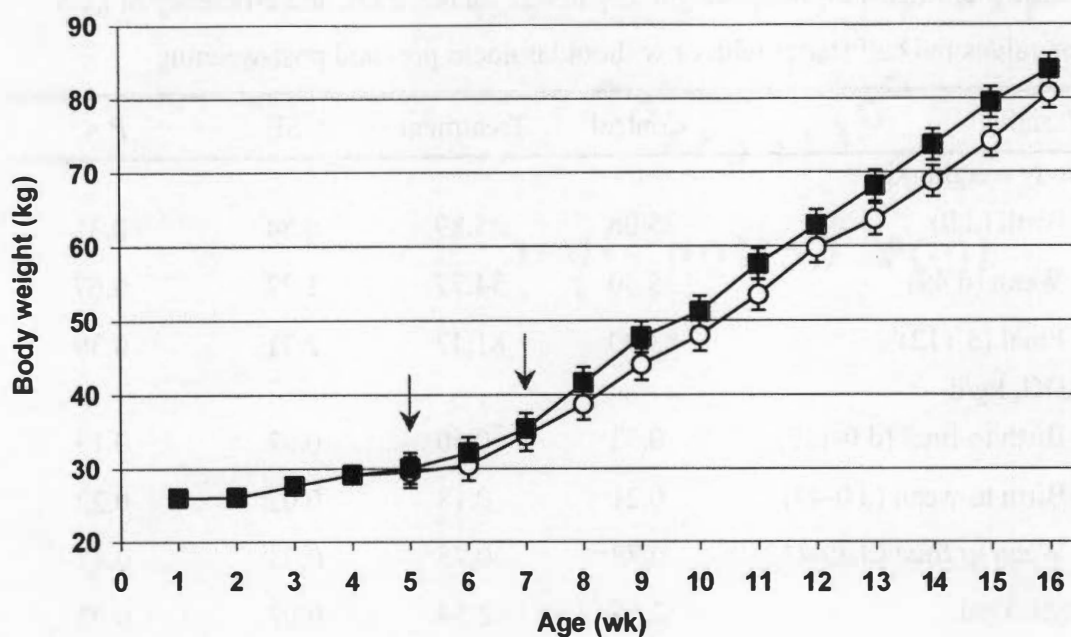


Figure 3.1. Effects of calf starter supplemented with (○) or without lasalocid (■) on weekly body weights. Arrows indicate initiation of starter ration at wk 5 and cessation of milk replacer ration at wk 7. Weeks 1 to 4 are means $\pm$ SE for 22 calves. From wk 5 to 16 effect of diet was significant ($P = 0.10$) and all values are means ($n = 11$ per treatment) $\pm$ SE.

Blood Metabolites

Glucose and non-esterified fatty acids. Concentrations of blood glucose were not significantly affected by treatment, but wk effect was significant (Figure 3.2). Analysis of variance for diet and diet $\times$ week interaction were not significant. There was no post-weaning effect of supplementation on glucose observations. Concentrations for both treatments decreased significantly with age reaching a nadir at wk 7 and 8 (2.5 and 2.7 mmol/L, respectively), which coincided with weaning. Concentrations began to increase and by wk 15 and 16 (3.6 mmol/L) were similar to levels observed at wk 1 (3.7 mmol/L).

Concentrations of plasma NEFA were not significantly affected by post-weaning ionophore supplementation (Figure 3.3). Both diet and week effects were significant ($P < 0.01$), but diet $\times$ week interaction was not significant. While there was no significance detected between CON and TRT at individual wk, there was a large drop between wk 5 and 8 (274.7 and 158.8 $\mu\text{mol/L}$, respectively). Like glucose this nadir coincided with the cessation of the milk replacer rations. Concentrations of NEFA pre-weaning (wk 1-7) were significantly greater than those taken post-weaning (wk 8-16; $P < 0.05$).

Ketones. Concentrations of ACAC were highly variable and although week effect was significant there was no trend observed (Figure 3.4). There were no observed differences between diets or pre- and post-weaning. The concentration of ACAC was static and the expected increase in concentrations beginning at wk 5 to 7 in association with starter intake was not distinguishable.

Circulating concentrations of BHBA were more dynamic than ACAC across the 16-wk experimental period (Figure 3.5). The effect of week was significant, but neither

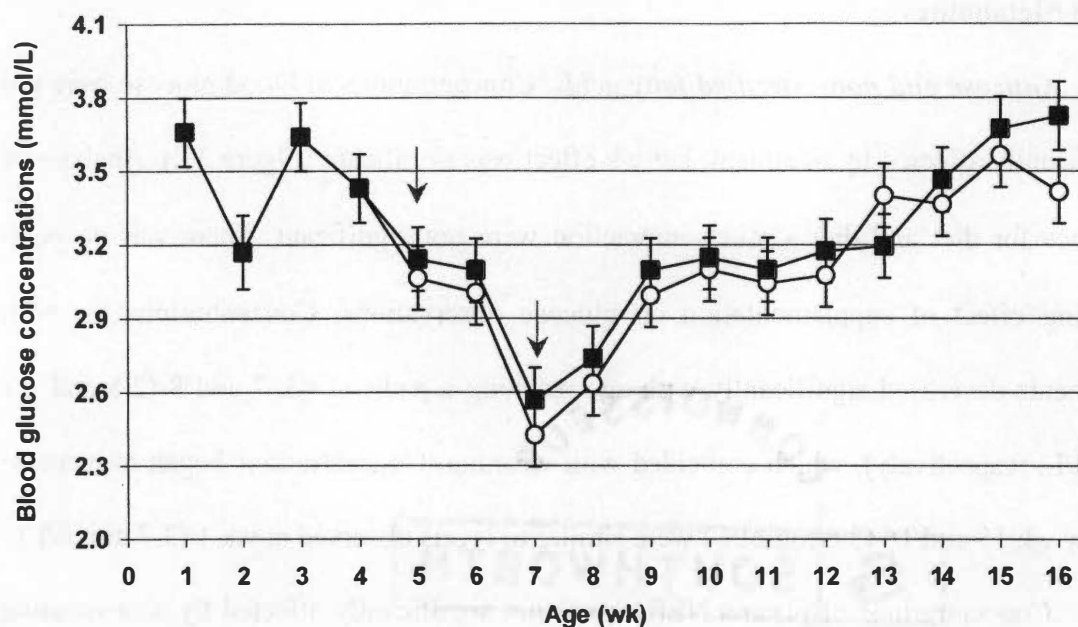


Figure 3.2. Effects of calf starter supplemented with (○) or without lasalocid (■) on weekly glucose concentrations. Arrows indicate initiation of starter ration at wk 5 and cessation of milk replacer ration at wk 7. Weeks 1 to 4 are means $\pm$ SE for 22 calves. From wk 5 to 16 all values are means ($n = 11$ per treatment) $\pm$ SE.

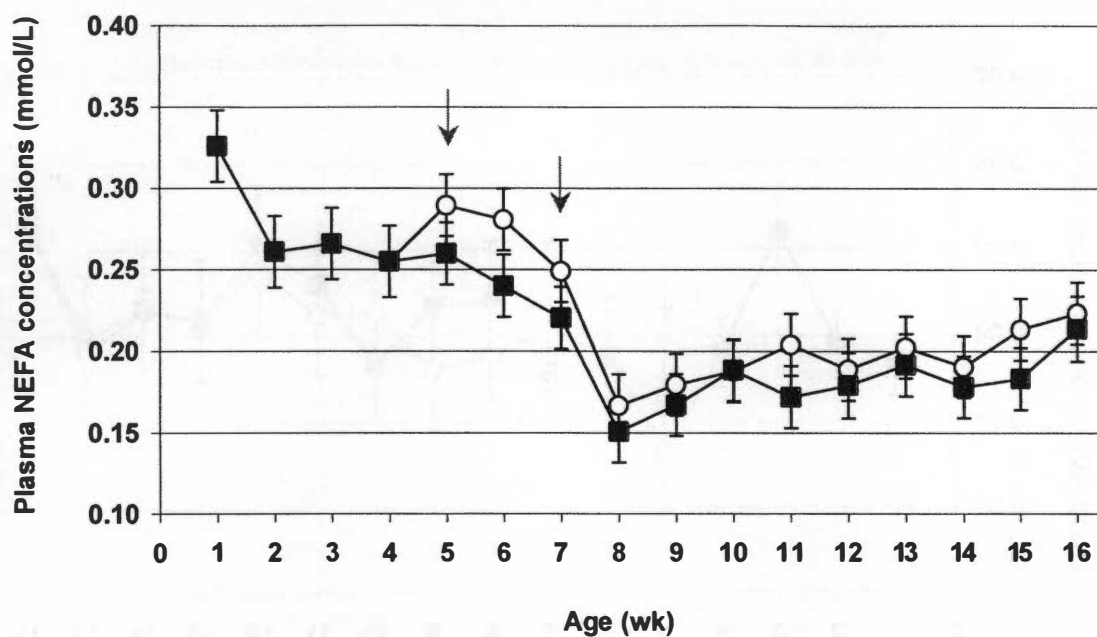


Figure 3.3. Effects of calf starter supplemented with (○) or without lasalocid (■) on weekly non-esterified fatty acid (NEFA) concentrations. Arrows indicate initiation of starter ration at wk 5 and cessation of milk replacer ration at wk 7. Weeks 1 to 4 are means $\pm$ SE for 22 calves. From wk 5 to 16 effect of diet was significant ($P < 0.05$) and all values are means ($n = 11$ per treatment) $\pm$ SE.

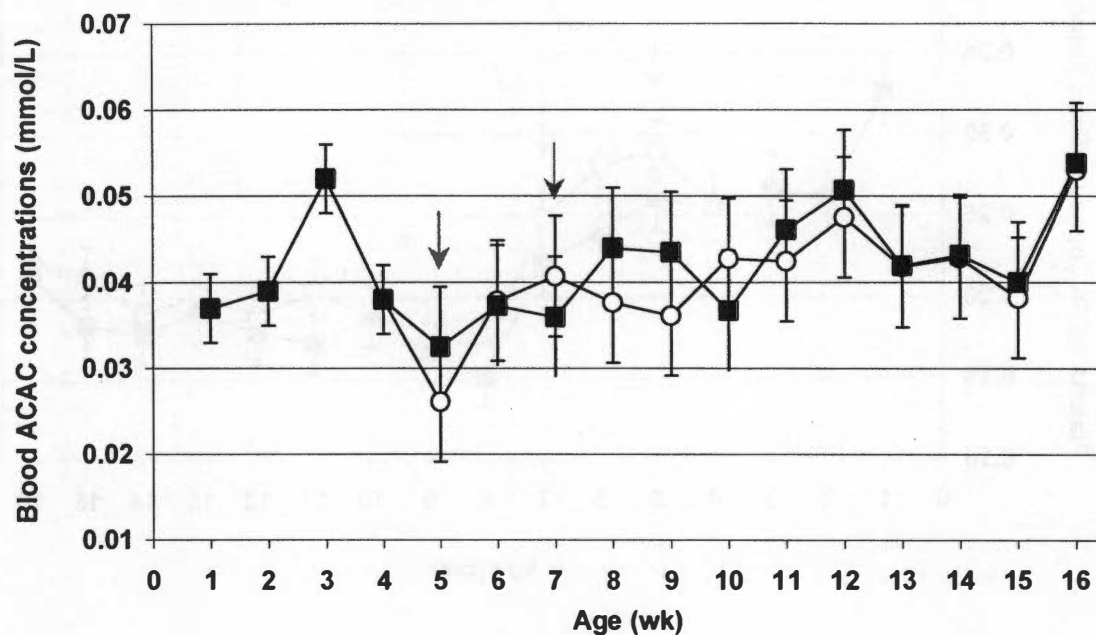


Figure 3.4. Effects of calf starter supplemented with (○) or without lasalocid (■) on weekly acetoacetate (ACAC) concentrations. Arrows indicate initiation of starter ration at wk 5 and cessation of milk replacer ration at wk 7. Weeks 1 to 4 are means $\pm$ SE for 22 calves. From wk 5 to 16 all values are means ($n = 11$ per treatment) $\pm$ SE.

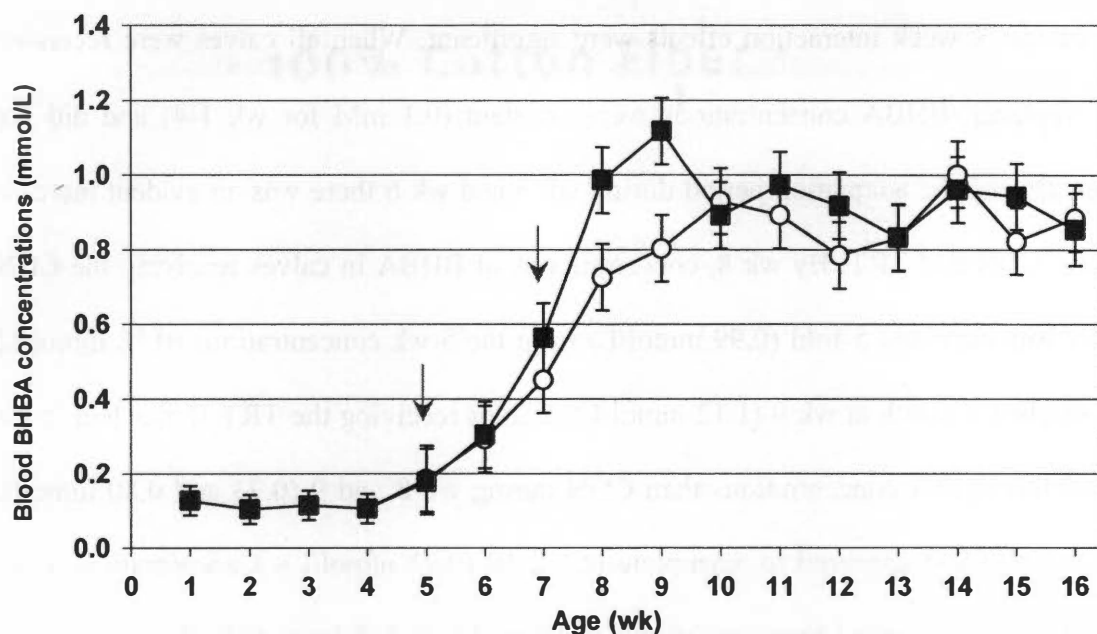


Figure 3.5. Effects of calf starter supplemented with (○) or without lasalocid (■) on weekly β -hydroxybutyrate (BHBA) concentrations. Arrows indicate initiation of starter ration at wk 5 and cessation of milk replacer ration at wk 7. Weeks 1 to 4 are means $\pm$ SE for 22 calves. From wk 5 to 16 all values are means ($n = 11$ per treatment) $\pm$ SE.

diet or diet $\times$ week interaction effects were significant. When all calves were receiving milk replacer, BHBA concentrations were constant (0.1 mM for wk 1-4) and did not differ. During the adaptation period during wk 5 and wk 6 there was an evident increase in both CON and TRT. By wk 8, concentrations of BHBA in calves receiving the CON starter had increased 5-fold (0.99 mmol/L) from the 5-wk concentrations (0.18 mmol/L) and reached a zenith at wk 9 (1.12 mmol/L). Calves receiving the TRT starter had lower ($P < 0.05$) BHBA concentrations than CON during wk 8 and 9 (0.73 and 0.80 mmol/L respectively) and appeared to asymptote at wk 10 (0.93 mmol/L). Concentrations from wk 10 to wk 16 ranged between 0.8 and 1.0 mmol/L and did not differ between wk or diet.

Volatile fatty acids. Plasma concentrations of acetate measured across 16 wk are presented in Figure 3.6. Both effects of diet and week were significant for acetate, but diet $\times$ week interaction was not. Prior to wk 5 concentrations of acetate were stable staying at or below 0.2 mmol/L for all calves. After dry feed intake was initiated (wk 5), an increase similar to BHBA was observed. At weaning (wk 7) acetate concentrations of calves receiving CON starter (0.54 mmol/L) were greater than calves receiving TRT (0.35 mmol/L; $P < 0.10$). Concentrations of acetate (Figure 3.6) continued to increase after weaning in a similar manner, but CON was not significantly greater than TRT until wk 13 ($P < 0.05$). Like BHBA (Figure 3.5), the post-weaning increase in acetate concentrations for TRT group appeared to be delayed and have a lower maximum concentration relative to CON. The highest acetate concentrations were measured at wk 10 (0.80 mmol/L) and 13 (0.93 mmol/L) for TRT and CON, respectively.

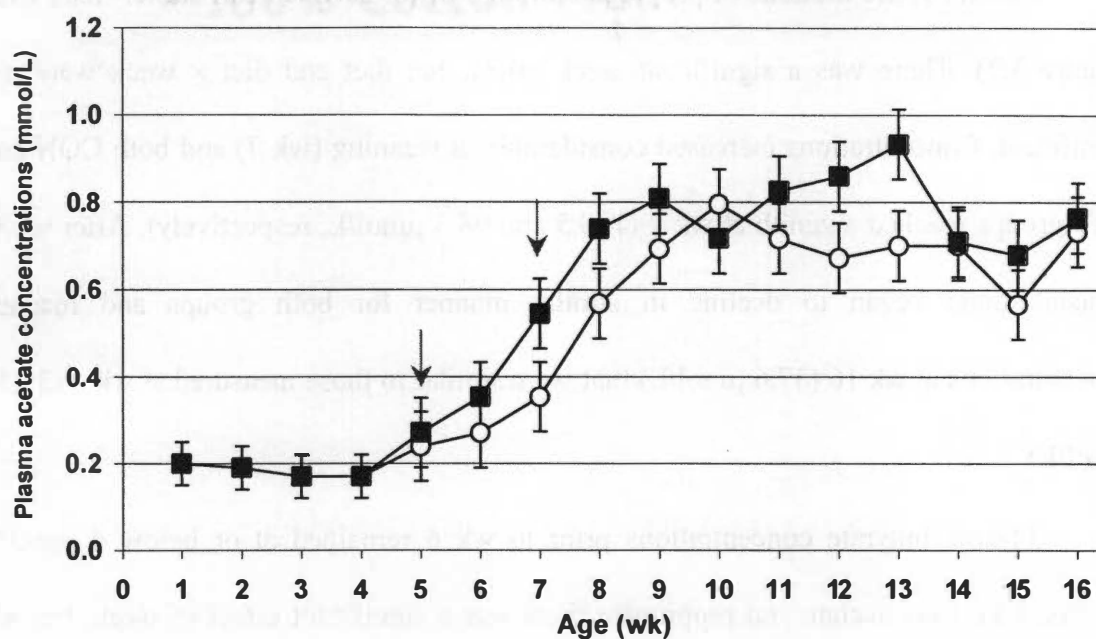


Figure 3.6. Effects of calf starter supplemented with (○) or without lasalocid (■) on weekly acetate concentrations. Arrows indicate initiation of starter ration at wk 5 and cessation of milk replacer ration at wk 7. Weeks 1 to 4 are means $\pm$ SE for 22 calves. From wk 5 to 16 effect of diet was significant ($P < 0.05$) and all values are means ($n = 11$ per treatment) $\pm$ SE.

Plasma concentrations of propionate did not differ between calf starter treatments (Figure 3.7). There was a significant week effect, but diet and diet $\times$ week were not significant. Concentrations increased considerably at weaning (wk 7) and both CON and TRT groups reached a zenith at wk 9 (110.5 and 98.3 $\mu\text{mol/L}$, respectively). After wk 9, concentrations began to decline in similar manner for both groups and reached concentrations at wk 16 (37.6 $\mu\text{mol/L}$) that were similar to those measured at wk 5 (37.59 $\mu\text{mol/L}$).

Plasma butyrate concentrations prior to wk 6 remained at or below 4 $\mu\text{mol/L}$ (Figure 3.8). Like acetate and propionate there was a significant effect of week, but not for diet or diet $\times$ week. During the adaptation period, between wk 5 and wk 7, there was a five-fold increase in butyrate for CON (1.6 to 11.2 $\mu\text{mol/L}$), but only a two-fold increase in TRT (2.6 to 6.0 $\mu\text{mol/L}$). Butyrate concentrations at wk 7 were greater in CON than TRT ($P = 0.09$). Week 7 butyrate concentrations in TRT were decreased from those measured at wk 6 and may be responsible for the observed difference. Maximum plasma butyrate for the CON group was measured at wk 9 (17.3 $\mu\text{mol/L}$), while TRT concentrations peaked at wk 10 (14.0 $\mu\text{mol/L}$). Similar to plasma propionate, butyrate concentrations began a decline after each acme and continued in parallel to wk 16.

Lactate and pyruvate. Concentrations of blood lactate and pyruvate had a significant week effect, but diet effects and diet $\times$ week interactions were not significant for either metabolite (Figures 3.9 and 3.10, respectively). The effect of weaning generated no visible response in either metabolite. Lactate and pyruvate concentrations

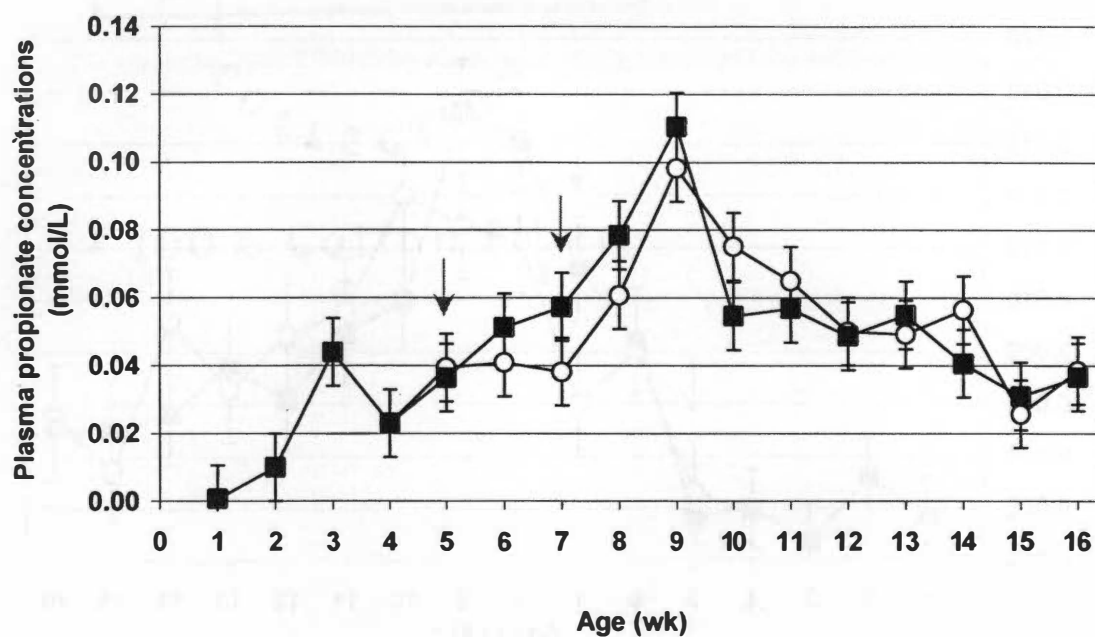


Figure 3.7. Effects of calf starter supplemented with (○) or without lasalocid (■) on weekly propionate concentrations. Arrows indicate initiation of starter ration at wk 5 and cessation of milk replacer ration at wk 7. Weeks 1 to 4 are means $\pm$ SE for 22 calves. From wk 5 to 16 all values are means ($n = 11$ per treatment) $\pm$ SE.

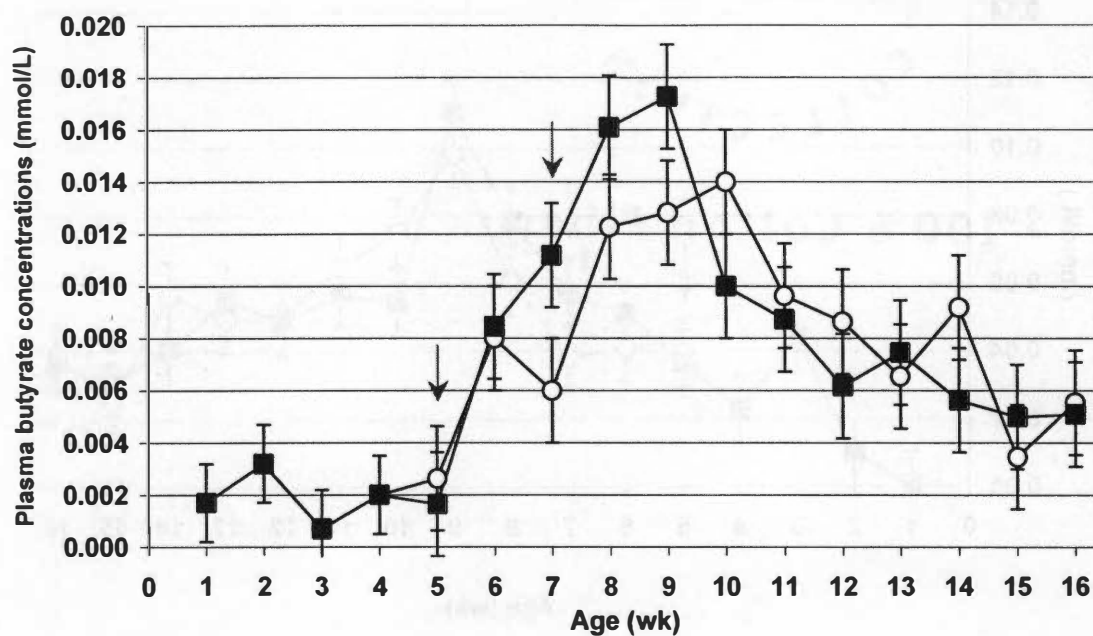


Figure 3.8. Effects of calf starter supplemented with (○) or without lasalocid (■) on weekly butyrate concentrations. Arrows indicate initiation of starter ration at wk 5 and cessation of milk replacer ration at wk 7. Weeks 1 to 4 are means $\pm$ SE for 22 calves. From wk 5 to 16 all values are means ($n = 11$ per treatment) $\pm$ SE.

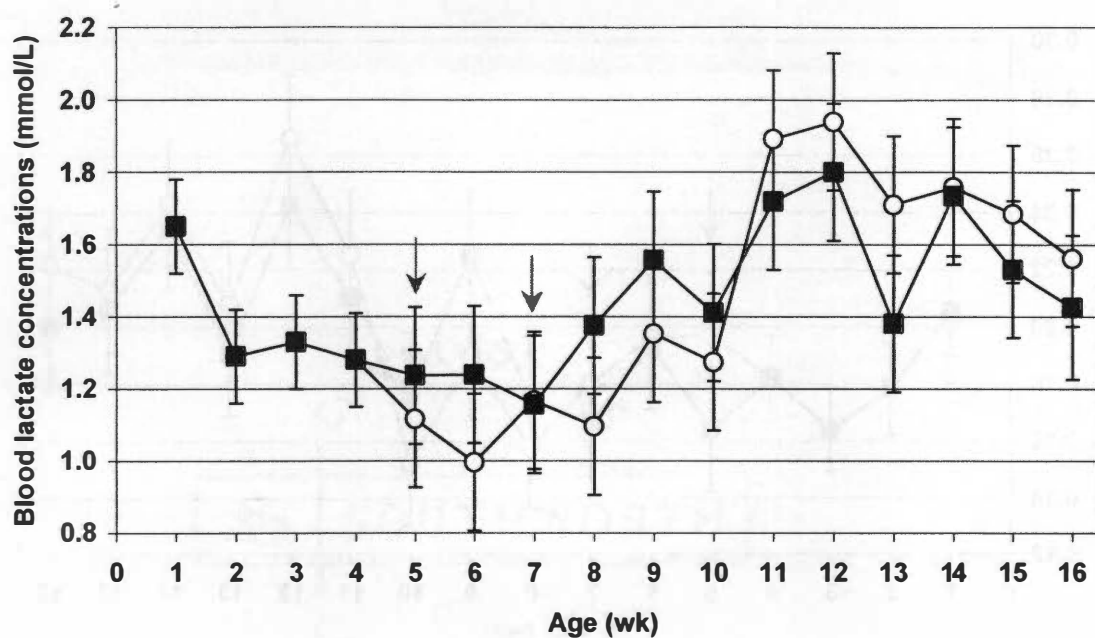


Figure 3.9. Effects of calf starter supplemented with (○) or without lasalocid (■) on weekly lactate concentrations. Arrows indicate initiation of starter ration at wk 5 and cessation of milk replacer ration at wk 7. Weeks 1 to 4 are means $\pm$ SE for 22 calves. From wk 5 to 16 all values are means ($n = 11$ per treatment) $\pm$ SE.

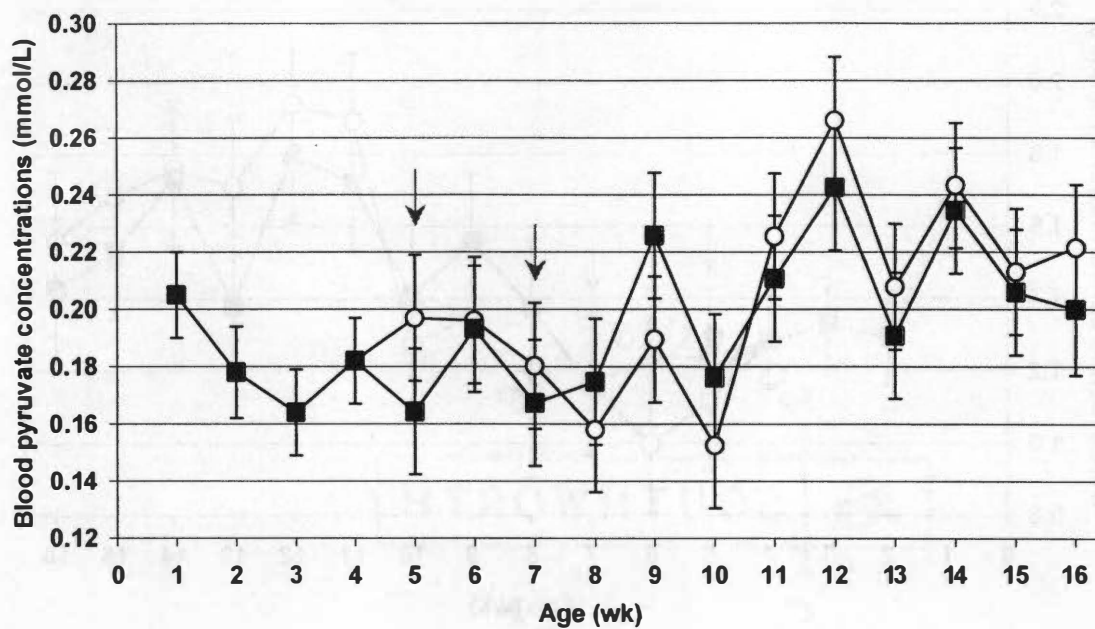


Figure 3.10. Effects of calf starter supplemented with (○) or without lasalocid (■) on weekly pyruvate concentrations. Arrows indicate initiation of starter ration at wk 5 and cessation of milk replacer ration at wk 7. Weeks 1 to 4 are means $\pm$ SE for 22 calves. From wk 5 to 16 all values are means ($n = 11$ per treatment) $\pm$ SE.

were highest during wk 12 (1.87 and 0.25 mmol/L, respectively) and lactate concentrations were generally six to eight-fold greater than pyruvate across the 16 wk period. Lactate concentrations appeared to remain elevated from wk 12 to wk 16, a period when propionate concentrations were declining.

Insulin and glucagon. Plasma concentrations of insulin were variable across all 16 wk of the experiment (Figure 3.11). The CON group had dynamic changes in pre-weaning concentrations when there was no dietary treatment. The TRT group had an obvious decline from wk 1 (22.8 pmol/L) to a nadir at wk 7 (1.4 pmol/L). The concentrations of TRT insulin increased after wk 7 and reached a maximum at wk 16 (37.0 pmol/L).

Measured plasma concentrations of glucagon were less variable than insulin and exhibited a response to the dietary regime change at weaning (Figure 3.12). There was a significant week effect, but diet and diet $\times$ week were not significant. Concentrations measured in both CON and TRT had similar changes across the 16 wk period. Glucagon was at the lowest concentration at wk 5 (13.6 pmol/L) and then sharply increased to a maximum concentration for wk 8, 9, and 10 (25.6, 26.3, and 24.5 pmol/L, respectively). Prior to wk 5, the ratio of insulin and glucagon was approximately one to one, but glucagon became the greater circulating peripheral hormone post-weaning and the ratio was below one.

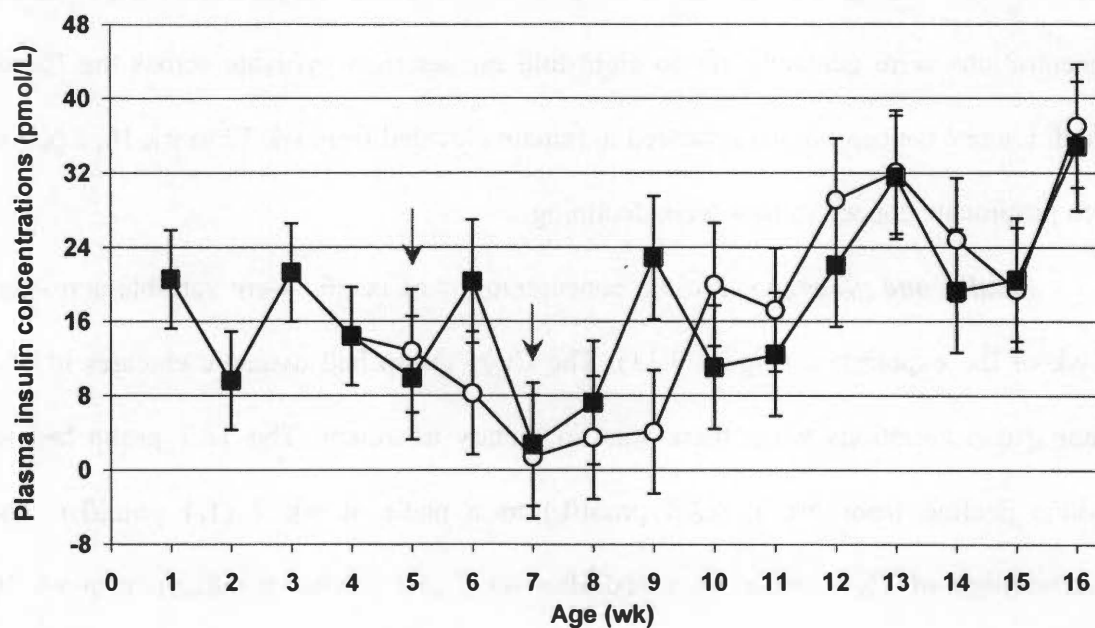


Figure 3.11. Effects of calf starter supplemented with (○) or without lasalocid (■) on weekly insulin concentrations. Arrows indicate initiation of starter ration at wk 5 and cessation of milk replacer ration at wk 7. Weeks 1 to 4 are means $\pm$ SE for 22 calves. From wk 5 to 16 all values are means ($n = 11$ per treatment) $\pm$ SE.

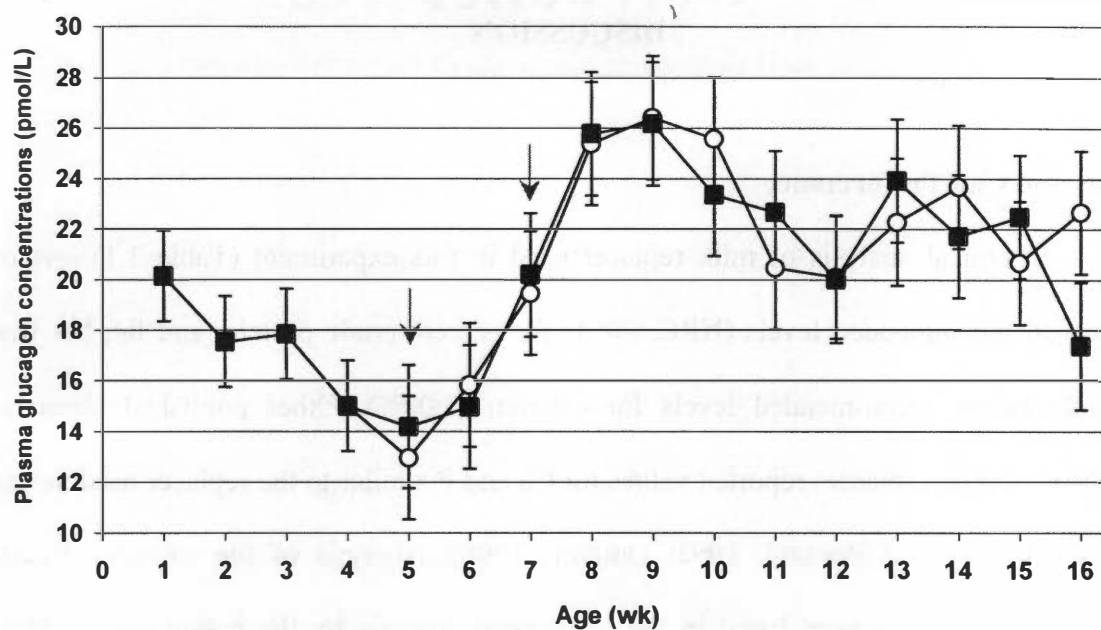


Figure 3.12. Effects of calf starter supplemented with (○) or without lasalocid (■) on weekly glucagon concentrations. Arrows indicate initiation of starter ration at wk 5 and cessation of milk replacer ration at wk 7. Weeks 1 to 4 are means $\pm$ SE for 22 calves. From wk 5 to 16 all values are means ($n = 11$ per treatment) $\pm$ SE.

DISCUSSION

Diets and Calf Performance

Chemical analysis of milk replacer used in this experiment (Table 3.1) met or exceeded recommended levels (NRC, 2001) for percent crude protein, and fat, but was slightly below recommended levels for calcium (1.00%). Other published chemical analysis of milk replacers reported values for Ca and P similar to the replacer used in this study (Quigley and Bernard, 1992; Quigley, 1996). Analysis of the current replacer corresponded with ranges listed in the guaranteed analysis by the manufacturer. Milk replacer quality was not a concern, as protein content consisted of various forms of milk protein (whey) and measured crude fiber was below 0.2% (Davis and Drackley, 1998).

Chemical analysis of both calf starters (Table 3.1) met or exceeded recommended levels (NRC, 2001) and both starters were similar in components that corresponded with the ranges listed in the guaranteed analysis of the manufacturer. Gross energy values for both starters were comparable to the 4.49 Mcal/kg reported by the NRC (2001). The NDF and ADF values for starter were substantially greater than the recommended 12.8% and 11.6%, respectively (NRC, 2001), but still fell within the acceptable range (ADF 6-20% and NDF 15-25%; Davis and Drackley, 1998). The starters used in this experiment were complete feeds containing high-fiber byproducts designed to stimulate ruminal development and to be fed as the sole feed.

Previous performance studies summarized by Chalupa (1980) demonstrated that lasalocid and monensin produced a small reduction of intake, an increase in ADG, and improved feed efficiency (intake/gain) compared to controls. These observations,

however, were made primarily from feedlot cattle. Using Holstein calves, Anderson et al. (1988) reported that calves fed lasalocid from 4-d of age to 12-wk of age had a greater feed intake and weight gain after 5-wk of age. Conversely, Eicher-Pruett et al. (1992) reported an increase in daily gain with lasalocid supplementation to be greatest during the first six weeks. Quigley et al. (1992) and Steen et al. (1992) observed no effects of lasalocid administration on BW, ADG, or feed efficiency in Holstein bull calves and Holstein heifers, respectively. This agrees with the results presented in Table 3.2, generated from twice the number of calves. Birth weights and weaning weights (Table 3.2) were similar to other studies (25 and 34 kg) using Jersey calves (Quigley, 1996; Stanley et al., 2002).

The above inconsistencies in reported effects of lasalocid supplementation on gain and intake is likely a result of differences in supplementation dosage, supplementation vehicle, and experimental timelines. All three studies (Anderson et al., 1988, Eicher-Pruett et al., 1992; Quigley et al., 1992) supplemented in liquid and solid diets, but Quigley et al. (1992) also supplemented via rumen cannula. Weaning occurred at 3 wk of age ($n = 22$), when total dry feed reach 1.3% of birth weight ($n = 40$), and at 8 wk of age ($n = 12$), respectively. Dosage of lasalocid could be another effect causing the findings to differ. Eicher-Pruett et al. (1992) concluded that ionophore concentrations greater than 1.0 mg/kg of BW were the most effective and should be delivered in all three feeds (milk replacer, pre-starter, and starter). Quigley et al. (1992) only included lasalocid in milk replacer and calf starter and used 1.0 mg/kg of BW (not greater than 1.0 mg/kg as suggested by Eicher-Pruett and co-workers; did not use a pre-starter). In the current study, lasalocid was supplemented only in the calf starter and the dosage was dependent

on intake of the animal (75 mg/kg as-fed or 83 mg/kg of DM). Concentrations supplemented in this study, were close to the amount supplemented by Anderson et al. (1988) in pre-starter (88 mg/kg as fed) and starter (44 mg/kg as fed). However, Anderson et al. (1988) reported that there were periods where calves were consuming two feed types, both supplemented with ionophore likely generating a larger combined daily dosage. Eicher-Pruiett et al. (1992) also reported that lasalocid supplemented only in pre-starter and starter combined and starter alone did not perform as well as controls or calves receiving lasalocid in all three diet types and related it to palatability. This observation agrees with results of the current study, where TRT calves were always slightly lower than CON in weekly BW measures (Figure 3.1), although not always significantly and DMI was the same.

Blood Metabolites

Preruminant blood glucose concentrations (5.3 mmol/L; lambs; Leat, 1970) are higher than those of mature ruminants (2.7 mmol/L; sheep; Bergman, 1971), but decrease to adult values *circa* 3 months of age. This fall appears to be independent of ruminal development or diet changes and is probably caused by alimentary hypoglycemia associated with absence of milk and loss of glucose from erythrocytes. Pre-weaning blood glucose concentrations in the present study were low by these standards. While the lowest concentrations approximated 2.5 mmol/L, the pre-weaning concentrations ranged from 3.3 – 3.8 and did not equate with other reports.

Greenwood et al. (2002) reported glucose concentrations between 5.0 and 7.0 mmol/L for two-wk old lambs. Quigley et al. (1991a) reported glucose concentrations

between 6.3 and 5.8 mmol/L for calves one and two weeks old and a nadir of 4.2 mmol/L at 9 weeks. Stanley et al. (2002) reported blood glucose concentrations of 4.7 and 4.4 mmol/L at 1 and 2 wk of age for Jersey calves and 5.1 and 4.8 mmol/L for Holstein calves. At 16 wk of age, reported concentrations of glucose in Holstein calves were between 4.5 and 5.0 mmol/L (Quigley and Bernard, 1992; Quigley et al., 1991a). Quigley and Bernard (1992) sampled calves zero hours after feeding, similar to the peri-prandial sampling of the current study, and glucose concentrations were slightly lower than other post-prandial measures in the experiment. These values for zero-hr glucose were presented graphically and exhibit a similar shaped curve, to that of the present study (Figure 3.2) with a dip in concentrations around weaning and a gradual increase to wk 16. Lower than normal glucose concentrations in the current study appear to be constant across all 16 wk and can probably be attributed to the time of sampling in relation to feeding and the 24-hr shipment period that transpired prior to analysis. This decrease in glucose concentrations appeared constant for all measures and suggests that detection of significant differences related to the effects of age and diet should be unaffected. Consequently, the fact that no dietary effect was observed on blood glucose is presumably not a Type II error.

Propionate is a potent glucogenic compound and is the only VFA that is a glucose precursor (Bergman, 1971; 1990). Thus, as a calf undergoes ruminal maturation, dwindling concentrations of dietary glucose are replaced by glucogenic compounds like propionate. Propionate concentrations presented in Figure 3.7 resemble an inverted version of the glucose concentrations in Figure 3.2. Glucose concentrations had reached the low point of the 16-wk study at wk 7 and at this time propionate concentrations were

increasing and peaked shortly thereafter. There were no treatment differences detected in circulating propionate concentrations in this study or by Quigley et al. (1992). Anderson et al. (1988) reported small, but significant increases in ruminal propionate with lasalocid supplementation. This however, was the ruminal concentration. The rumen epithelium metabolizes propionate into lactate, pyruvate, carbon dioxide, and amino acids prior to release into portal circulation (Bergman, 1990). The liver then removes most propionate not extracted by the ruminal epithelium of ruminating sheep or calves (Bergman and Wolff, 1971; Weigand et al., 1972) and preruminant calves (Donkin and Armentano, 1995) leaving little to enter peripheral circulation. This probably contributed to the low concentrations (< 0.12 mmol/L) measured throughout this study and in other reports (Quigley et al., 1992; and Quigley and Bernard, 1992). The sudden increase in plasma propionate concentration in Figure 3.7 coincides exactly with weaning and is comparable to other reports in calves (Quigley et al., 1991b).

As propionate is absorbed from the rumen a varying percentage is converted to lactate and pyruvate (Weigand et al., 1972; Weekes and Webster, 1975). In the preruminant lamb or calf, the ability to metabolize lactate to pyruvate is significantly greater than that in post-weanling lambs and calves and adult sheep (Ballard and Oliver, 1965; Savan et al., 1986; Donkin and Armentano, 1995). Circulating concentrations of lactate (Figure 3.9) or pyruvate (Figure 3.10) do not appear to be affected by developing rumen function (Bartley et al., 1966; Quigley and Bernard, 1992) or ionophore. Concentrations of lactate at 3 - 4 wk of age in this study (1.3 mmol/L) agree with concentrations in the vena cava (1.0 - 1.3 mmol/L) measured in calves of the same age by Ortigues et al. (1996). Lactate concentrations across 16- wk of age in calves differed

slightly from results presented by Quigley and Bernard (1992). They reported a gradual decline (wk 1 = 2.7 to wk 16 = 1.3 mmol/L) in lactate concentrations as calves aged, but Figure 3.9 shows a lower initial concentration than Quigley and Bernard and then a slight increase post-weaning. The apparent increase of lactate and pyruvate after wk 10 appeared to coincide with a decline in propionate concentrations occurring during the same time period. A possible explanation would be the substantially reduced rate of hepatic gluconeogenesis from lactate in the ruminant calf (Donkin and Armentano, 1995) due to decreased lactate dehydrogenase and pyruvate carboxylase activity (Table 2.1).

Circulating concentrations of NEFA are negatively correlated with intake of solid feed by the preruminant calf (Quigley, 1996). This is presumably in response to the shift in nutrient composition from high fat and low carbohydrate to a diet low in fat and high in carbohydrate. Similar to glucose concentrations, NEFA declined with age. Measured plasma NEFA concentrations in this experiment (Figure 3.3) agree with other reports of pre- (Stanley et al., 2002; Ortigues et al., 1996; Quigley and Bernard, 1992) and post-weaning concentrations (Quigley and Bernard, 1992; Leat, 1970). In contrast to the current study, Quigley et al. (1992) reported significantly lower post-weaning concentrations of NEFA in calves receiving lasalocid in milk replacer and rumen cannula than control calves (0.088, 0.101, and 0.133 mmol/L, respectively). These values and the reported pre-weaning values were consistently lower than pre- and post-weaning values reported in this and other experiments.

Acetate derived from rumen fermentation is largely unused by hepatic parenchyma, making acetate the largest source of dietary energy available to extrasplanchnic tissues (Armentano, 1992; Bergman, 1990). Thus, peripheral measures of

circulating acetate relate more closely to intake than propionate or butyrate. Acetate constituted over 90% of VFA measured during the study and the patterns exhibited in Figure 3.6 are similar to those reported by Quigley et al. (1991b). The significantly greater acetate concentrations at wk 7 and wk 13 in CON calves suggest that lasalocid may have had an effect by mitigating the rate that VFA concentrations increased in TRT calves. This is an observation that could have easily been lost for butyrate or propionate due to their extensive metabolism and removal by rumen epithelium and liver.

Butyrate concentrations in Figure 3.8, however, did show a delayed response similar to acetate with CON calves having greater concentrations than TRT when weaned at 7 wk of age. Due to extensive rumen epithelial metabolism of butyrate to ketones, changes in plasma butyrate following weaning more closely resembled those described for propionate. Butyrate concentrations reported by Quigley et al. (1991b) were greater than those presented in Figure 3.8 and did not decline post-weaning. However, using data from the same study, but published separately, Quigley et al. (1991a) reported BHBA concentrations in late-weaned calves (which were on a comparable time line as this study) approximately 200 $\mu\text{mol/L}$ lower than those presented in Figure 3.5. Butyrate has been estimated to be the source of 80 – 95% of β -hydroxybutyrate released by the rumen (Leng and West, 1969).

β -Hydroxybutyrate is the reduced form of acetoacetate and is the main circulating ketone (Bergman, 1971). Measured concentrations of BHBA in this study correspond to that published for preruminants (Varnam et al., 1978b; Quigley et al., 1991a) and were below 0.2 mmol/L prior to initiation of dry feed intake. After wk 5 there was an abrupt increase in BHBA concentrations for both diet groups. Ruminant concentrations of

BHBA at 8 and 9 wk of age were significantly higher in CON than TRT (Figure 3.5) and agreed with the significantly greater concentration of BHBA reported by Quigley et al. (1992) in control calves compared to those receiving lasalocid. The effect lasalocid had on BHBA concentrations over the 16-wk period resembled the same patterns described for butyrate and acetate, with the ionophore mitigating the sharp increase in metabolites resulting from the onset of rumen fermentation. Quigley et al. (1992) also reported lower post-weaning ruminal butyrate concentrations in calves receiving lasalocid supplement over controls. This may explain the observed effect in the reduced circulating BHBA concentrations in ionophore supplemented calves. Conversely, Krehbiel et al. (1992) demonstrated that an increasing ruminal butyrate concentration was associated with an increase in portal-drained viscera flux of ACAC, but not BHBA. This is likely due to the mitochondrial location of BHBA dehydrogenase in rumen epithelium and its saturation at high levels of butyrate (Leighton et al., 1983).

Pre-weaning ACAC concentrations (Figure 3.4) were similar to those described by Quigley et al. (1991a), but slightly lower than those reported in lambs by Varnam et al. (1978b) and in calves by Quigley et al. (1992). There was a lack of response to weaning and the increase in alimentary ketogenesis of ACAC was not apparent at wk 7. Acetoacetate concentrations actually appeared to stay the same in contrast to the reported postweaning increase to 0.025 to 0.05 mmol/L (Quigley et al, 1992; Quigley et al., 1991a; Varnam et al., 1978b). Like the glucose concentrations, the absence of an expected increase in ACAC concentrations post-weaning may have been a result of the 24-hr shipping period prior to analysis. Although the samples were kept cold during

shipping, enough time transpired for spontaneous decarboxylation to acetone to occur in quantities sufficient to affect sample quality for this metabolite (Bergman, 1971).

Ruminants generally seem more insulin resistant compared to non-ruminants (Brockman, 1986; Weekes, 1991) and the role of glucagon is less clear in ruminants. The action of glucagon is opposite that of insulin and stimulates gluconeogenesis. The ratio of insulin to glucagon may be more important in stimulating gluconeogenesis than the individual concentrations of insulin and glucagon. The adult ruminant liver is glucogenic instead of glycogenic and is less sensitive to acute changes in insulin concentration (Fahey and Berger, 1993). Hepatocytes from ruminating calves were less sensitive to glucagon and lost all sensitivity to insulin during the transition from preruminant to ruminant (Donkin and Armentano, 1995).

Insulin measurements in the present study were variable and difficult to interpret (Figure 3.11) but, glucagon concentrations increased at weaning (Figure 3.12). Stanley et al. (2002) reported post-weaning glucagon concentrations in 8 wk old Jersey calves of 24.96 pmol/L that agree with findings of this experiment. This increase of glucagon to a maximum at 8 – 10 wk of age, coincides with the nadir of glucose, the decrease in NEFA (indicating no hepatic oxidation of lipid reserves), and the peak in propionate and butyrate concentrations. This reflects the calves' overall shift towards a dependency on gluconeogenesis as it develops into a functioning ruminant.

CONCLUSIONS

Measurements of blood metabolites in young calves demonstrated changes characteristic of rumen development and metabolism. Declines in glucose and non-esterified fatty acids along with rapid increases in blood volatile fatty acids and ketones with dry feed intake occurred as expected. Feeding calf starter supplemented with the ionophore lasalocid at levels used to prevent coccidiosis in calves during pre-weaning adaptation period and post-weaning delayed maximum acetate and butyrate concentrations and concomitantly lowered peak β -hydroxybutyrate concentrations. Consumption of starter containing lasalocid had no detectable improvement on the performance characteristics of average daily gain or feed efficiency and seemed to result in slightly lower weekly body weights. Thus, feeding a calf starter supplemented with lasalocid for coccidiosis does not appear to have any advantageous effects on post-weaning growth or efficiency, but may have a effect on metabolic transition at weaning. This may facilitate the adaptation from a preruminant to a ruminant metabolism by reducing the potential for energy loss through sudden influx of volatile fatty acids and concomitant ketones.

PART IV

NET NUTRIENT FLUX DURING PRE- AND POST-WEANING GROWTH IN DAIRY CALVES

ABSTRACT

Dairy calf weaning is associated with increases in ketone levels that exceed measured rates of utilization in adults and present a potential energy loss that may be mitigated by ionophore. To assess effects of weaning and ionophore on net portal-drained viscera (PDV), hepatic (HEP), and total splanchnic tissue (TSP) fluxes in dairy calves, glucose, acetoacetate (ACAC), BHBA, NEFA, VFA, lactate, pyruvate and insulin (INS), and glucagon (GLN) concentrations and PDV, HEP, and TSP fluxes were determined on d 35, 56, 84, and 112 in Jersey bull calves weaned at d 49. From d 3 - 34 all calves (n = 19) were fed 454 g/d milk replacer. After sampling on d 35, calves were randomly assigned to a commercial pelleted starter without (CON) or with lasalocid (TRT; 83 mg/kg DM) *ad libitum* plus replacer until d 48. From d 49-112 calves received only CON or TRT *ad libitum*. Calves were equipped with catheters in artery (A), portal (P), and mesenteric veins (V) at wk 3 to 4. Calf number varied by treatment and sample day due to catheter patency (calves = 3 to 5) and limited interpretations on HEP and TSP net fluxes. Blood flow was measured by continual infusion of para-aminohippurate into V (1.5% at 0.764 mL/min) and 6 serial samples taken at 30-min intervals simultaneously from A and P. Portal blood flow increased ($P < 0.05$) with age, but did not differ between CON and TRT. Glucose was released pre-weaning and extracted post-weaning by PDV, but not affected by ionophore. Conversely, pre-weaning uptake of NEFA changed to post-weaning release, but this change was delayed to d 84 in TRT. Flux of ACAC and BHBA in CON and TRT went from pre-weaning uptake to post-weaning PDV release that peaked at d 84, but peak release of ACAC was lower and BHBA tended to be lower

in TRT ($P = 0.07, 0.15$). Portal release of VFA increased with age, but was similar to ketones with both butyrate and propionate PDV release lower at d 84 in TRT ($P < 0.1$). Importantly, glucagon was greater in CON than TRT at d 84 ($P < 0.05$). Significant changes in metabolic profile and net PDV flux of transition calves were demonstrated and ionophore appears to moderate alimentary output at a post-weaning period (d 84) where ketone concentrations have potential to exceed whole animal capacity for utilization.

INTRODUCTION

A defining characteristic of adult rumen epithelial tissue is the production of ketones and in the fed animal it is the primary ketogenic tissue (Heitmann et al., 1987). The ketogenic capacity of rumen epithelium is minimal from birth until weaning (Baldwin and Jesse, 1992), as such the liver is the primary source of ketone production. At weaning the rumen undergoes profound morphological and metabolic changes. Although the mechanisms are not entirely understood, a strong relationship exists between dry matter intake and ketogenic potential of the ruminal epithelium (Baldwin and Jesse, 1992; Quigley et al., 1991a). The oxidation of butyrate to carbon dioxide and ketones by the rumen epithelium of adults is concentration dependent, but seems to be saturable when very high (5.0 mM) concentrations are encountered *in vitro* (Baldwin and McLeod, 2000). Peripheral tissue uptake and utilization of acetoacetate (ACAC) and β -hydroxybutyrate (BHBA) measured *in vivo* are concentration dependent until concentrations greater than 2.0 mM are reached (Bergman, 1971; Leng, 1965).

Homeorhesis at weaning is not well understood and the potential for a metabolic lag from imbalance of opposing mechanisms of production and utilization could exist.

The ruminant liver is the mediator between supply of portal nutrients and that which is available to peripheral tissues. In adults, hepatic ketogenesis is constant and the liver is refractory to acute insulin and glucagon inputs (Donkin et al., 1997). The preruminant liver is glycolytic, and gluconeogenesis is highly regulated (Leat, 1970). The hepatic capacity for gluconeogenesis from propionate appears to be similar in preruminant and ruminant calves (Donkin and Armentano, 1995) and should not be a constraint on early weaning. Ortigues et al. (1996), using multicatheterized preruminant calves, demonstrated a 20% maximum contribution of lactate to hepatic gluconeogenesis. This is attenuated in the ruminant calf (Donkin and Armentano, 1995). Also, hepatic and hindquarter metabolism of glucose was constant and did not appear to be subject to any circadian rhythm (Ortigues et al., 1996).

Many of the available reports concerning shifts in calf metabolism at weaning are single-site, venous samples. The portal release of metabolites may substantially increase, but a proportionate hepatic extraction could result in only slight changes in the overall circulating concentrations (Heitmann and Fernandez, 1986). Thus, many of the actual changes occurring in relation to ruminal development would be less evident or concealed in measures taken from a single venous sample. There is an absence of information regarding interorgan metabolic dynamics of splanchnic tissues in calves around the time of weaning. Therefore, the objective of this study was to determine the effects of weaning and post-weaning supplementation of lasalocid on the net portal-drained viscera (PDV),

hepatic, and total splanchnic flux of energy and alternate nutrients and insulin and glucagon.

MATERIALS AND METHODS

Animals and Management

Calves and housing. All procedures were conducted under protocols that were approved by the University of Tennessee Animal Care and Use Committee (protocol # 1004). Nineteen Jersey bull calves were obtained from the University of Tennessee Knoxville and Dairy Experiment Stations (Lewisburg, TN). Calves were left on-site until three days post-partum for adequate intake of colostrum and castration and were then transported to the Joseph E. Johnson Animal Research and Teaching Unit (JARTU) at the Knoxville Experiment Station. Calves were housed in individual pens (4 x 8 ft.) on rubber mat flooring for duration of the experiment. Pens were cleaned twice daily (am and pm) and prior to cleaning calves were moved to clean pens on the opposite side of the room. This permitted daily exercise for the animal and prevented exposure to the cleaning process. The temperature of the room was set at 21.0°C ($\pm$ 3.0°C) and artificial lighting was provided from 0600 to 1800 h.

Diets and feeding. Calves had *ad libitum* access to fresh water and trace-mineralized salt (Champions Choice; Cargill Inc., Minneapolis, MN) for the duration of the study. Starting at 3 d of age, all calves received 227 g commercial milk replacer brought to an approximate volume of 2 L with H₂O (12% solids; w/v; Instant Nursing Formula NT Medicated, Land O'Lakes Animal Milk Products Co., Fort Dodge, IA) twice

daily at 0800 and 1630 hours from nipple bottles. At 21 d of age the milk replacer ration was increased to 284 g per 2 L H₂O (15% solids; w/v). Milk replacer ration was withheld the morning of surgery and the calf was offered PM ration upon successful recovery. The first of four flux studies was conducted at 5 weeks of age (d 35) and upon completion a commercially prepared high fiber calf starter (CO-OP Calf Primer-I and Calf Primer-I-B, Tennessee Farmers Cooperative, Lavergne, TN) was offered for *ad libitum* consumption. At this time, the calf was still receiving milk replacer twice daily, but ration was reduced to 227 g/2 L. Starting at week six (d 42), only the AM replacer ration was offered and at week seven (d 49) the calves were weaned. Calves often acquired scours at least once during their time on the study. Calves with scours were treated with an commercial oral electrolyte solution (Revitilyte, Vets Plus, Inc., Knapp, WI) that was fed between the AM and PM milk replacer feedings (1200). No surgeries or flux studies were performed on calves with scours, but were rescheduled when animals recovered.

Calf starter diets were commercially prepared and pelleted without (CON) or with lasalocid (TRT) supplement (83 mg/kg DM). Calves were sequentially assigned to either CON (n = 12) or TRT (n = 7) starter at birth. Starter was fed in a single ration at 0800. When a calf consumed an entire previous days ration, the ration was increased by 105% of previous days consumption. Feed rations and refusals were recorded daily.

Surgery

Pre-operative procedures and anesthesia. A pre-surgical subcutaneous dose of Penicillin Procaine-G (U.S. VET, Hanford Pharmaceuticals, Syracuse, NY) was administered 4 hr before surgery. Because the calves were receiving only milk replacer at

the time of the surgery, only the AM ration the day of surgery was withheld. Calves were fasted at least 16 hours prior to surgery to prevent emesis and other gastrointestinal disturbances. Water and trace mineralized salt blocks were removed during this period. Prior to surgery calves were initially anesthetized with halothane-oxygen mixture (5% halothane at 2.0 L/min O₂) using a facemask. Immediately after loss of mandible tension and swallowing reflex, calves were intubated with an endotracheal tube (9.5 mm i.d.; Mallinckrodt Inc., St. Louis, MO) through which surgical level anesthesia was introduced and maintained (at 2% halothane at 2.0 L/min O₂). Lack of pedal reflex, eye dilation and position, and finally, lack of response to scalpel were used as determinants of surgical level of anesthesia.

Surgical procedures. Between weeks 3 and 4 post-partum calves were surgically equipped with chronic, indwelling-catheters equipped with silastic cuffs (See Appendix 1 for complete description of catheters) in the mesenteric, portal, and hepatic veins and mesenteric artery using techniques first developed by E. N. Bergman (Katz and Bergman, 1969b) and modified by Zanzalari et al., 1989.

Calves were placed in left lateral recumbency and the area from the sixth lumbar vertebra to the 12th thoracic vertebra and from the lumbar vertebra shelf to the linea alba was clipped, shaved, scrubbed with iodine, and rinsed with alcohol. A 30-cm celiotomy 3-cm caudal and paracostal to the 13th rib was made beginning 3 cm below the 2nd lumbar vertebra. The skin and subcutaneous, external oblique, transverse abdominal and internal oblique muscles were dissected individually. Prior to catheter implantation the remnant of the umbilical vein was ligated. Major branches of portal and hepatic veins were located by palpation on the caudal surface of the left lateral lobe and accessed via puncture

incision through the parenchyma and into the veins. Catheters were introduced into both the portal and hepatic veins. Portal catheter location was confirmed by palpation of the porta-hepatis at the epiploic foramen (foramen of Winslow). Hepatic catheter location was confirmed by noting the movement of an intentionally placed air bubble within the catheter in response to respiration and heart rate. Upon removal of air bubble the catheters were secured by triple ligation directly to the hepatic parenchyma just caudal to each cuff with #3.0 cardiovascular non-degradable silk (Ethicon, Inc. Somerville, NJ).

The omentum was pulled cranially, exposing a segment of the small intestine and accessible branches of the common mesenteric vein and artery were identified (See Appendix 2 for description of artery catheter). The artery and vein were elevated and cleaned of fascia. During this procedure a topical application of lidocaine was used to prevent shriveling of the vein. Both vessels were ligated caudal to the intended site of insertion with #0 non-degradable silk and a temporary stasis was applied 3 cm cranial to the ligation by gently elevating the vessel with #0 silk. While elevated, a small incision was made in both vessels between the stasis and ligation. Catheters were introduced through the incision and past the stasis until the silastic cuffs were inside the vessels. The vessels were triple ligated at positions cranial, medial, and just caudal to the cuffs with #0 non-degradable silk.

Hepatic, portal, and mesenteric catheters were exteriorized in the dorsolumbar region via a sterile skin needle and inserted into a denim pouch that was attached between the 1st and 3rd lumbar vertebrae with tag cement. Catheters were flushed and filled with sodium heparin (1000 USP units/mL; Elkins-Sinn, Cherry Hill, NJ). Individual muscle layers were closed using a continuous Ford interlocking suture of #1.0 chromic gut

(Ethicon, Somerville, NJ) and skin was closed with an interrupted horizontal everted mattress suture of #0 silk.

Post-operative care. Following surgery and removal of the endotracheal tube, the calf was placed on its sternum in a padded recovery room and closely supervised until it was able to stand without assistance. A second dose of penicillin was administered following surgery and at 12-hour intervals for 4 days thereafter. Calves were also treated with flunixin meglumine (Banamine; Schering-Plough Animal Health Corp., Union, NJ), a potent analgesic, following surgery and the following day to minimize discomfort. All external suture lines were treated daily with liberal application of an antimicrobial paste. Body temperature was recorded for all animals, twice daily post-surgery until they completed the experiment.

Measurements

Blood sampling. Net flux studies of metabolites across portal-drained viscera, liver, total splanchnic tissues and hindquarters were conducted at 5, 8, 12, and 16 weeks post-partum (2 weeks pre-weaning and 1, 5, and 9 weeks post-weaning). Care was taken to keep the animals calm during sampling and took place in the normal pen and accustomed environment of the calf. The animal had normal access to feed and water relative to the particular sample period during sampling. On experiment days the calf was weighed and sampling began one hour post-prandial. Prior to sampling, a 23 mL pulse-dose (one half of one hours infused amount) of para-aminohippuric acid (PAH) was infused into the mesenteric vein catheter. Following the pulse-dose, PAH was infused for one hr (1.5% w/v, at 0.764 mL/min). The infusion of PAH was done with a KD Scientific

infusion pump (model 200; New Hope, PA) and a 50-mL glass syringe (Becton Dickinson, Franklin Lakes, NJ). Following the one-hour equilibration period, six serial 15-mL blood samples were taken simultaneously from the mesenteric artery and portal and hepatic veins at 30-minute intervals. Infusion of PAH was continuous until blood sampling was completed. Samples were collected in 20-mL syringes that contained 400 μ L of Na₂EDTA (6%). Because the normal anticoagulant used to maintain catheter patency is known to activate lipoprotein lipase and generate inaccurate measure of NEFA, catheters contents were replaced with only physiological saline between samples. Whole blood samples were kept on ice until analyzed, and samples for ketones, lactate, pyruvate, glucose, and PAH were deproteinized (see chemical analyses below) on site prior to transport to the laboratory.

Body weights and feed samples. Prior to feeding, calves were weighed weekly for the entirety of the study. Body weight measures were also recorded at birth, weaning, termination, and each blood sample day. All diets in this study were commercially prepared and were obtained in a single shipment with the same lot number. Feeds (milk replacer and calf starters) were sampled monthly and composited for proximate analysis.

Chemical Analyses

Blood samples. Analysis for arterial packed-cell volume was done as samples were collected using a micro-hematocrit centrifuge and microcapillary reader (International Equipment Co., Needham Heights, MA). Whole blood samples were analyzed for concentrations of PAH, glucose, L-(+)-lactate, pyruvate, ACAC, and BHBA. Spectrophotometric analyses for PAH, glucose, ACAC, and BHBA were

performed same day as sampling. Deproteinized lactate and pyruvate samples were centrifuged ($1600 \times g$ at 4°C) and supernatants were collected and frozen (-20°C) for analysis within two weeks. Plasma was harvested from the remaining blood sample following centrifugation at $1600 \times g$ at 4°C then frozen (-20°C) and stored for later analysis of NEFA, VFA, glucagon, and insulin.

Para-aminohippuric acid (Appendix 10) was measured as described in Kaufman and Bergman (1971). Analyses for BHBA and ACAC (Appendices 4 and 5) were done using enzymatic assays of Williamson and Mellanby (1974) and Mellanby and Williamson (1974). L-(+)-lactate and pyruvate (Appendices 7 and 8) were determined using procedures modified from the Sigma kits for lactate and pyruvate (#726-UV and #826-UV, Sigma Chemical Co., St. Louis, MO). Analysis of NEFA (Appendix 9) utilized the WAKO NEFA-C kit (WAKO Chemical USA, Dallas, TX). Blood glucose (Appendix 6) was determined using the Sigma glucose kit (#510; Sigma Chemical Co., St. Louis, MO). Plasma glucagon and insulin were determined using the double antibody radioimmunoassay and the Coat-A-Count[®] insulin kits and protocols from Diagnostics Products Corporation (Los Angeles, CA). For the hormones, intra-assay errors greater than 10% were not accepted and samples were rerun. Plasma VFA analysis (Appendix 11) used the procedure described by Reynolds et al. (1986) and modified by Quigley et al. (1991b) for capillary column, and Benson et al., (2002) with a Hewlett Packard gas chromatograph (5890A), auto-sampler (7637A), integrator (3393A), and a J&W Scientific 530 μm HP-FFAP capillary column (Agilent Technologies Inc., Palo Alto, CA).

Feed samples. Composite dietary samples of CON and TRT starter were ground through a 1-mm screen in a Wiley Mill (Author H. Thomas Co., Philadelphia, PA) and analyzed (Table 4.1) with composite milk replacer sample for DM, N (model FP-2000 nitrogen analyzer, LECO, St. Joseph, MI), GE (model 1241 adiabatic calorimeter, Parr Instruments, Moline, IL) ether extract (AOAC, 1999), NDF and ADF (ANKOM Technology, 2003a; 2003b; ANKOM 200 series fiber analyzer, ANKOM Technology Corp. Macedon, NY), and minerals (Unicam Solaar 969, atomic absorption spectrometer, Cambridge, UK).

Calculations

Equations used to determine blood flow, net flux rates, and extraction ratios have been published in full by Heitmann and Fernandez (1986) and Heitmann (1989) and are detailed in Appendix 2. Blood flows were determined using the indicator dilution method via PAH infusion. Blood flow in portal and hepatic veins were measured directly and hepatic artery flow was determined by taking the difference in portal and hepatic measurements. Plasma flow was calculated by multiplying the whole blood flows by the measured plasma percent. Net fluxes across the PDV, liver, and total splanchnic tissues were calculated as a product of the venous-arterial concentration difference multiplied by the respective blood or plasma flow. Total splanchnic flux is simply the sum of the PDV and hepatic fluxes, but was calculated separately. Values reported are net fluxes and represent a combination of metabolite uptake and release. These values are not unidirectional flux rates and cannot differentiate absorption, interconversion and release

Table 4.1. Measured chemical composition of feeds^a

Component ^b	Milk Replacer	Control	Treatment ^c
DM, %	93.77	91.01	90.98
Crude Protein, %	22.69	19.85	19.09
Ether extract, %	19.55	2.87	2.89
NDF, %	0.39	33.18	34.45
ADF, %	0.14	17.71	18.67
Ash, %	12.46	7.75	7.75
Calcium, g/kg	7.04	8.82	8.54
Phosphorus, g/kg	9.95	7.93	7.51
Magnesium, g/kg	1.62	3.66	3.64
Potassium, g/kg	28.21	12.89	12.80
Gross Energy, Mcal/kg	4.96	4.34	4.33

^aMilk replacer was Instant Nursing Formula (Land O'Lakes, Fort Dodge, IA) and calf starters were Calf Primer I = control and Calf Primer I-B = treatment (Tennessee Farmers Cooperative, Lavergne, TN).

^bComposition on a dry matter basis.

^cTreatment calf starter formulated to contained lasalocid supplement at 83 mg/kg DM.

of a metabolite from *de novo* production and release of a metabolite. A positive net flux indicates net release or production of a metabolite and a negative net flux indicates net uptake or tissue utilization of a metabolite by the specific tissue (Heitmann, 1989).

Statistical Analyses

This experiment was originally designed as a randomized complete block that blocked calves in groups of two by birth date and weight and incorporated age at sampling as a repeated measures treatment design. Difficulties associated with catheter implantation, maintenance of catheter patency, and calf mortality limited the ability to obtain samples from arterial, portal and hepatic catheters at each of the four sampling periods for both control and treatment calves within a block. This resulted in a considerable number of incomplete blocks or variety of different combinations of catheters sampled between calves within a block. Also, sparse data across the four sampling ages resulted in non-convergence of the maximum likelihood solution for covariance parameter estimates when week was used as a repeated measure. Thus, block effect was dropped from the model and age at sampling (week) was used as a fixed factor instead of a repeated measure. Data for total splanchnic and hepatic flux was analyzed as described below, but number of experimental units for these determinations is likely too low to safely make any conclusions without committing Type II error.

All statistical analyses were conducted using mixed model procedures of SAS (version 8.2; SAS Inst., Inc., Cary, NC). Data for blood variables, rates of metabolism (net flux), and weekly body weights were analyzed as a completely randomized design. Data obtained from each sampling site were analyzed separately. Analyses of variance

for measured variables were performed with effects of diet (starter with or without lasalocid), age (week), and diet $\times$ age in the model. The statistical model used was:

$$Y_{ijk} = \mu + D_i + W_j + D*W_{ij} + R(D*W)_{ijk}$$

where:

Y_{ijk} = dependent variable,

μ = overall mean,

D_i = effect of diet i ,

W_j = effect of week j (age at sampling),

$D*W_{ij}$ = effect of diet by week interaction,

$R(D*W)_{ijk}$ = effect of calf (replicate) k nested within diet by week (residual error).

Individual means presented are averages of the six serial blood samples (number of observations) taken and averaged across all replicates (n = # of calves) for each metabolite for each week. Because of the unbalanced number of observations, all data are presented as least square means $\pm$ SE. Significant treatment interactions were separated using least significant difference features in SAS. Effects were considered significant at $P \leq 0.05$ and are discussed as having a tendency to be significant at $P \leq 0.10$.

Similar to factors described above, data for birth (d 0), d 35, weaning (d 49), d 56, d 84, and term (112 d) weights; pre-weaning, post-weaning, and overall ADG; post-weaning feed:gain efficiency, and DMI were analyzed as a completely randomized design for effect of dietary treatment. Class variable was diet, but there was no week

effect included in the analysis of these data. Consequently, the error term for analysis of these variables was calf nested within dietary treatment.

RESULTS

Due to problems with calf mortality and catheter patency, calf numbers vary between measured and calculated values. Included in the study are only data from calves that were healthy and consuming a consistent daily ration. Data presented in the results and corresponding discussion will pertain primarily to portal-drained viscera (PDV) data. Data for net flux of metabolites across hepatic (HEP) and total splanchnic (TSP) tissue beds will be presented, but discussed in less detail due the low number of experimental units limiting statistical interpretation of the data. When a negative net flux, or uptake of a metabolite occurred, the extraction ratio for that metabolite was calculated, but due the sparsity of the data set these calculations could not be analyzed and are not included.

Analysis of chemical composition of milk replacer and calf starter supplemented with (treatment; TRT) or without (control; CON) lasalocid is in Table 4.1. All components for all feeds matched the ranges specified by the manufacturer and did not vary between calf starters. The same feeds were used in this experiment that were used in the previous experiment and agreed with results presented for diets in Chapter 3.

Body Weights, Dry Matter Intake, and Efficiency of Gain

All calves had similar birth, weaning, day 35 BW (Table 4.2) and wk 1 -5 BW (Figure 4.1). This suggests that no pre-existing differences between calves influenced the

Table 4.2. Effects of age and calf starter with (treatment) or without (control) lasalocid on body weights, dry matter intake, gain, and efficiency of gain for calves fed pre- and post-weaning.

Variable ¹	Control ²	SEM	Treatment ³	SEM	<i>P</i> < ⁴
Body weight, kg					
Birth (d 0)	26.88	1.06	26.38	1.44	NS
Day 35	32.20	0.83	31.55	1.08	NS
Wean (d 49)	36.55	0.97	36.34	1.27	NS
Day 56	40.10	1.29	39.07	1.69	NS
Day 84	61.19	2.19	58.24	2.87	NS
Final (d 112)	81.48	3.43	78.85	4.49	NS
ADG, kg/d					
Birth to final (d 0-112)	0.48	0.03	0.47	0.04	NS
Birth to wean (d 0-49)	0.19	0.02	0.20	0.03	NS
Wean to final (d 49-112)	0.71	0.04	0.67	0.06	NS
DMI, kg/d ⁵	2.07	0.14	1.83	0.19	NS
Feed:Gain ⁵	2.94	0.11	2.74	0.15	NS

¹Measures for variables birth and d 35 weights occurred prior to initiation of control and treatment starter diets on d 35 and represent pre-weaning period of milk replacer diet.

²Control means and SE calculated using *n* = 11 for birth weight, ADG (birth to final), and ADG (birth to wean) and *n* = 12 for all remaining variables.

³Treatment means and SE calculated using *n* = 6 for birth weight, ADG (birth to final), and ADG (birth to wean) and *n* = 7 for all remaining variables.

⁴Probability of greater *F* for the effect of dietary treatment on variable; not significant (NS) at *P* > 0.10.

⁵Calculated from post-weaning period data only, thus represents period from wean to finish.

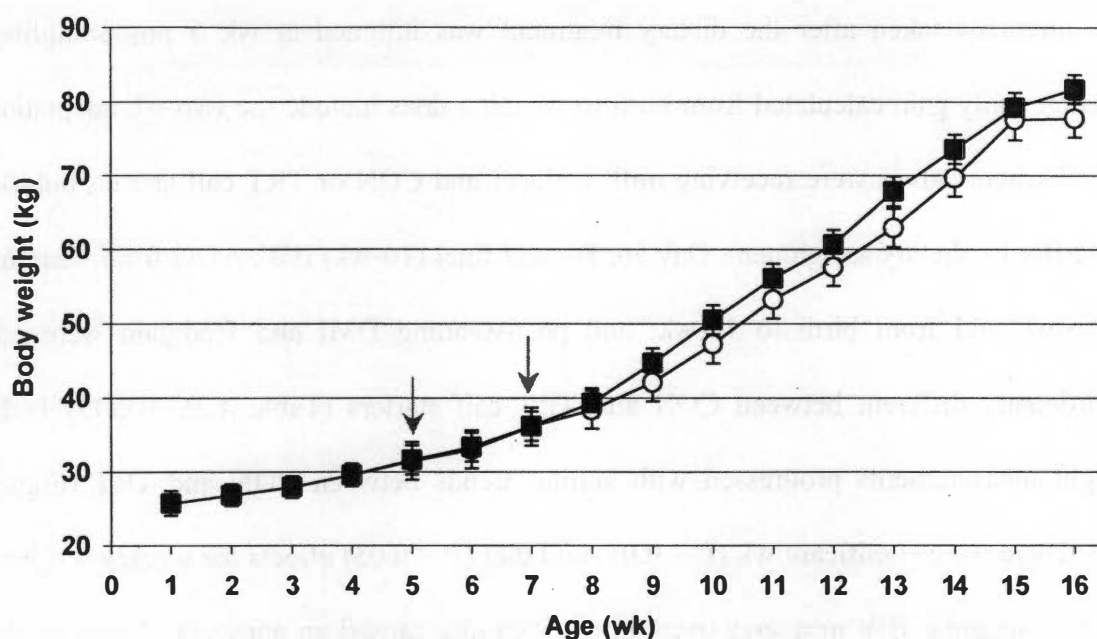


Figure 4.1. Effects of calf starter supplemented with (○; $n = 7$) or without lasalocid (■; $n = 12$) on weekly body weights. Arrows indicate initiation of starter ration at wk 5 and cessation of milk replacer ration at wk 7. All values are means $\pm$ SE and from wk 5 to 16 effect of diet was significant ($P < 0.05$).

measurements taken after the dietary treatment was initiated at wk 5 post-sampling. Average daily gain calculated from birth to weaning does include the two-wk adaptation period where calves were receiving milk replacer and CON or TRT calf starter, but did not differ by dietary assignment. Day 56, 84, and final (16-wk) BW; ADG from weaning to 16-wk and from birth to 16-wk; and post-weaning DMI and feed:gain were not significantly different between CON and TRT calf starters (Table 4.2). Weekly body weight measurements progressed with similar trends between CON and TRT (Figure 4.1). There were significant wk ($P < 0.01$) and diet ($P < 0.05$) effects for weekly weights. Prior to weaning BW measures overlapped. Weaning caused an apparent change in the slope after wk 7 and the lines diverged slightly and TRT remained slightly lower and progressed parallel with CON to wk-16. However, body weight measures did not significantly differ between CON and TRT ($P < 0.05$) at any point during the experiment (Figure 4.1).

Blood Flow and Packed-Cell Volume

Means and standard errors for portal and hepatic veins and hepatic artery are summarized in Table 4.3. Plasma percent values for flow rates and packed-cell volumes can be found in Appendix 3 (Table A3.1). Arterial packed-cell volume differed between CON and TRT prior to initiation of dietary treatment, but not after weaning. There is no apparent physiological or experimental explanation for this difference. Post-weaning packed-cell volume increased across Periods 2, 3, and 4.

Blood flow through the portal vein increased with age ($P < 0.01$) and almost doubled between sampling Periods 1 and 2 and continued to increase through Period 4.

Table 4.3. Means and standard errors for blood flow (L/h) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Blood flow and dietary assignment	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Portal vein, L/h ²					0.49	< 0.01	0.62
Control	145.5 ^d ± 19.9	223.3 ^c ± 22.2	377.4 ^{ab} ± 22.2	416.8 ^a ± 25.7			
Treatment	117.3 ^d ± 22.2	236.3 ^c ± 19.9	342.6 ^b ± 22.2	422.9 ^a ± 25.7			
Hepatic vein, L/h ³					0.02	< 0.01	0.02
Control	229.8 ^{cd} ± 37.3	265.7 ^{cd} ± 37.3	336.3 ^{bc} ± 52.7	— —			
Treatment	177.3 ^d ± 37.3	393.2 ^b ± 37.3	499.9 ^a ± 37.3	— —			
Hepatic artery, L/h ⁴					0.13	0.22	0.11
Control	76.2 ^{ab} ± 56.5	58.2 ^b ± 56.5	— —	— —			
Treatment	72.4 ^b ± 39.9	213.9 ^a ± 39.9	106.2 ± 39.9	— —			

^{a,b,c,d}Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

²Control starter: Period 1 (n = 5), Periods 2 and 3 (n = 4), and Period 4 (n = 3). Treatment starter: Periods 1 and 3 (n = 4), Period 2 (n = 5), and Period 4 (n = 3).

³Control starter: Periods 1 and 2 (n = 2) and Period 3 (n = 1). Treatment starter: Periods 1 through 3 (n = 2).

⁴Control starter: Periods 1 and 2 (n = 1). Treatment starter: Periods 1 through 3 (n = 2).

There was no effect of ionophore supplementation on portal vein blood flow. Like the portal vein, blood flow through the hepatic vein in TRT calves was significantly affected by age ($P < 0.01$) and demonstrated a marked increase in flow (L/hr) between Periods 1 and 2. Rates of blood flow presented in Table 4.4 were adjusted by body weight (L/hr per kg). Only the pre- and post-weaning (Period 1 and Period 2, respectively) portal blood flow remained different ($P < 0.05$). The post-weaning blood flow rates no longer were significantly different and suggests that at weaning there is an increase in blood flow to gut tissues that stabilizes post-weaning, perhaps due to rapid growth in the rumen and other splanchnic tissues.

Arterial Concentrations

Concentrations of blood and plasma metabolites and hormones measured from hepatic and portal veins are presented in Appendix 3 (Tables A3.2 – A3.4 and A3.5 – A3.7, respectively). All arterial concentrations of blood and plasma metabolites and hormones were significantly affected by age (Tables 4.5, 4.6, and 4.7; respectively). There were no significant differences between CON and TRT arterial concentrations during the pre-weaning measurements of Period 1 when all calves were receiving milk replacer. With the exception of glucose, NEFA, and insulin, the arterial concentrations of ketones, lactate, VFA, and glucagon all increased from Period 1 to Period 2 ($P < 0.05$). Arterial concentrations for glucose and NEFA (and for TRT calves insulin) decreased from Period 1 to Period 2 ($P < 0.05$). Glucose (Table 4.5) was lowest 1-wk post-weaning (Period 2) for CON and TRT and concentrations for both CON and TRT increased ($P < 0.05$) to those measured in Periods 3 and 4. Arterial concentrations of NEFA (Table 4.6)

Table 4.4. Means and standard errors for blood flow adjusted for body weight (L/h per kg) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Blood flow and dietary assignment	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Portal vein, L/h per kg ²					0.41	< 0.01	0.68
Control	4.3 ^{bc} ± 0.3	5.2 ^{ab} ± 0.3	5.8 ^a ± 0.4	5.2 ^{ab} ± 0.4			
Treatment	3.7 ^c ± 0.3	5.4 ^a ± 0.3	5.5 ^a ± 0.3	5.0 ^{ab} ± 0.4			
Hepatic vein, L/h per kg ³					0.05	0.27	0.07
Control	7.1 ^{ab} ± 0.9	6.3 ^b ± 0.9	5.4 ^b ± 1.3	— —			
Treatment	6.2 ^b ± 0.9	9.7 ^a ± 0.9	7.9 ^{ab} ± 0.9	— —			
Hepatic artery, L/h per kg ⁴					0.12	0.5	0.15
Control	2.4 ^{ab} ± 1.5	1.4 ^b ± 1.5	— —	— —			
Treatment	2.6 ^{ab} ± 1.1	5.3 ^a ± 1.1	1.6 ± 1.1	— —			

^{a,b,c,d}Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

²Control starter: Period 1 (n = 5), Periods 2 and 3 (n = 4), and Period 4 (n = 3). Treatment starter: Periods 1 and 3 (n = 4), Period 2 (n = 5), and Period 4 (n = 3).

³Control starter: Periods 1 and 2 (n = 2) and Period 3 (n = 1). Treatment starter: Periods 1 through 3 (n = 2).

⁴Control starter: Periods 1 and 2 (n = 1). Treatment starter: Period 1 through 3 (n = 2).

Table 4.5. Means and standard errors for arterial concentration of metabolites (mmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Glucose					0.80	< 0.001	0.41
Control	4.10 ^a ± 0.07	2.82 ^c ± 0.08	3.31 ^b ± 0.07	3.48 ^b ± 0.10			
Treatment	4.18 ^a ± 0.10	2.71 ^c ± 0.08	3.41 ^b ± 0.09	3.34 ^b ± 0.11			
Acetoacetate					0.002	< 0.001	0.12
Control	0.019 ^e ± 0.002	0.052 ^a ± 0.002	0.040 ^b ± 0.002	0.032 ^{cd} ± 0.003			
Treatment	0.014 ^e ± 0.003	0.038 ^{bc} ± 0.002	0.038 ^{bcd} ± 0.003	0.029 ^d ± 0.003			
β-hydroxybutyrate					0.19	< 0.001	0.66
Control	0.084 ^c ± 0.082	1.451 ^{ab} ± 0.088	1.523 ^a ± 0.082	1.236 ^b ± 0.108			
Treatment	0.089 ^c ± 0.097	1.238 ^b ± 0.088	1.436 ^{ab} ± 0.097	1.178 ^b ± 0.125			
Lactate					0.21	< 0.001	0.001
Control	0.55 ^{cd} ± 0.08	1.19 ^a ± 0.08	0.91 ^b ± 0.08	0.79 ^{bc} ± 0.10			
Treatment	0.47 ^d ± 0.10	0.77 ^{bc} ± 0.08	1.17 ^a ± 0.09	0.71 ^{bcd} ± 0.12			
Pyruvate					< 0.001	< 0.001	0.03
Control	0.17 ^b ± 0.01	0.23 ^a ± 0.01	0.23 ^a ± 0.01	0.23 ^a ± 0.02			
Treatment	0.15 ^b ± 0.02	0.15 ^b ± 0.01	0.17 ^b ± 0.02	0.23 ^a ± 0.02			

^{a,b,c,d,e}Means within row and dietary between treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

²Control starter: Periods 1 and 3 ($n = 7$), Period 2 ($n = 6$), and Period 4 ($n = 4$). Treatment starter: Period 1 glucose and lactate ($n = 4$), acetoacetate, β-hydroxybutyrate, and pyruvate ($n = 5$); Period 2 ($n = 6$), Period 3 ($n = 5$), and Period 4 ($n = 3$).

Table 4.6. Means and standard errors for arterial plasma concentration of metabolites (mmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
NEFA					0.25	< 0.001	0.47
Control	0.866 ^a ± 0.036	0.131 ^b ± 0.039	0.231 ^b ± 0.036	0.118 ^b ± 0.047			
Treatment	0.883 ^a ± 0.042	0.216 ^b ± 0.039	0.201 ^b ± 0.042	0.186 ^b ± 0.055			
Acetate					0.34	< 0.001	0.05
Control	0.199 ^e ± 0.063	1.849 ^a ± 0.075	1.226 ^d ± 0.068	1.461 ^{bc} ± 0.084			
Treatment	0.199 ^e ± 0.075	1.570 ^b ± 0.068	1.332 ^{cd} ± 0.075	1.428 ^{bcd} ± 0.097			
Propionate					< 0.001	< 0.001	< 0.001
Control	0.008 ^d ± 0.011	0.259 ^a ± 0.012	0.154 ^b ± 0.011	0.079 ^c ± 0.014			
Treatment	0.006 ^d ± 0.013	0.153 ^b ± 0.012	0.108 ^c ± 0.013	0.083 ^c ± 0.016			
Butyrate					0.03	< 0.001	0.35
Control	0.002 ^e ± 0.004	0.075 ^a ± 0.005	0.052 ^b ± 0.004	0.035 ^{cd} ± 0.006			
Treatment	0.003 ^e ± 0.005	0.058 ^b ± 0.005	0.045 ^{bc} ± 0.005	0.026 ^d ± 0.007			

^{a,b,c,d,e}Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

²Control starter: Periods 1 and 3 (n = 7), Period 2 for acetate (n = 5) and NEFA, propionate, and butyrate (n = 6); and Period 4 (n = 4).
Treatment starter: Periods 1 and 3 (n = 5), Period 2 (n = 6), and Period 4 (n = 3).

Table 4.7. Means and standard errors for arterial plasma concentration of hormones (pmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Insulin					0.13	< 0.001	0.02
Control	20.12 ^c ± 4.61	18.77 ^c ± 4.98	46.03 ^a ± 4.72	38.73 ^{ab} ± 6.23			
Treatment	29.11 ^{bc} ± 5.55	3.81 ^d ± 4.98	49.47 ^a ± 5.55	17.66 ^{cd} ± 7.04			
Glucagon					0.04	< 0.001	0.19
Control	14.38 ^c ± 1.49	24.66 ^b ± 1.60	20.51 ^{bcd} ± 1.50	16.51 ^{de} ± 1.96			
Treatment	18.50 ^{cde} ± 1.96	30.91 ^a ± 1.60	21.39 ^{bc} ± 1.76	15.60 ^{de} ± 2.27			

^{a,b,c,d,e} Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹ Probability of a greater F statistic.

² Control starter: Periods 1 and 3 ($n = 7$), Periods 2 ($n = 6$), and Period 4 ($n = 4$). Treatment starter: Period 1 glucagon ($n = 4$) and insulin ($n = 5$); Period 2 ($n = 6$), Period 3 ($n = 5$), and Period 4 ($n = 3$).

did not differ between CON and TRT and significantly decreased from over 800 $\mu\text{mol/L}$ (Period 1) to concentrations below 250 $\mu\text{mol/L}$ in Period 2 where they stayed for the remainder of the experiment.

Arterial concentrations of lactate (Table 4.5) were affected by weaning in both CON and TRT and increased from Period 1 to Period 2 ($P < 0.05$). The diet $\times$ age interaction was also significant for lactate concentrations and concentrations measured in CON calves were greater in Period 2 than TRT ($P < 0.05$). Period 3 lactate concentrations were the inverse with greater lactate concentration from TRT calves than CON ($P < 0.05$). Pyruvate concentrations (Table 4.5) increased after Period 1 in CON and did not differ thereafter, but concentrations in TRT calves did not significantly increase until Period 4 where they matched concentrations in CON calves.

Arterial concentrations of ACAC and BHBA (Table 4.5) were below 0.02 and 0.09 mmol/L during Period 1. There was a significant diet effect for ACAC ($P < 0.05$) and there tended to be one for BHBA ($P = 0.19$). Period 2 concentrations of ACAC were greater ($P < 0.05$) in CON than TRT calves and BHBA was numerically, but not statistically greater. In CON calves, ACAC and BHBA concentrations peaked at Period 2 and 3 (0.05 and 1.5 mmol/L, respectively) and declined each sampling period thereafter. In TRT calves, maximum ACAC concentrations were observed during Periods 2 and 3 and BHBA concentrations did not significantly differ post-weaning.

Acetate, propionate, and butyrate arterial concentrations (Table 4.6) were all greater in CON than TRT calves 1 wk post-weaning at Period 2 ($P < 0.05$). Unlike acetate and butyrate concentrations which did not differ between CON and TRT at

Periods 3 and 4, propionate concentrations were still significantly greater in CON at Period 3 but were similar to TRT with CON at Period 4.

Insulin arterial concentrations (Table 4.7) in CON did not change until an increase at Period 3, but TRT calves insulin concentration decreased at Period 2 and was lower than CON ($P < 0.05$). Conversely, glucagon concentrations at Period 2 were significantly greater in TRT than CON. Insulin and glucagon concentrations at Periods 3 and 4 were generally not different by period or diet.

Venous-arterial Concentration Differences

Portal –arterial concentration differences are presented in Tables 4.8, 4.9, and 4.10 for blood and plasma metabolites and hormones respectively. Due to low sample numbers, tables containing hepatic-arterial and hepatic-portal concentration differences are located in Appendix 3 in Tables A3.8 – A3.10 and A3.11 – A3.13, respectively. Student's *t*-tests were used to determine if the portal-arterial (PA) concentration differences differed from zero which would be an indication of significant net flux. Glucose PA differences were significant from zero at Period 1, but not different in Periods 2 – 4. Non-esterified fatty acid PA difference (Table 4.9) was not significant from zero at any of the four sample periods. Conversely, lactate, acetate, insulin, and glucagon PA differences were significantly different from zero in all four sampling periods. Portal-arterial differences for ketones, propionate, and butyrate were all significantly different from zero at Periods 2 – 4, but not for Period 1. Pyruvate was the only metabolite that had significance in one dietary treatment and not the other. Pyruvate

Table 4.8. Means and standard errors for portal venous-arterial concentration differences of metabolites (mmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Glucose					0.14	< 0.001	0.99
Control	0.213 ^a ± 0.025	-0.022 ^b ± 0.029	-0.033 ^b ± 0.028	-0.029 ^b ± 0.032			
Treatment	0.185 ^a ± 0.028	-0.059 ^b ± 0.025	-0.066 ^b ± 0.026	-0.052 ^b ± 0.033			
Acetoacetate					0.37	< 0.001	0.03
Control	-0.002 ^e ± 0.002	0.026 ^{cd} ± 0.002	0.038 ^a ± 0.002	0.035 ^{ab} ± 0.002			
Treatment	-0.002 ^e ± 0.002	0.025 ^d ± 0.002	0.030 ^{bc} ± 0.002	0.038 ^a ± 0.002			
β-hydroxybutyrate					0.40	< 0.001	0.78
Control	-0.008 ^c ± 0.012	0.077 ^b ± 0.014	0.204 ^a ± 0.014	0.180 ^a ± 0.016			
Treatment	-0.013 ^c ± 0.012	0.083 ^b ± 0.012	0.185 ^a ± 0.012	0.165 ^a ± 0.016			
Lactate					0.20	0.013	0.51
Control	0.090 ^{abc} ± 0.02	0.068 ^{bc} ± 0.023	0.092 ^{abc} ± 0.023	0.119 ^{ab} ± 0.026			
Treatment	0.117 ^{ab} ± 0.02	0.050 ^c ± 0.020	0.138 ^a ± 0.02	0.144 ^a ± 0.026			
Pyruvate					< 0.001	< 0.001	0.81
Control	0.010 ^{ab} ± 0.007	-0.035 ^{cd} ± 0.008	-0.045 ^{cd} ± 0.008	-0.058 ^d ± 0.009			
Treatment	0.024 ^a ± 0.007	-0.007 ^b ± 0.007	-0.028 ^c ± 0.007	-0.036 ^{cd} ± 0.009			

^{a,b,c,d,e}Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

²Control starter: Period 1 (n = 5), Periods 2 and 3 (n = 4), and Period 4 (n = 3). Treatment starter: Period 1 (n = 5) except glucose (n = 4); Periods 2 and 3 (n = 5), and Period 4 (n = 3).

Table 4.9. Means and standard errors for portal venous-arterial plasma concentration differences of metabolites (mmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
NEFA					0.99	0.15	0.79
Control	-0.007 ± 0.013	0.010 ± 0.014	0.008 ± 0.014	0.018 ± 0.016			
Treatment	-0.010 ± 0.013	-0.005 ± 0.013	0.017 ± 0.013	0.027 ± 0.016			
Acetate					0.17	< 0.001	0.45
Control	0.086 ^e ± 0.031	0.527 ^c ± 0.034	0.620 ^{abc} ± 0.034	0.719 ^a ± 0.040			
Treatment	0.085 ^e ± 0.031	0.433 ^d ± 0.031	0.616 ^{bc} ± 0.031	0.683 ^{ab} ± 0.041			
Propionate					< 0.001	< 0.001	< 0.001
Control	0.028 ^c ± 0.018	0.616 ^a ± 0.020	0.607 ^a ± 0.020	0.508 ^b ± 0.024			
Treatment	0.024 ^c ± 0.018	0.461 ^b ± 0.018	0.484 ^b ± 0.018	0.586 ^a ± 0.024			
Butyrate					< 0.001	< 0.001	0.06
Control	0.016 ^c ± 0.007	0.124 ^a ± 0.008	0.123 ^a ± 0.008	0.104 ^{ab} ± 0.009			
Treatment	0.018 ^c ± 0.007	0.096 ^b ± 0.007	0.088 ^b ± 0.007	0.088 ^b ± 0.009			

^{a,b,c,d,e} Means within row and dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

²Control starter: Period 1 (n = 5), Periods 2 and 3 (n = 4), and Period 4 (n = 3). Treatment starter: Periods 1 through 3 (n = 5) and Period 4 (n = 3).

Table 4.10. Means and standard errors for portal venous-arterial plasma concentration differences of hormones (pmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Insulin					0.27	< 0.001	0.48
Control	10.24 ^{bc} ± 2.36	8.51 ^{cd} ± 2.64	17.36 ^a ± 2.70	16.77 ^{ab} ± 3.05			
Treatment	5.64 ^{cd} ± 2.40	3.12 ^d ± 2.36	18.0 ^a ± 2.36	17.84 ^{ab} ± 3.05			
Glucagon					0.09	< 0.001	0.03
Control	1.576 ^d ± 0.500	1.622 ^{cd} ± 0.559	4.482 ^a ± 0.559	3.594 ^{ab} ± 0.646			
Treatment	2.127 ^{bcd} ± 0.559	1.278 ^d ± 0.500	1.951 ^{cd} ± 0.500	3.217 ^{abc} ± 0.646			

^{a,b,c,d,e} Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹ Probability of a greater F statistic.

² Control starter: Period 1 (n = 5), Periods 2 and 3 (n = 4), and Period 4 (n = 3). Treatment starter: Period 1 (n = 5) except glucagon (n = 4); Periods 2 and 3 (n = 5), and Period 4 (n = 3).

PA was not significantly different than zero for Period 1 CON calves and Period 2 TRT calves. All remaining periods had PA that were significantly different than zero.

Portal-Drained Visceral Metabolism

All net portal-drained visceral (PDV) flux of blood and plasma metabolites had a significant age effect (Tables 4.11, 4.12; respectively). There was a net PDV release of glucose during Period 1 only and subsequent measures did not differ with age or calf starter. Lactate and acetate also had net PDV release, but unlike glucose, net release of lactate and acetate continued to increase across sampling periods with CON calves having a greater numerical net release of acetate than TRT. Calves receiving the lasalocid had higher net PDV release of lactate at Period 3 than CON calves ($P < 0.05$). During Periods 3 and 4, TRT calves had a net PDV uptake of pyruvate (Table 4.11) that was 50% lower than in CON calves ($P < 0.05$). Net PDV flux of propionate and butyrate (Table 4.12) and ACAC and BHBA (Table 4.11) during Period 3 was greater in CON than TRT ($P < 0.05$), but by Period 4 net release VFA and ketones no longer differed between diets.

The net release of insulin and glucagon (Table 4.13) did not differ between Periods 1 and 2, but an increase in PDV release measured in Periods 3 and 4 was greater than Periods 1 and 2 ($P < 0.05$). For insulin flux, there was only a significant age effect ($P < 0.001$), but for glucagon effects for diet, age and diet $\times$ age interaction were significant. Net PDV release of glucagon was significantly lower in TRT than CON calves during Period 3, but like the metabolites above no differences were detected at Period 4.

Table 4.11. Means and standard errors for net portal-drained visceral flux of blood metabolites (mmol/h) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Glucose					0.39	< 0.001	0.81
Control	31.85 ^a ± 5.31	-8.97 ^b ± 5.93	-14.30 ^b ± 5.93	-9.14 ^b ± 6.85			
Treatment	34.81 ^a ± 6.85	-16.45 ^b ± 5.31	-21.28 ^b ± 5.93	-12.70 ^b ± 6.85			
Acetoacetate					0.14	< 0.001	0.15
Control	-0.19 ^d ± 1.04	6.15 ^c ± 1.16	15.56 ^a ± 1.16	15.76 ^a ± 1.34			
Treatment	-0.13 ^d ± 1.16	5.92 ^c ± 1.04	11.04 ^b ± 1.16	15.44 ^a ± 1.34			
β-hydroxybutyrate					0.03	< 0.001	0.33
Control	-1.10 ^d ± 6.38	24.83 ^c ± 7.13	87.04 ^a ± 7.13	77.81 ^{ab} ± 8.23			
Treatment	-1.19 ^d ± 7.13	20.73 ^c ± 6.38	64.13 ^b ± 7.13	60.61 ^b ± 8.23			
Lactate					0.08	< 0.001	0.11
Control	12.19 ^b ± 5.44	21.99 ^b ± 6.08	27.96 ^b ± 6.21	46.74 ^a ± 7.02			
Treatment	15.16 ^b ± 6.08	15.19 ^b ± 5.44	48.62 ^a ± 6.08	60.93 ^a ± 7.02			
Pyruvate					< 0.001	< 0.001	0.36
Control	1.24 ^a ± 2.36	-10.20 ^b ± 2.59	-18.66 ^c ± 2.59	-27.28 ^d ± 2.99			
Treatment	3.81 ^a ± 2.59	-1.82 ^a ± 2.32	-9.37 ^b ± 2.59	-15.68 ^{bc} ± 2.99			

^{a,b,c,d,e} Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹ Probability of a greater F statistic.

² Control starter: Period 1 (n = 5), Periods 2 and 3 (n = 4), and Period 4 (n = 3). Treatment starter: Periods 1 and 3 (n = 4) except Period 1 glucose (n = 3); Period 2 (n = 5), and Period 4 (n = 3).

Table 4.12. Means and standard errors for net portal-drained visceral flux of plasma metabolites (mmol/h) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
NEFA					0.36	0.007	0.32
Control	0.50 ^{bc} ± 1.52	3.78 ^{ab} ± 1.67	2.46 ^{abc} ± 1.67	5.92 ^a ± 1.93			
Treatment	-0.51 ^{bc} ± 1.67	-0.89 ^c ± 1.50	3.47 ^{abc} ± 1.67	6.15 ^a ± 1.93			
Acetate					0.02	< 0.001	0.64
Control	8.56 ^e ± 14.13	97.95 ^d ± 15.79	181.06 ^{bc} ± 15.79	235.55 ^a ± 18.24			
Treatment	6.26 ^e ± 15.79	71.40 ^d ± 14.13	146.02 ^c ± 15.79	195.63 ^{ab} ± 18.76			
Propionate					0.13	< 0.001	0.07
Control	2.93 ^d ± 11.84	108.26 ^{bc} ± 13.24	169.69 ^a ± 13.24	164.51 ^a ± 15.29			
Treatment	1.60 ^d ± 13.24	79.72 ^c ± 11.84	120.41 ^b ± 13.24	185.38 ^a ± 15.29			
Butyrate					0.004	< 0.001	0.16
Control	1.51 ^d ± 2.67	20.23 ^c ± 2.94	36.55 ^a ± 2.94	32.17 ^{ab} ± 3.39			
Treatment	1.23 ^d ± 2.94	16.82 ^c ± 2.63	24.18 ^{bc} ± 2.94	23.49 ^{bc} ± 3.39			

^{a,b,c,d,e} Means within row and dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹ Probability of a greater F statistic.

² Control starter: Period 1 (n = 5), Periods 2 and 3 (n = 4), and Period 4 (n = 3). Treatment starter: Periods 1 and 3 (n = 4), Period 2 (n = 5), and Period 4 (n = 3).

Table 4.13. Means and standard errors for net portal-drained visceral flux of plasma hormones (nmol/h) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Insulin					0.37	< 0.001	0.58
Control	1.25 ^b ± 0.54	1.54 ^b ± 0.60	4.64 ^a ± 0.62	4.51 ^a ± 0.72			
Treatment	0.60 ^b ± 0.62	0.53 ^b ± 0.54	4.01 ^a ± 0.62	5.23 ^a ± 0.70			
Glucagon					0.02	< 0.001	0.03
Control	0.17 ^c ± 0.11	0.22 ^{bc} ± 0.12	1.12 ^a ± 0.12	1.12 ^a ± 0.14			
Treatment	0.18 ^{bc} ± 0.12	0.17 ^{bc} ± 0.11	0.49 ^b ± 0.12	0.94 ^a ± 0.14			

^{a,b,c,d,e}Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

²Control starter: Period 1 (n = 5), Periods 2 and 3 (n = 4), and Period 4 (n = 3). Treatment starter: Periods 1 and 3 (n = 4), Period 2 (n = 5), and Period 4 (n = 3).

Liver Metabolism

Interpretation of hepatic flux is restricted due to low numbers of experimental units. However, the data that is presented in Tables 4.14, 4.15, and 4.16 for blood and plasma metabolites and plasma hormones, respectively, does appear to be physiologically normal. There was a net uptake of ACAC and butyrate by the hepatic tissues and a net release of BHBA at Period 2 that was similar in CON and TRT calves (Table 4.14 and 4.15). The net hepatic ACAC and butyrate uptake and BHBA release seemed to be increasing with age in TRT calves as Period 3 fluxes were numerically greater than Period 2. This is supported by the observed increases in net portal supply of ketones and butyrate increasing at Period 3 (Table 4.11 and 4.12).

Similarly there was a net hepatic uptake of lactate and propionate at Period 2 that can account for much of net release of glucose from the liver (Tables 4.14 and 4.15). This is more evident in Period 3 TRT data which had a numerically greater net hepatic release of lactate and propionate (Tables 4.14 and 4.15; $119 \text{ mmol/hr of propionate}/2 + 65 \text{ mmol/hr lactate}/2 = 92$; 101% hepatic glucose release). Period 1 net hepatic release of glucose was not numerically different from that measured in Period 2 for TRT calves. Periods 2 and 3 had apparent precursors of propionate and lactate, but there was no propionate supplied in Period 1. Therefore, in the preruminant the portal supply of dietary glucose accounted for 50% (Table 4.11) and the hepatic uptake of lactate can account for another 25% (Table 4.14) of the hepatic glucose release in TRT calves.

Table 4.14. Means and standard errors for net liver flux of blood metabolites (mmol/h) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Glucose					0.2	0.71	0.55
Control	34.17 ± 20.67	51.84 ± 20.67	— —	— —			
Treatment	68.76 ± 15.27	64.66 ± 14.62	90.79 ± 14.62	— —			
Acetoacetate					0.11	< 0.001	0.74
Control	3.21 ^a ± 1.84	-5.78 ^{bc} ± 2.60	— —	— —			
Treatment	-0.71 ^{ab} ± 1.50	-8.34 ^c ± 1.84	-15.28 ^d ± 1.84	— —			
β-hydroxybutyrate					0.49	< 0.001	0.06
Control	9.10 ^{cd} ± 7.12	23.95 ^{bc} ± 10.07	— —	— —			
Treatment	-0.31 ^d ± 5.82	44.16 ^b ± 7.12	74.68 ^a ± 7.12	— —			
Lactate					0.83	0.84	0.22
Control	-2.22 ± 19.88	-32.31 ± 26.92	— —	— —			
Treatment	-32.58 ± 15.99	-11.23 ± 19.03	-65.13 ± 19.03	— —			
Pyruvate					0.001	0.003	0.003
Control	1.04 ^b ± 4.06	25.99 ^a ± 4.97	— —	— —			
Treatment	-0.08 ^b ± 2.95	-0.14 ^b ± 3.51	6.88 ^b ± 3.51	— —			

^{a,b,c,d,e}Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

²Control starter: Period 1 (n = 2) except glucose (n = 1) and Period 2 (n = 1). Treatment starter: Period 1 (n = 3) except glucose (n = 2) and Periods 2 and 3 (n = 2).

Table 4.15. Means and standard errors for net liver flux of plasma metabolites (mmol/h) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
NEFA					0.13	0.20	0.03
Control	14.38 ^a ± 6.92	-10.52 ^b ± 8.93	— —	— —			
Treatment	-12.28 ^b ± 5.47	-5.61 ^b ± 6.31	-32.27 ^c ± 6.31	— —			
Acetate					0.006	0.008	0.58
Control	-28.71 ^b ± 13.12	1.98 ^b ± 17.77	— —	— —			
Treatment	2.82 ^b ± 10.26	48.97 ^a ± 12.56	-14.75 ^b ± 12.56	— —			
Propionate					< 0.001	< 0.001	< 0.001
Control	-4.67 ^a ± 2.89	-121.91 ^c ± 4.09	— —	— —			
Treatment	0.54 ^a ± 2.36	-42.58 ^b ± 2.89	-119.23 ^c ± 2.89	— —			
Butyrate					0.002	< 0.001	0.02
Control	-1.24 ^a ± 0.83	-11.72 ^c ± 1.12	— —	— —			
Treatment	-0.34 ^a ± 0.65	-6.76 ^b ± 0.79	-21.77 ^d ± 0.79	— —			

^{a,b,c,d,e} Means within row and dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹ Probability of a greater F statistic.

² Control starter: Period 1 (n = 2) and Period 2 (n = 1). Treatment starter: Period 1 (n = 3) and Periods 2 and 3 (n = 2).

Table 4.16. Means and standard errors for net liver flux of plasma hormones (nmol/h) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Insulin					0.01	0.22	0.34
Control	-1.44 ^b ± 1.11	-1.77 ^b ± 1.56	— —	— —			
Treatment	2.78 ^a ± 0.90	0.12 ^{ab} ± 1.11	-3.64 ^b ± 1.11	— —			
Glucagon					0.47	0.61	0.24
Control	0.20 ± 0.29	-0.01 ± 0.39	— —	— —			
Treatment	-0.39 ± 0.23	0.13 ± 0.28	0.12 ± 0.28	— —			

^{a,b,c,d,e}Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

²Control starter: Period 1 (n = 2) and Period 2 (n = 1). Treatment starter: Period 1 (n = 3) and Periods 2 and 3 (n = 2).

Total Splanchnic Flux

As an expression of the combined effects of liver and PDV metabolism, only the net total splanchnic flux of lactate was not significant across sampling periods 1, 2, 3 (Table 4.17). Net splanchnic release of BHBA, acetate, propionate, butyrate and glucagon all increased in response to weaning ($P < 0.05$; Tables 4.18 and 4.19). Net splanchnic uptake of acetoacetate increased in response to weaning, whereas lactate appeared to decline. During Period 2 CON calves had a greater net splanchnic release of VFA than TRT calves ($P < 0.05$), but by Period 3 there were no observed differences attributable to the supplementation of lasalocid.

DISCUSSION

The results of lasalocid supplementation on BW and performance characteristics (Figure 4.1 and Table 4.2) did not demonstrate any differences and these findings agree with those described in Chapter 3. While no statistical comparisons can be made between the two experiments, overall and pre-weaning ADG were numerically the same, but post-weaning ADG appeared slightly lower in this experiment. This may have been due to calves in this experiment undergoing surgery. Also, this experiment had numerically lower DMI and feed efficiency than the previous experiment and may have been a result of calves in this experiment being housed in an controlled environment versus calves in the previous experiment housed outdoors during winter months.

Table 4.17. Means and standard errors for net total splanchnic flux of blood metabolites (mmol/h) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Glucose					0.17	0.001	0.12
Control	75.45 ^b ± 19.64	42.86 ^{bc} ± 19.64	45.07 ^{bc} ± 27.77	— —			
Treatment	133.86 ^a ± 19.64	23.78 ^c ± 16.03	76.26 ^b ± 19.64	— —			
Acetoacetate					0.54	0.04	0.73
Control	-0.54 ± 0.74	-2.56 ± 0.74	-1.58 ± 1.05	— —			
Treatment	-0.93 ± 0.61	-2.31 ± 0.61	-2.57 ± 0.74	— —			
β-hydroxybutyrate					0.15	< 0.001	< 0.001
Control	8.65 ^d ± 9.55	61.32 ^{bc} ± 9.55	83.29 ^b ± 13.50	— —			
Treatment	-1.52 ^d ± 7.79	38.85 ^c ± 7.79	151.19 ^a ± 9.54	— —			
Lactate					0.69	0.31	0.72
Control	-21.78 ± 14.02	-16.35 ± 14.02	6.88 ± 19.83	— —			
Treatment	-26.99 ± 11.45	-8.94 ± 11.45	-9.58 ± 14.02	— —			
Pyruvate					0.01	0.02	0.04
Control	-4.58 ^b ± 2.99	0.41 ^{ab} ± 2.99	-15.12 ^c ± 4.24	— —			
Treatment	4.28 ^a ± 2.52	-1.60 ^{ab} ± 2.45	-2.23 ^{ab} ± 2.99	— —			

^{a,b,c,d,e} Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹ Probability of a greater F statistic.

² Control starter: Periods 1 and 2 (n = 2) and Period 3 (n = 1). Treatment starter: Periods 1 and 2 (n = 3) except Period 1 glucose (n = 2) and Period 3 (n = 2).

Table 4.18. Means and standard errors for net total splanchnic flux of plasma metabolites (mmol/h) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
NEFA					0.69	0.005	0.81
Control	-7.24 ^{ab} ± 5.86	-5.45 ^{ab} ± 5.86	-23.52 ^{bc} ± 8.28	— —			
Treatment	-11.48 ^{ab} ± 5.07	-3.15 ^a ± 4.78	-27.37 ^c ± 5.86	— —			
Acetate					0.91	<0.001	0.08
Control	7.34 ^d ± 14.04	109.16 ^b ± 14.04	123.44 ^{ab} ± 19.86	— —			
Treatment	10.03 ^d ± 11.46	72.11 ^c ± 11.46	153.60 ^a ± 14.04	— —			
Propionate					0.02	< 0.001	0.002
Control	-0.52 ^e ± 2.75	22.97 ^a ± 2.75	17.65 ^{ab} ± 3.89	— —			
Treatment	2.31 ^{de} ± 2.24	7.54 ^{cd} ± 2.24	13.08 ^{bc} ± 2.75	— —			
Butyrate					0.24	< 0.001	0.09
Control	0.54 ^c ± 0.95	7.92 ^a ± 0.95	7.26 ^{ab} ± 1.35	— —			
Treatment	1.16 ^c ± 0.78	4.78 ^b ± 0.78	6.93 ^{ab} ± 0.95	— —			

^{a,b,c,d,e} Means within row and dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹ Probability of a greater F statistic.

² Control starter: Periods 1 and 2 (n = 2) and Period 3 (n = 1). Treatment starter: Periods 1 and 2 (n = 3) and Period 3 (n = 2).

Table 4.19. Means and standard errors for net total splanchnic flux of plasma hormones (nmol/h) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Insulin					0.05	0.03	0.34
Control	1.70 ^{bc} ± 1.09	0.13 ^c ± 1.09	1.03 ^{abc} ± 1.69	— —			
Treatment	3.34 ^{ab} ± 0.89	0.26 ^c ± 0.89	4.78 ^a ± 1.09	— —			
Glucagon					0.14	< 0.001	0.54
Control	0.11 ^c ± 0.24	0.60 ^{bc} ± 0.24	1.64 ^a ± 0.33	— —			
Treatment	0.05 ^c ± 0.20	0.39 ^c ± 0.19	1.02 ^{ab} ± 0.24	— —			

^{a,b,c,d,e}Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

²Control starter: Periods 1 and 2 (n = 2) and Period 3 (n = 1). Treatment starter: Periods 1 and 2 (n = 3) and Period 3 (n = 2).

Blood Flow

The lowest portal blood flow rates were observed in Period 1 (pre-weaning) at 145.5 and 117.3 L/h for CON and TRT when calves were only receiving milk replacer. These values agreed with the previously published preruminant portal blood flow rates of 159.8 (mean of 24 hourly measurements; Ortigues et al., 1995), 145.8 (Carr and Jacobson, 1968), and 141.2 L/hr (Durand et al., 1988). Ortigues et al. (1995) also reported hourly blood flow rates across a 24-hr period for hepatic vein and hepatic artery of 182.4, and 22.6 L/hr, respectively. These data agree with our preruminant hepatic vein flow rates of 177.3 and 229.3 L/hr for CON and TRT, respectively. Hepatic artery flow is calculated by subtracting the measured portal flow from the measured hepatic flow and therefore requires a complete set of functioning catheters. The hepatic artery flow rate in this study (Table 4.3) was higher than the previously reported data and had a large standard error due to the low number of calves that had all three catheters functioning simultaneously.

As calves increased in size, the portal blood flow increased significantly with each period resulting in a greater flow rate than the preceding measure (Table 4.3). Interestingly, when this data was corrected for body weight (Table 4.4), only Period 1 remained significantly different from the other sample periods. This is most likely a response to the rapid growth of gut tissues in response to the peri-weaning diet change. Huntington et al. (1990) presented portal blood flow data from several different techniques and species corrected for BW. The average mature bovine portal blood flow measured using PAH dilution was 44 mL/min/kg. The values reported in this study for Period 4, when converted and averaged between CON and TRT was 84.9 mL/min/kg,

nearly twice that reported by Huntington et al. (1990). If the previously reported adult portal flow rate was converted from BW to metabolic body weight the average flow was $185.2 \text{ mL/min/kg}^{0.75}$ and the value for the current study was $258.2 \text{ mL/min/kg}^{0.75}$. Thus, when corrected for BW or metabolic BW measured portal flow was still greater in this study than other reports from adults. The bovine portal flow rates determined by PAH presented by Huntington et al. (1990) were measured in much larger cattle, as the smallest weight reported at 211 kg, but the largest BW measured in the current study was during Period 4 (wk 16) and 81 kg. It is likely that this observed difference in reported portal flow rates is an effect of calves in the current study slowly adjusting their flow rates on a BW or metabolic BW basis as muscle growth catches up with the rapid increase in splanchnic tissue growth.

The absence of an ionophore related effect on blood flow was anticipated. Previous research using high-concentrate diets supplemented with monensin and salinomycin reported only a tendency for increased portal flow with ionophore supplementation ($n = 3$), but a significant decrease when monensin supplement was removed from a diet of chopped alfalfa hay ($n = 2$; Harmon et al., 1988). When high-concentrate diets were supplemented with monensin no effects on portal blood flow were reported ($n = 4$; Harmon and Avery, 1987). Ionophore supplementation in the current study did not affect blood flow rates.

Arterial Concentrations

Arterial concentrations are an important component in the calculation of net flux of a nutrient. When discussed alone they have the same limitations as the results from

jugular blood sampling presented and discussed for the experiment in Chapter 3 and would be repetitious. As such, they will currently be discussed as a component of net nutrient fluxes below. However, it is worth briefly noting agreement between the two experiments. Supporting the previous experiment in this study, glucose concentrations reached a nadir that coincided with weaning. Concentrations of NEFA were significantly reduced following weaning and were representative of adult concentrations reported for cattle and sheep (Bergman, 1971). Acetate, propionate and butyrate all increased substantially post-weaning and were significantly lower in TRT than CON for all three VFA at Period 2. These concentrations exhibited similar patterns as age increased, but concentrations were substantially greater in this experiment than in the previous experiment or other published values (Quigley et al., 1991b). β -hydroxybutyrate concentrations were similar in trends between the two experiments. The current experiment had larger measured concentrations and is probably due to the decreased time until samples were analyzed and subsequently less spontaneous decarboxylation to acetone. Concentrations of individual calves measured in this experiment exceeded 2.0 mmol/L on several occasions and in one individual, concentrations exceeded 3.0 mmol/L (although, it did not exhibit any adverse symptoms of ketosis). This was interesting, since these blood concentrations exceed the published limits for peripheral tissue utilization of ketones (Bergman, 1971; Leng, 1965) and the levels at which an adult would be exhibiting clinical symptoms of ketosis (Bergman, 1984). However, these calves did not demonstrate the concomitant hypoglycemia observed in adults with hyperketonemia.

Portal-Drained Viscera and Liver Metabolism

Glucose and glucose precursors – Periods 1 and 2. Net glucose flux shifted from a net portal release of glucose during Period 1 (Table 4.11) to no significant portal-venous difference (Table 4.8) and thus, no significant uptake or release during Period 2, 3, and 4. This is indicative of development of the gut, as absorption of glucose from the mature gut on a fiber diet is negligible in ruminants (Bergman et al., 1970) and the PDV will actually have a net utilization of glucose. The adult ruminant liver is responsible for 85% of the total glucose production (Bergman et al., 1970). The liver removes little or no glucose since there is minimal quantities arriving via portal or hepatic arterial blood. This is apparent by the low glucokinase activities of in the ruminant liver (Leat, 1970; Ballard and Oliver, 1969) and a half maximal velocity (half saturated) that is equal to about 10 mM (Lehninger, 1993).

In the preruminant however, the gut appears to be the main glucose producing organ and the liver has high activities of glycolytic enzymes (Table 2.1). Lactate is the primary glucose precursor used by the preruminant liver, which has a better ability to convert lactate to glucose than adults (Donkin and Armentano, 1995). In this regard, Period 1 portal supply of glucose (31.9 mmol/hr) can explain about 100% of hepatic glucose release in CON (34.2 mmol/L). Unlike CON, the hepatic glucose release in TRT calves (68.8 mmol/hr) was 2-fold the net portal release (34.8 mmol/hr). There also was a large net hepatic extraction of lactate in TRT (-32.6 mmol/hr) that could account for another 15% of hepatic glucose release in TRT calves. The difference in net flux of insulin across the liver observed during Period 1 (Table 4.16) between CON and TRT (-1.44 and 2.78 nmol/hr) could also be contributing to the observed difference in net

hepatic release of glucose. Due to the significant post-prandial changes report in glucose and lactate concentrations in preruminants (Ortigue et al., 1996; Quigley and Bernard, 1992) the small number of calves used in these determinations is likely a causal factor.

The major glucose precursors are propionate, lactate/pyruvate, alanine, and glycerol. The most important substrate for hepatic glucose production in fed ruminants is propionate which can account for over 50% glucose synthesis (reviewed by Brockman, 1993). Hepatic propionyl-CoA synthase has a high $V_{\max}$ (maximum velocity) and a low K_m (half maximum velocity; Ash and Baird, 1973) and little propionate ever reaches hepatic venous blood (Bergman and Wolff, 1971). This study numerically demonstrated (Table 4.15) a net hepatic removal of propionate (TRT = -42.6 mmol/hr) post-weaning that could account for 33% of the hepatic glucose release (TRT = 64.7 mmol/hr).

Post-weaning lactate is the secondary precursor, responsible for 15 – 20% of hepatic glucose production (Van der Walt et al., 1983), and this experiment demonstrates $\approx$ 30% contribution of lactate to glucose (Table 4.14). Donkin and Armentano (1995) demonstrated that the addition of propionate to ruminant hepatocytes caused a reduction in lactate incorporation into glucose. This observation is probably the result of preference of propionate over lactate as substrate for glucose production and the decreased activities of lactate dehydrogenase and pyruvate carboxylase in the adult liver (Table 2.1). Net hepatic release of glucose at Period 2 in this study was 51.8 and 64.7 mmol/L for CON and TRT. These values are lower than other reports of net hepatic glucose release in mature cattle (Huntington, 1990). Net portal release of propionate and lactate can account for over 100% of CON and almost 50% of TRT hepatic glucose release. Amino acids

would likely make up any remaining contributions to glucose not accounted for in the metabolites measured in this study.

Net portal nutrient flux in Period 3. The reported effects of ionophores include the modification of rumen bacterial populations that result in the secondary effect of altered molar ratios of VFA available, typified by an increase in propionate at the expense of acetate and butyrate (Russell and Strobel, 1989; Bergen and Bates, 1984). Due to varying rates of rumen epithelial metabolism of individual VFA during absorption, rumen production is not necessarily reflected in PDV release. The effects of ruminal ionophore on rumen concentrations of VFA may not be reflected in the net portal flux of these nutrients. A limitation of the technique used in this study is that intracellular activity and intracellular dilution of a metabolite from intracellular metabolism cannot be determined (Brockman, 1993; Heitmann, 1989). This could be overcome by the use of isotope dilutions to permit the determination of unidirectional fluxes and the fate of a metabolite by measuring the appearance of the label in its metabolic products (Brockman, 1993; Heitmann, 1989). This technique, while very informative, is very complex and time consuming and was beyond the physical capacity of personnel involved with this experiment.

In the presence of increasing ruminal butyrate concentrations, there appears to be an increase in ACAC, but not BHBA production and portal appearance (Krehbiel et al., 1992; Baldwin and Jesse, 1996). Krehbiel and coworkers (1992) accounted for 26% of ruminally infused butyrate that appeared in portal blood. This suggested that 74% was metabolized to ACAC and BHBA, but they could only account for 14% of ruminally infused butyrate appearing as ACAC and BHBA. More recently, the portal appearance of

butyrate plus the unidirectional PDV flux of BHBA accounted for 62% of intraruminally infused butyrate (Kristensen et al., 2000b). The portal recovery of intraruminally infused propionate was between 62% and 85% (Kristensen et al., 2000a, 2000b). Possible explanations for the missing carbon in the above studies is oxidation of butyrate to carbon dioxide by rumen epithelial cells, or sequestering of infused ruminal VFA and by rumen microbes (Kristensen et al., 2000a). Because the current study did not measure ruminal VFA concentrations, conclusions regarding the metabolic origin of portal released metabolites are more speculative.

Large and rapid increases in post-weaning ketones have been previously demonstrated (Quigley et al., 1991a; Quigley et al., 1992; Baldwin and Jesse, 1992), but was either the result of single-site samples or *in vitro* cell incubations. This study observed large rapid increases in ketone concentrations in all veins and arteries sampled and to the knowledge of the author, this is the first attempt to measure net ketone fluxes peri-weaning. Portal flux of ACAC and BHBA for fed, non-pregnant, non-lactating sheep average 2.94 and 11.5 mmol/hr, respectively (Heitmann et al., 1987), which was substantially lower than net portal appearance of ACAC and BHBA in 12 wk old calves reported in the current experiment (CON - 15.6 & 87.04; TRT - 11.0 & 48.6 mmol/L). However, for growing beef steers, net portal release of BHBA was 55 mmol/hr (Huntington and Eisemann, 1988). In Period 3 net portal releases of ACAC, BHBA, and butyrate were greater in CON than TRT (Table 4.11). This supports previous work that reported similar reductions in PDV release of BHBA in lasalocid supplemented wethers (Housewright et al., 1994) and suggests modification of substrates being absorbed from the lumen of the rumen and metabolized by the epithelium.

As much as 90% of butyrate absorbed by the rumen epithelium is metabolized to ketones (Bergman, 1990) and is an effect of butyryl-CoA synthetase having the highest activity of the activating enzymes for VFA in the rumen epithelium (Ash and Baird, 1973). The activity of the hepatic isozyme of butyryl-CoA is substantial and the remaining butyrate that escapes metabolism by rumen epithelium enters via the portal blood and is normally extracted by the liver. This study demonstrated net portal release of butyrate at 24.2 mmol/h in TRT (CON = 36.6 mmol/L) and net hepatic extraction of -21.8 mmol/hr for TRT calves.

In the presence of butyrate, there are large reductions in rumen epithelial propionyl-CoA synthetase activity observed *in vitro* due to butyrate inhibition of propionyl-CoA synthetase (Harmon et al., 1991; Ash and Baird, 1973). The significantly greater net portal appearance of butyrate in CON calves may partially explain some of the other differences seen during this sampling period. Greater net PDV release of butyrate implies that more butyrate was absorbed and thus available for ketogenesis and would explain the significantly greater PDV release of ACAC and BHBA during this period. If the ionophore was affecting net nutrient release into the portal vein, a decrease in ruminal butyrate concentrations and absorption could lower the inhibition of rumen epithelial propionyl-CoA synthase in the rumen epithelium. Reduction of this inhibition may permit more absorbed propionate to be metabolized to lactate by the rumen epithelium and may explain why calves receiving the ionophore had a higher net portal release of lactate, but a lower net portal release of propionate Period 3 compared to CON. Conversely, a significant increase in net PDV release of propionate when supplementing monensin and salinomycin was demonstrated by Harmon et al. (1988), but no effect on propionate

release in response to monensin supplementation was seen by Harmon and Avery (1987). This study was differed in ionophore supplemented and diet type than the current experiment and may not be an adequate comparison.

Total Splanchnic Metabolism

The PDV (gastrointestinal tract –excluding the lower colon and rectum, pancreas, spleen, and associated omental and mesenteric fat) and liver tissues constitute total splanchnic tissues (TSP). Metabolites released by the TSP are representative of what is available to peripheral tissues after gut tissues have extracted their energetic cost of service from the nutrient supply (Reynolds, 2002). In the case of net TSP release of VFA in this experiment, the apparent increases across sampling periods in net portal release was not matched by net hepatic extraction (Table 4.18), resulting in an increase in net TSP release of VFA. This is in contrast to reports for dairy cows (Reynolds et al., 2003), but were similar to reports of net total splanchnic release of VFA in growing beef steers (Huntington and Eisemann, 1988). Calves in the current study were also growing and the differences in net TSP release of VFA are likely the result of physiological status (growing vs. lactating) and how the liver may be partitioning nutrients to meet the different demands of lactation and growth.

CONCLUSIONS

The net portal appearance of ketones increased quickly with respect to diet changes at weaning and metabolite concentrations changed with age and weaning as

previously reported. Maximum net portal release of ketones was measured in calves 12 weeks old, but maximums for VFA continued on to the 16 week sample period. A significant effect of lasalocid was observed on net portal release of nutrients in growing calves, with reduced releases of VFA, acetoacetate, and β -hydroxybutyrate release in ionophore supplemented calves. Our hypothesis was that any reduction in alimentary ketogenesis and circulating ketones below levels of maximum utilization by peripheral tissues would improve growth and feed efficiency. However, there was no obvious growth or feed efficiency improvement. Thus, calves supplemented with ionophore at feed-grade levels typically used to treat coccidiosis may have additional effects on nutrient proportions appearing in the rumen. The lack of any performance response related to the reduction of circulating ketones may be due to developing calves having a greater ability to peripherally metabolize ketones than the rates reported for adults.

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1. SOUTHWORTH

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APPENDICES

APPENDIX 1

DESCRIPTION OF CATHETERS

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1. Hepatic and portal vein catheters (~1.2 mL capacity):

- Length of 100 cm Tygon microbore tubing ($0.050 \times 0.090''$; i.d. $\times$ o.d.; Norton Performance Plastics, Akron, OH)
- Silastic cuffs (1 cm) at 4, 7, and 10 cm from tip end (Dow Corning Silastic Silicone tubing, $0.062 \times 0.125''$; i.d. $\times$ o.d.; Helix Medical, Inc., Carpinteria, CA)
- Cuffs are tapered at the end facing tip end of catheter to facilitate insertion
- Silastic tip (1.5 cm) tip wedged on to end of Tygon (with or without bevel)

2. Mesenteric vein catheter (~0.9 mL capacity):

- Length of 110 cm Tygon microbore tubing ($0.040 \times 0.070''$; i.d. $\times$ o.d.; Norton Performance Plastics, Akron, OH)
- Silastic cuffs (1 cm) at 10 and 11.5 cm from tip end (Dow Corning Silastic Silicone tubing, $0.062 \times 0.125''$; i.d. $\times$ o.d.; Helix Medical, Inc., Carpinteria, CA)
- Cuffs are tapered at the end facing tip end of catheter to facilitate insertion
- No silastic tip

3. Mesenteric artery catheter (~1.3 mL capacity):

- Length of 110 cm Tygon microbore tubing ($0.050 \times 0.090''$; i.d. $\times$ o.d.; Norton Performance Plastics, Akron, OH)

- Silastic cuffs (1 cm) at 4 and 5.5 cm from tip end (Dow Corning Silastic Silicone tubing, 0.062 × 0.125"; i.d. × o.d.; Helix Medical, Inc., Carpentaria, CA)
- Cuffs are tapered at the end facing tip end of catheter to facilitate insertion
- No silastic tip

NOTES:

1. All catheters were soaked in 7% TDMAC Heparin (Polysciences, Inc., Warrington, PA) for 5 min. The TDMAC was also aspirated the length of the catheter during this 5-min interval.
2. Typically all catheters have a 1.5 cm silastic tip, but due to the small vessel size the tips for mesenteric catheters had to be removed to allow implantation.
3. The typical amount catheter inserted into the mesenteric artery in previous surgeries is 20 cm and in adults. However, insertion of this length of catheter into calves used in the present experiment had one of two results: a quality catheter with good patency or occlusion of the common mesenteric artery would cause a portion of small intestine to necrose and the calf would die (necropsy n = 3).
 - To minimize calf mortality a compromise was made and only 4-cm of catheter was inserted into the artery. A branch connecting to separate arteries (|--|) was always the preferred site of insertion and seemed to minimize potential for occlusion of blood supply.

4. If catheters are to be autoclaved with surgical instruments, use silastic grade medical tubing. If Tygon-type tubing is used, catheters must be sterilized with ethylene oxide (without hot air drying) or a comparable cold-sterilization agent.

APPENDIX 2

EQUATIONS USED TO CALCULATE BLOOD FLOW AND NET FLUX

EQUATIONS USED TO CALCULATE BLOOD FLOW AND NET FLUX

1. Whole blood flow rates through splanchnic tissues is calculated using the indicator-dilution method:

$$F = I^{PAH} / (C_v^{PAH} - C_a^{PAH})$$

F = whole blood flow through the tissue in L/min

I^{PAH} = the infusion rate of PAH in optical density (OD) units/min

C_v^{PAH} = the PAH OD units/L in the venous drain

C_a^{PAH} = the PAH OD units/L in the arterial input

- Portal and hepatic blood flows will be measured directly and hepatic arterial flow will be calculated as the difference.
 - Plasma flow will be obtained by subtracting that portion of the flow represented by the packed cell volume.
2. Net fluxes of each metabolite across the individual tissues are calculated using the following equations:

$$\text{Portal-drained viscera flux} = PF * (C_p - C_a)$$

$$\text{Hepatic flux} = PF * (C_h - C_p) + AF * (C_h - C_a)$$

$$\text{Total splanchnic flux} = HF * (C_h - C_a)$$

- PF , HF , and AF = the whole blood or plasma flow rates (L/min) in portal and hepatic veins and hepatic artery

- C_p , C_h , and C_a = the concentrations in portal and hepatic veins and hepatic artery of each metabolite in whole blood or plasma in some units of concentration/L
- A positive net flux indicates net release and a negative net flux indicates net uptake of a metabolite by the specific tissue.

APPENDIX 3

ACCESSORY TABLES FOR PART IV

Table A3.1. Means and standard errors for plasma flow (L/h) and arterial packed-cell volume in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Plasma flow and dietary assignment	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Portal vein, L/h²					0.19	< 0.01	0.72
Control	111.2 ^d ± 14.1	166.8 ^c ± 15.7	270.0 ^{ab} ± 15.7	294.2 ^a ± 18.2			
Treatment	84.6 ^d ± 15.7	166.7 ^c ± 14.1	241.7 ^b ± 15.7	290.2 ^a ± 18.2			
Hepatic vein, L/h³					0.03	< 0.01	0.01
Control	175.0 ^{cd} ± 25.7	198.9 ^{cd} ± 25.7	234.7 ^{bc} ± 36.3	— —			
Treatment	130.4 ^d ± 25.7	272.3 ^b ± 25.7	357.3 ^a ± 25.7	— —			
Hepatic artery, L/h⁴					0.17	0.24	0.11
Control	60.7 ^{ab} ± 37.9	45.2 ^b ± 37.9	— —	— —			
Treatment	52.7 ^b ± 26.8	145.5 ^a ± 26.8	76.5 ± 26.8	— —			
Packed-cell volume, %⁵					< 0.01	< 0.01	0.08
Control	26.2 ^c ± 0.4	26.7 ^{de} ± 0.4	29.2 ^b ± 0.4	30.8 ^a ± 0.5			
Treatment	27.6 ^{cd} ± 0.5	28.7 ^{bc} ± 0.4	29.7 ^{ab} ± 0.5	30.9 ^a ± 0.6			

^{a,b,c,d,e} Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

²Control starter: Period 1 (n = 5), Periods 2 and 3 (n = 4), and Period 4 (n = 3). Treatment starter: Period 1 and 3 (n = 4), Period 2 (n = 5), and Period 4 (n = 3).

³Control starter: Period 1 and 2 (n = 2) and Period 3 (n = 1). Treatment starter: Periods 1 through 3 (n = 2).

⁴Control starter: Period 1 and 2 (n = 1). Treatment starter: Period 1 and 2 (n = 2).

⁵Control starter: Periods 1 and 2 (n = 6), Period 3 (n = 7), and Period 4 (n = 4). Treatment starter: Periods 1 and 3 (n = 5), Period 2 (n = 6), and Period 4 (n = 3).

Table A3.2. Means and standard errors for hepatic concentration of metabolites (mmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Glucose					0.32	< 0.001	0.03
Control	4.44 ^a ± 0.07	3.09 ^d ± 0.09	3.44 ^c ± 0.13	3.70 ^{bc} ± 0.13			
Treatment	4.50 ^a ± 0.07	2.84 ^e ± 0.07	3.64 ^c ± 0.07	3.98 ^{bc} ± 0.13			
Acetoacetate					0.39	< 0.001	0.07
Control	0.015 ^{de} ± 0.002	0.047 ^a ± 0.003	0.022 ^{cde} ± 0.004	0.024 ^{cd} ± 0.004			
Treatment	0.014 ^e ± 0.002	0.036 ^b ± 0.002	0.028 ^c ± 0.002	0.022 ^{cde} ± 0.004			
β-hydroxybutyrate					0.40	< 0.001	0.65
Control	0.091 ^c ± 0.137	1.861 ^a ± 0.168	1.610 ^a ± 0.238	0.875 ^b ± 0.238			
Treatment	0.081 ^c ± 0.119	1.512 ^a ± 0.137	1.586 ^a ± 0.137	0.821 ^b ± 0.238			
Lactate					< 0.001	< 0.001	< 0.001
Control	0.494 ^b ± 0.053	0.948 ^a ± 0.065	0.865 ^a ± 0.091	0.500 ^b ± 0.091			
Treatment	0.452 ^b ± 0.046	0.457 ^b ± 0.053	0.520 ^b ± 0.053	0.544 ^b ± 0.091			
Pyruvate					< 0.001	< 0.001	0.02
Control	0.171 ^{cd} ± 0.019	0.304 ^a ± 0.024	0.309 ^{ab} ± 0.033	0.216 ^{bc} ± 0.033			
Treatment	0.131 ^d ± 0.017	0.162 ^{cd} ± 0.019	0.154 ^{cd} ± 0.019	0.176 ^{cd} ± 0.033			

^{a,b,c,d,e} Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹ Probability of a greater F statistic.

² Control starter: Period 1 (n = 3), Period 2 (n = 2), and Periods 3 and 4 (n = 1). Treatment starter: Period 1 (n = 4) except glucose (n = 3), Periods 2 and 3 (n = 3), and Period 4 (n = 1).

Table A3.3. Means and standard errors for hepatic plasma concentration of metabolites (mmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
NEFA					0.84	< 0.001	0.53
Control	0.908 ^a ± 0.063	0.093 ^b ± 0.077	0.268 ^b ± 0.109	0.114 ^b ± 0.109			
Treatment	0.876 ^a ± 0.054	0.237 ^b ± 0.063	0.235 ^b ± 0.062	0.084 ^b ± 0.109			
Acetate					0.003	< 0.001	0.46
Control	0.250 ^d ± 0.031	2.204 ^a ± 0.113	2.041 ^{ab} ± 0.153	1.617 ^{bc} ± 0.136			
Treatment	0.232 ^d ± 0.026	1.671 ^{bc} ± 0.079	1.694 ^{bc} ± 0.081	1.381 ^c ± 0.126			
Propionate					< 0.001	< 0.001	< 0.001
Control	0.007 ^e ± 0.011	0.313 ^a ± 0.078	0.246 ^{ab} ± 0.098	0.085 ^c ± 0.057			
Treatment	0.010 ^e ± 0.010	0.160 ^b ± 0.046	0.103 ^c ± 0.037	0.034 ^d ± 0.037			
Butyrate					< 0.001	< 0.001	0.001
Control	0.005 ^d ± 0.007	0.121 ^a ± 0.008	0.084 ^b ± 0.012	0.019 ^{cd} ± 0.012			
Treatment	0.009 ^d ± 0.006	0.072 ^b ± 0.007	0.043 ^c ± 0.007	0.007 ^d ± 0.012			

^{a,b,c,d,e} Means within row and dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹ Probability of a greater F statistic.

² Control starter: Period 1 (n = 3), Period 2 (n = 2), and Periods 3 and 4 (n = 1). Treatment starter: Period 1 (n = 4), Periods 2 and 3 (n = 3), and Period 4 (n = 1).

Table A3.4. Means and standard errors for hepatic plasma concentration of hormones (pmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Insulin					0.29	< 0.001	0.34
Control	36.25 ^b ± 7.97	27.18 ^{bc} ± 9.76	79.20 ^a ± 15.11	29.69 ^{bc} ± 13.80			
Treatment	43.27 ^b ± 6.89	5.00 ^c ± 7.97	65.14 ^a ± 7.97	25.92 ^{bc} ± 13.80			
Glucagon					0.74	< 0.001	0.05
Control	19.43 ^c ± 2.81	36.74 ^a ± 3.44	36.27 ^{ab} ± 4.86	20.77 ^c ± 4.86			
Treatment	19.84 ^c ± 2.43	33.94 ^{ab} ± 2.81	26.99 ^{bc} ± 2.81	35.99 ^{ab} ± 4.86			

^{a,b,c,d,e} Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹ Probability of a greater F statistic.

² Control starter: Period 1 (n = 3), Period 2 (n = 2), and Periods 3 and 4 (n = 1). Treatment starter: Period 1 (n = 4), Periods 2 and 3 (n = 3), and Period 4 (n = 1).

Table A3.5. Means and standard errors for portal concentration of metabolites (mmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Glucose					0.74	< 0.001	0.93
Control	4.35 ^a ± 0.09	2.66 ^c ± 0.10	3.30 ^b ± 0.09	3.30 ^b ± 0.10			
Treatment	4.44 ^a ± 0.10	2.61 ^c ± 0.10	3.33 ^b ± 0.10	3.32 ^b ± 0.13			
Acetoacetate					0.003	< 0.001	0.37
Control	0.016 ^d ± 0.003	0.079 ^a ± 0.004	0.077 ^{ab} ± 0.003	0.075 ^{abc} ± 0.004			
Treatment	0.014 ^d ± 0.003	0.065 ^c ± 0.004	0.068 ^{bc} ± 0.004	0.068 ^{abc} ± 0.005			
β-hydroxybutyrate					0.706	< 0.001	0.22
Control	0.079 ^b ± 0.088	1.538 ^a ± 0.097	1.424 ^a ± 0.088	1.554 ^a ± 0.097			
Treatment	0.080 ^b ± 0.088	1.448 ^a ± 0.097	1.621 ^a ± 0.097	1.342 ^a ± 0.125			
Lactate					0.62	< 0.001	0.003
Control	0.623 ^c ± 0.077	1.016 ^b ± 0.084	0.897 ^b ± 0.077	1.002 ^b ± 0.084			
Treatment	0.618 ^c ± 0.084	0.886 ^b ± 0.084	1.306 ^a ± 0.084	0.850 ^{bc} ± 0.109			
Pyruvate					0.03	0.011	0.07
Control	0.189 ^{ab} ± 0.012	0.199 ^a ± 0.013	0.163 ^{bc} ± 0.012	0.193 ^{ab} ± 0.013			
Treatment	0.184 ^{ab} ± 0.012	0.135 ^c ± 0.013	0.144 ^c ± 0.013	0.197 ^{ab} ± 0.017			

^{a,b,c,d,e}Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

²Control starter: Periods 1 and 3 ($n = 6$) and Periods 2 and 4 ($n = 5$). Treatment starter: Period 1 ($n = 6$) except glucose and lactate ($n = 5$); Periods 2 and 3 ($n = 5$), and Period 4 ($n = 3$).

Table A3.6. Means and standard errors for portal plasma concentration of metabolites (mmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
NEFA					0.20	< 0.001	0.82
Control	0.867 ^a ± 0.043	0.208 ^b ± 0.043	0.169 ^b ± 0.039	0.170 ^b ± 0.043			
Treatment	0.940 ^a ± 0.039	0.202 ^b ± 0.043	0.218 ^b ± 0.043	0.213 ^b ± 0.055			
Acetate					0.03	< 0.001	0.29
Control	0.287 ^c ± 0.094	2.226 ^b ± 0.103	2.038 ^b ± 0.094	2.616 ^a ± 0.103			
Treatment	0.283 ^c ± 0.094	2.081 ^b ± 0.103	1.948 ^b ± 0.103	2.203 ^b ± 0.133			
Propionate					0.01	< 0.001	< 0.001
Control	0.036 ^d ± 0.032	0.759 ^a ± 0.035	0.780 ^a ± 0.032	0.626 ^{bc} ± 0.035			
Treatment	0.030 ^d ± 0.032	0.614 ^{bc} ± 0.035	0.592 ^c ± 0.035	0.708 ^{ab} ± 0.045			
Butyrate					0.02	< 0.001	0.41
Control	0.019 ^d ± 0.012	0.197 ^a ± 0.013	0.154 ^{bc} ± 0.012	0.151 ^{bc} ± 0.013			
Treatment	0.021 ^d ± 0.012	0.161 ^{ab} ± 0.013	0.133 ^{bc} ± 0.013	0.113 ^c ± 0.017			

^{a,b,c,d,e} Means within row and dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹ Probability of a greater F statistic.

² Control starter: Periods 1 and 3 ($n = 6$) except NEFA for Period 1 ($n = 5$) and Periods 2 and 4 ($n = 5$). Treatment starter: Period 1 ($n = 6$), Periods 2 and 3 ($n = 5$), and Period 4 ($n = 3$).

Table A3.7. Means and standard errors for portal plasma concentration of hormones (pmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Insulin					0.16	< 0.001	0.04
Control	30.51 ^b ± 7.55	20.91 ^{bc} ± 8.27	66.83 ^a ± 7.55	72.09 ^a ± 8.56			
Treatment	40.02 ^b ± 7.66	7.69 ^c ± 8.27	73.61 ^a ± 8.27	35.50 ^b ± 10.68			
Glucagon					0.79	< 0.001	0.01
Control	18.28 ^d ± 1.80	26.75 ^b ± 1.97	23.42 ^{bc} ± 1.80	26.43 ^b ± 1.97			
Treatment	21.53 ^{bcd} ± 1.97	32.68 ^a ± 1.97	23.34 ^{bcd} ± 1.97	18.82 ^{cd} ± 2.55			

^{a,b,c,d,e}Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

²Control starter: Periods 1 and 3 (n = 6) and Periods 2 and 4 (n = 5). Treatment starter: Period 1 (n = 6) except glucagon (n = 5); Periods 2 and 3 (n = 5), and Period 4 (n = 3).

Table A3.8. Means and standard errors for hepatic venous-arterial concentration differences of metabolites (mmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Glucose					0.96	0.003	0.98
Control	0.276 ^a ± 0.035	0.157 ^{bc} ± 0.043	0.138 ^{abc} ± 0.061	— —			
Treatment	0.275 ^{ab} ± 0.043	0.152 ^c ± 0.035	0.150 ^c ± 0.043	— —			
Acetoacetate					0.51	0.005	0.88
Control	-0.003 ^{ab} ± 0.002	-0.010 ^c ± 0.002	-0.005 ^{abc} ± 0.003	— —			
Treatment	-0.002 ^a ± 0.002	-0.008 ^{bc} ± 0.002	-0.005 ^{abc} ± 0.002	— —			
β-hydroxybutyrate					0.37	< 0.001	0.01
Control	0.028 ^d ± 0.024	0.236 ^b ± 0.028	0.246 ^{ab} ± 0.040	— —			
Treatment	0.006 ^d ± 0.024	0.123 ^c ± 0.023	0.318 ^a ± 0.028	— —			
Lactate					0.41	0.19	0.19
Control	-0.082 ± 0.022	-0.068 ± 0.027	0.015 ± 0.038	— —			
Treatment	-0.016 ± 0.024	-0.042 ± 0.022	-0.023 ± 0.027	— —			
Pyruvate					0.02	0.04	0.10
Control	-0.024 ^{ab} ± 0.007	-0.0003 ^a ± 0.009	-0.047 ^b ± 0.013	— —			
Treatment	-0.008 ^a ± 0.008	-0.004 ^a ± 0.008	-0.006 ^a ± 0.009	— —			

^{a,b,c,d,e} Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹ Probability of a greater F statistic.

² Control starter: Period 1 (n = 3), Period 2 (n = 2), and Period 3 (n = 1). Treatment starter: Periods 1 and 2 (n = 3) except Period 1 glucose (n = 2), and Period 3 (n = 2).

Table A3.9. Means and standard errors for hepatic venous-arterial plasma concentration differences of metabolites (mmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
NEFA					0.36	0.03	0.99
Control	-0.043 ^{ab} ± 0.018	-0.026 ^{ab} ± 0.022	-0.091 ^b ± 0.031	— —			
Treatment	-0.027 ^{ab} ± 0.018	-0.013 ^a ± 0.018	-0.072 ^b ± 0.022	— —			
Acetate					0.01	< 0.001	0.01
Control	0.045 ^c ± 0.047	0.558 ^a ± 0.058	0.519 ^a ± 0.082	— —			
Treatment	0.040 ^c ± 0.047	0.257 ^b ± 0.047	0.452 ^a ± 0.058	— —			
Propionate					< 0.001	< 0.001	< 0.001
Control	-0.001 ^d ± 0.009	0.109 ^a ± 0.011	0.072 ^b ± 0.015	— —			
Treatment	0.007 ^d ± 0.009	0.023 ^{cd} ± 0.009	0.038 ^{bc} ± 0.011	— —			
Butyrate					0.012	< 0.001	0.011
Control	0.001 ^d ± 0.003	0.035 ^a ± 0.004	0.031 ^{ab} ± 0.005	— —			
Treatment	0.005 ^d ± 0.003	0.018 ^c ± 0.003	0.021 ^{bc} ± 0.004	— —			

^{a,b,c,d,e}Means within row and dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

²Control starter: Period 1 (n = 3), Period 2 (n = 2), and Period 3 (n = 1). Treatment starter: Periods 1 and 2 (n = 3), and Period 3 (n = 2).

Table A3.10. Means and standard errors for hepatic venous-arterial plasma concentration differences of hormones (pmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Insulin					0.14	0.03	0.48
Control	4.59 ^b ± 2.38	-1.89 ^b ± 3.04	4.30 ^{ab} ± 4.51	— —			
Treatment	4.70 ^{ab} ± 2.38	1.28 ^b ± 2.38	12.08 ^a ± 2.91	— —			
Glucagon					< 0.001	< 0.001	0.002
Control	0.45 ^{cd} ± 0.67	3.29 ^b ± 0.82	7.04 ^a ± 1.17	— —			
Treatment	0.50 ^c ± 0.67	-1.43 ^d ± 0.67	2.73 ^b ± 0.82	— —			

^{a,b,c,d,e}Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

²Control starter: Period 1 (n = 3), Period 2 (n = 2), and Period 3 (n = 1). Treatment starter: Periods 1 and 2 (n = 3), and Period 3 (n = 2).

Table A3.11. Means and standard errors for hepatic-portal venous concentration differences of metabolites (mmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Glucose					0.39	0.001	0.19
Control	0.07 ^{bc} ± 0.043	0.174 ^{ab} ± 0.061	— —	— —			
Treatment	-0.033 ^c ± 0.037	0.196 ^a ± 0.043	0.186 ^a ± 0.043	— —			
Acetoacetate					0.003	< 0.001	< 0.001
Control	-0.003 ^a ± 0.003	-0.021 ^b ± 0.004	— —	— —			
Treatment	-0.001 ^a ± 0.002	-0.041 ^c ± 0.003	-0.036 ^c ± 0.003	— —			
β-hydroxybutyrate					0.61	< 0.001	0.04
Control	0.031 ^{bc} ± 0.015	0.078 ^{ab} ± 0.022	— —	— —			
Treatment	0.006 ^c ± 0.011	0.120 ^a ± 0.015	0.126 ^a ± 0.015	— —			
Lactate					0.60	0.49	0.77
Control	-0.138 ± 0.038	-0.121 ± 0.054	— —	— —			
Treatment	-0.128 ± 0.027	-0.088 ± 0.038	-0.168 ± 0.038	— —			
Pyruvate					< 0.001	< 0.001	< 0.001
Control	-0.003 ^b ± 0.009	0.094 ^a ± 0.012	— —	— —			
Treatment	-0.032 ^c ± 0.006	-0.001 ^b ± 0.009	0.018 ^b ± 0.009	— —			

^{a,b,c,d,e}Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

²Control starter: Period 1 (n = 2) and Period 2 (n = 1). Treatment starter: Period 1 (n = 4) except Period 1 glucose (n = 3), and Period 2 and 3 (n = 2).

Table A3.12. Means and standard errors for hepatic-portal venous plasma concentration differences of metabolites (mmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
NEFA					0.78	0.18	0.35
Control	0.019 ± 0.028	-0.050 ± 0.040	— —	— —			
Treatment	-0.018 ± 0.020	-0.030 ± 0.028	-0.091 ± 0.028	— —			
Acetate					0.33	0.81	0.74
Control	-0.021 ± 0.022	-0.023 ± 0.032	— —	— —			
Treatment	-0.053 ± 0.016	-0.039 ± 0.024	-0.130 ± 0.024	— —			
Propionate					< 0.001	< 0.001	< 0.001
Control	-0.026 ^a ± 0.010	-0.591 ^d ± 0.015	— —	— —			
Treatment	-0.016 ^a ± 0.007	-0.380 ^b ± 0.010	-0.483 ^c ± 0.010	— —			
Butyrate					< 0.001	< 0.001	< 0.001
Control	-0.012 ^a ± 0.003	-0.058 ^b ± 0.003	— —	— —			
Treatment	-0.013 ^a ± 0.002	-0.078 ^c ± 0.002	-0.080 ^c ± 0.002	— —			

^{a,b,c,d,e} Means within row and dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹ Probability of a greater F statistic.

² Control starter: Period 1 (n = 2) and Period 2 (n = 1). Treatment starter: Period 1 (n = 4) and Period 2 and 3 (n = 2).

Table A3.13. Means and standard errors for hepatic-portal venous plasma concentration differences of hormones (pmol/L) in pre-weaning and post-weaning dairy calves receiving calf starter with lasalocid (treatment) or without (control).

Variable and dietary assignment ²	Period and age (d)				Effect ¹		
	1 (35)	2 (56)	3 (84)	4 (112)	Diet (D)	Age (A)	D x A
Insulin					0.01	0.64	0.56
Control	-8.23 ^{ab} ± 2.41	-8.53 ^{ab} ± 3.41	— —	— —			
Treatment	-3.24 ^a ± 1.74	-0.51 ^a ± 2.41	-14.32 ^b ± 2.41	— —			
Glucagon					0.88	0.08	0.98
Control	-1.93 ± 0.90	-0.25 ± 1.28	— —	— —			
Treatment	-2.10 ± 0.64	-0.38 ± 0.90	-0.58 ± 0.90	— —			

^{a,b,c,d,e}Means within row and between dietary treatment not sharing like superscripts are different ($P < 0.05$).

¹Probability of a greater F statistic.

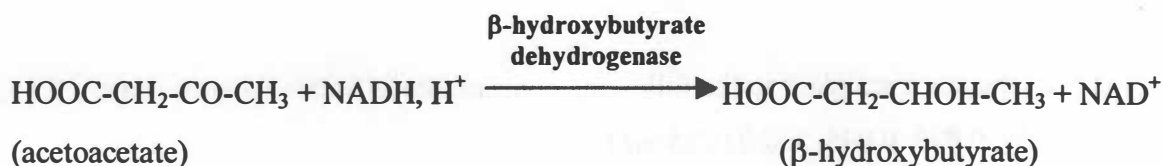
²Control starter: Period 1 (n = 2) and Period 2 (n = 1). Treatment starter: Period 1 (n = 4) and Period 2 and 3 (n = 2).

APPENDIX 4

ACETOACETATE ANALYSIS

ACETOACETATE ANALYSIS

This procedure is a modification of the method of Mellanby and Williamson (1974). The analysis is a spectrophotometric determination based upon the compound nicotinamide adenine dinucleotide (NAD^+) and its unique ability to absorb a specific quantity of light of a specific wavelength. Acetoacetate is indirectly determined from the concentration of NAD^+ through the following chemical reaction:



Acetoacetate is reduced to β-hydroxybutyrate in a 1 : 1 stoichiometry with the oxidation of the cofactor nicotinamide adenine dinucleotide (NADH) to NAD^+ in the presence of the enzyme β-hydroxybutyrate dehydrogenase (E.C. 1.1.1.30). Because of the 1 : 1 conversion, the change in absorption between the reduced and oxidized forms of nicotinamide adenine dinucleotide permits the calculation of acetoacetate.

REAGENTS

- A. 1.0 M Perchloric Acid (HClO_4)
- B. 1.0 M Potassium Hydroxide (KOH)
- C. 0.1 M Phosphate Buffer ($\text{pH} = 6.8$)

1. Monobasic Potassium Phosphate (KH_2PO_4): 1.36 g → 100 mL dH_2O
2. Dibasic Potassium Phosphate (K_2HPO_4): 1.74 g → 100 mL dH_2O
3. Mix equal volumes of each solution, check pH, and adjust if necessary with one or the other.

- D. 2.0 mM NADH (Sigma Chemical Co., cat. # N-8129; disodium salt; 98% purity; FW = 709.4): 0.010 g NADH $\rightarrow$ 6.0 mL dH₂O. **NOTE:** Make up on day of use and store in amber bottle at 2 - 6°C until needed.
- E. β -Hydroxybutyrate dehydrogenase (E.C. 1.1.1.30; Roche Diagnostics Corporation, Indianapolis, IN, cat. # 127-841; Grade II; 5 mg/mL).
- F. 1.5 mM Acetoacetate Standard (ACAC; Sigma Chemical Co., cat. # A-8509; Lithium salt; 90-95% purity): 0.01620 g ACAC $\rightarrow$ 100 mL dH₂O.
1. Dilute stock standard to the following concentrations for working standards: 0.15, 0.09, 0.075, 0.045, and 0.0225 mM.
 2. Use two zero concentration standards as blanks.
 3. Standards (and blanks) must be taken through entire procedure as small amounts of perchlorate will affect enzyme activity.
 4. Pipette into 50 mL polypropylene centrifuge tubes: 1.0 mL of standard and 1.0 mL of 1.0 M HClO₄.
 5. Neutralize with KOH to pH between 6.0 and 8.0 (Remember to record volume of buffer added when adjusting pH!!).
 6. Centrifuge at 1,500 x g for 15 min.
 7. Standard dilutions:
 - a. 0.15 mM $\rightarrow$ 2.0 mL of 1.5 mM ACAC plus 18.0 mL dH₂O
 - b. 0.09 mM $\rightarrow$ 12.0 mL of (a) plus 8.0 mL of dH₂O
 - c. 0.075 mM $\rightarrow$ 4.0 mL of (a) plus 4.0 mL of dH₂O
 - d. 0.045 mM $\rightarrow$ 10.0 mL of (b) plus 10.0 mL of dH₂O
 - e. 0.0225 mM $\rightarrow$ 10.0 mL of (d) plus 10.0 mL of dH₂O

SAMPLE COLLECTION AND PREPARATION

- A. All procedures leading up to the reading of the samples must be carried out at 2 - 6°C and samples must be analyzed the day of collection.

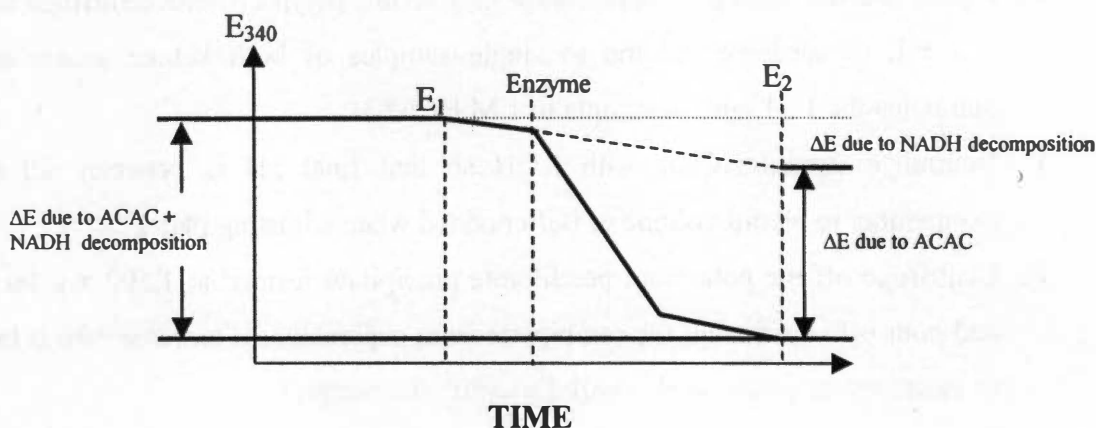
- B. Mix equal volumes of whole blood and 1 M HClO_4 immediately after sampling (e.g. – add 2 mL of whole blood to 15 mL centrifuge tube containing 2 mL of 1 M HClO_4), vortex.
- C. Centrifuge at 1,500 x g for approximately 15 min.
- D. Pipette desired volume of supernatant into 50 mL polypropylene centrifuge tube (e.g. – 2 mL is adequate volume to single samples of both ketone assays and also maintains the 1 : 1 ratio of sample to 1 M HClO_4)
- E. Neutralize excess HClO_4 with KOH so that final pH is between 6.0 and 8.0 (Remember to record volume of buffer added when adjusting pH!!).
- F. Centrifuge off the potassium perchlorate precipitate formed at 1,500 x g for 15 min and pour off supernatant (or can pipette from supernatant if extreme care is taken not to aspirate precipitate, as this will disqualify the sample).

ANALYSIS

- A. Pipette the following into 12 x 75 mm borosilicate culture tubes:
 - 1. 0.5 mL phosphate buffer
 - 2. Sample – 1.0 mL for fed animals (0.5 mL sample + 0.5 mL dH_2O for fasted animals)
 - 3. Standard – 1.0 mL
 - 4. 0.05 mL NADH
 - 5. Vortex
- B. Read E_1 at 340 nm.
- C. Add 0.005 mL of β -hydroxybutyrate dehydrogenase, vortex
- D. Incubate at room temperature for *ca.* 22 min.
- E. Read E_2 at 340 nm.

NOTES: NADH will slowly oxidize at room temperature, but at a relatively constant rate in all samples (see diagram). The zero concentration ACAC standards can be used to

control/compensate for this. Pipette NADH into all of the tubes at a set time interval (e.g. – 15 – 60 sec; typically about as fast as one can read samples in the spectrophotometer). Then read E_1 in all tubes at the same timed interval. Add the enzyme at the same intervals and incubate. Read the E_2 at the same timed intervals.



Alternatively, (less realistically) you can do the samples one at a time and take several E_1 and several E_2 readings to determine the rate of decay of NADH. In practice this takes longer and the creation of two “parallel” lines is very subjective (often the decay of NADH is low, but it must be taken into account).

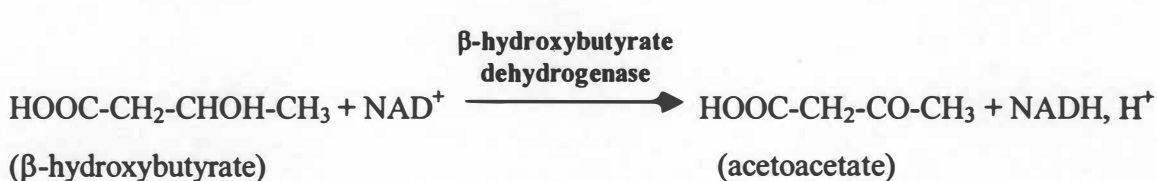
CALCULATIONS

- A. Calculate $E_1 - E_2$ for all blanks, standards, and samples.
- B. Calculate $\Delta E_{\text{std}} - \Delta E_{\text{blank}}$ and $\Delta E_{\text{sample}} - \Delta E_{\text{blank}}$
- C. Plot a standard curve of the known concentration vs. $\Delta E_{\text{std}} - \Delta E_{\text{blank}}$
- D. Extrapolate the concentrations of the samples using $\Delta E_{\text{sample}} - \Delta E_{\text{blank}}$ from the curve.
- E. Multiply by the appropriate dilution factors.

APPENDIX 5

β -HYDROXYBUTYRATE ANALYSIS

concentration of NADH through the following chemical reaction:



nicotinamide adenine dinucleotide permits the calculation of β -hydroxybutyrate.

REAGENTS

- A. 1.0 M Perchloric Acid (HClO_4)**
B. 1.0 M Potassium Hydroxide (KOH)
C. 0.1 M Tris-HCl buffer (pH = 8.5)

1. 2.42 g of Tris $\rightarrow$ *ca.* 50 mL with dH₂O (Sigma Chemical Co., cat.#, T-1378)
2. pH $\rightarrow$ 8.5 with 1 M HCl
3. Using dH₂O *q.s.* to 200 mL

- D. Hydrazine-Tris Buffer: Make up on day of use.**

1. 0.05 g EDTA
 2. 12.5 mL of 1M HCl
 3. 2.5 mL of hydrazine hydrate (Sigma Chemical Co., cat.# H-0883)
 4. *Q.s.* to 50 mL with Tris-HCl buffer
 5. Check pH, should be around 8.5.
- E. 15.0 mM NAD^+ (Sigma Chemical Co., cat. # N-6522; free acid; 98% purity; FW = 663.4): 0.03 g NAD^+ $\rightarrow$ 3.0 mL dH_2O . **NOTE:** Make up on day of use and store in amber bottle at 2 - 6°C until needed.
- F. β -Hydroxybutyrate dehydrogenase (E.C. 1.1.1.30; Roche Diagnostics Corporation, Indianapolis, IN, cat. # 127-841; Grade II; 5 mg/mL).
- G. 2.0 mM β -Hydroxybutyrate Standard (BHBA; Sigma Chemical Co., cat. # H-6501; Sodium salt; 98% pure): 0.0656 g BHBA $\rightarrow$ 250 mL dH_2O .
1. Dilute stock standard to the following concentrations for working standards: 2.0, 1.6, 1.2, 0.8, and 0.4 mM.
 2. Use two zero concentration standards as blanks.
 3. Standards (and blanks) must be taken through entire procedure as small amounts of perchlorate will affect enzyme activity.
 4. Pipette into 50 mL polypropylene centrifuge tubes: 1.0 mL of standard and 1.0 mL of 1.0 M HClO_4 .
 5. Neutralize with KOH to pH between 6.0 and 8.0 (Remember to record volume of buffer added when adjusting pH!!).
 6. Centrifuge at 1,500 x g for 15 min.
 7. Standard dilutions:
 - a. 2.0 mM $\rightarrow$ 20.0 mL of 2.0 mM BHBA plus 0.0 mL dH_2O
 - b. 1.6 mM $\rightarrow$ 16.0 mL of 2.0 mM BHBA plus 4.0 mL of dH_2O
 - c. 1.2 mM $\rightarrow$ 12.0 mL of 2.0 mM BHBA plus 8.0 mL of dH_2O
 - d. 0.8 mM $\rightarrow$ 8.0 mL of 2.0 mM BHBA plus 12.0 mL of dH_2O
 - e. 0.4 mM $\rightarrow$ 4.0 mL of 2.0 mM BHBA plus 16.0 mL of dH_2O

SAMPLE COLLECTION AND PREPARATION

- A. All procedures leading up to the reading of the samples must be carried out at 2 - 6°C and samples may be analyzed the day of collection or stored at -20°C for *ca.* one week.
- B. Mix equal volumes of whole blood and 1 M HClO₄ immediately after sampling (e.g. – add 2 mL of whole blood to 15 mL centrifuge tube containing 2 mL of 1 M HClO₄), vortex.
- C. Centrifuge at 1,500 x g for approximately 15 min.
- D. Pipette desired volume of supernatant into 50 mL polypropylene centrifuge tube (e.g. – 2 mL is adequate volume to single samples of both ketone assays and also maintains the 1 : 1 ratio of sample to 1 M HClO₄)
- E. Neutralize excess HClO₄ with KOH so that final pH is between 6.0 and 8.0 (Remember to record volume of buffer added when adjusting pH!!).
- F. Centrifuge off the potassium perchlorate precipitate formed at 1,500 x g for 15 min and pour off supernatant (or can pipette from supernatant if extreme care is taken not to aspirate precipitate, as this will disqualify the sample).

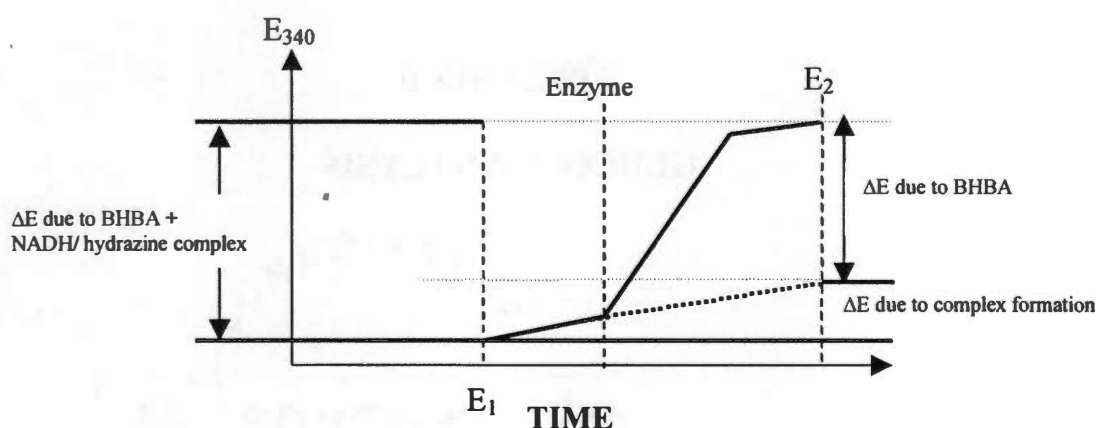
ANALYSIS

- A. Pipette the following into 12 x 75 mm borosilicate culture tubes:
 - 1. 0.5 mL hydrazine-tris buffer
 - 2. Sample or standard – 0.25 mL for fed animals (0.1 mL sample for fasted animals)
 - 3. Water – 0.75 mL for fed animals or standards (0.9 mL for fasted animals)
 - 4. 0.05 mL NAD⁺
 - 5. Vortex
- B. Read E₁ at 340 nm.
- C. Add 0.005 mL of β-hydroxybutyrate dehydrogenase, vortex

D. Incubate at room temperature for *ca.* 45 min.

E. Read E_2 at 340 nm.

NOTES: NADH and hydrazine slowly form a complex that absorbs at 340 nm. The hydrazine “captures” the NADH and prevents it from being converted back to the oxidized form. Therefore, a slow and constant increase in absorbance with occur, but at a relatively constant rate in all samples (see diagram). The zero concentration BHBA standards can be used to control/compensate for this. Pipette NAD^+ into all of the tubes at a set time interval (e.g. – 15 – 60 sec; typically about as fast as one can read samples in the spectrophotometer). Then read E_1 in all tubes at the same timed interval. Add the enzyme at the same intervals and incubate. Read the E_2 at the same timed intervals. This is pretty much like the ACAC assay, but in reverse.



CALCULATIONS

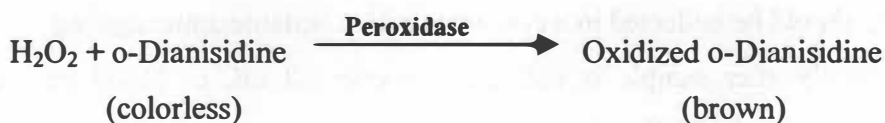
A. As for ACAC, except ΔE is calculated from $E_2 - E_1$.

APPENDIX 6

GLUCOSE ANALYSIS

GLUCOSE ANALYSIS

Determination of whole blood glucose was done using the Sigma Diagnostics glucose kit (#510; Sigma Diagnostics, St. Louis, MO). The procedure uses an enzymatic colorimetric that is based upon the following coupled enzymatic reactions:



REAGENTS

A. PGO Enzymes (500 units of glucose oxidase, 100 Purpurogalin units of Peroxidase and buffer salts; Sigma cat. # 510-6).

1. One capsule is diluted with dH₂O to 100 mL and stored in amber bottle at 2 – 6°C.

B. O-Dianisidine Dihydrochloride (Sigma cat. # 510-50).

1. Pre-weighed vial is diluted with dH₂O to 20 mL and stored at 2 – 6°C.
2. A 1.6 mL aliquot of this solution is added to the 100 mL of the PGO enzyme solution.
3. The combined Enzyme-Color Reagent Solution is stable up to 1 month refrigerated (2 – 6°C) unless turbidity or color forms.

- C. Glucose Standard Solution (100 mg/dL; Sigma cat. # 635-100). Consists of a solution of β -D-glucose, 100 mg/dL (5.56 mmol/L) in 0.1% benzoic acid and stored at 2 – 6°C.
- D. 0.3 N Barium Hydroxide solution (Sigma cat. # 14-3)
- E. 0.3 N Zinc Sulfate solution (Sigma cat. # 14-4)

SAMPLE COLLECTION AND PREPARATION

Blood samples should be collected in a container with a suitable anticoagulant.

1. Immediately after sample is collected, pipette 0.2 mL of blood into test tube containing 1.8 mL dH₂O and mix.
2. Add 1.0 mL barium hydroxide, vortex
3. Add 1.0 mL zinc sulfate, vortex and cap.
4. Tubes are then centrifuged at 3 x g for 15 min at 4 °C.

NOTES: It is recommended that the samples remain refrigerated until analysis steps and that analysis proceed as soon as possible. Blank and standard are prepared in a similar manner except 0.2 mL dH₂O and 0.2 mL of glucose standard are used, respectively, in place of blood.

ANALYSIS

Prepare blank, standard, and sample tubes in duplicate as follows:

1. Add 0.2 mL of supernatant from the respective centrifuged blank, standard, and sample tubes.
2. Add 2.0 mL of Enzyme-Color Reagent Solution to each tube, vortex
3. Incubate all tubes in a waterbath at 37°C for 30 min or at room temperature for 45 min. **NOTE:** Avoid direct exposure to sunlight or direct bright light.
4. Following incubation, remove all tubes from waterbath.

5. Read at 450 nm.
6. Readings should be completed within 30 min.

CALCULATIONS

Blood Glucose (mg/dL) = (absorbance of unknown/absorbance of standard)*100

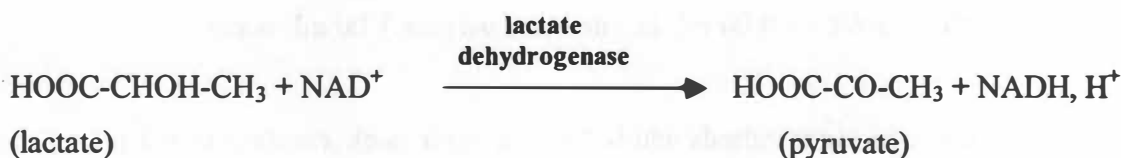
For conversion to mmol/L = glucose (mg/dL)/18

APPENDIX 7

LACTATE ANALYSIS

LACTATE ANALYSIS

This procedure is adapted from that of the Sigma Lactate Kit (Sigma Chemical Co., St. Louis, MO, cat. # 826-UV). The standard calibrations and procedures have been modified for use on a microtiter plate reader. The principle of this procedure is based on the conversion of lactate to pyruvate by lactate dehydrogenase (E.C.# 1.1.1.27) in the presence of NAD^+ :



When excess NAD is present, all lactate is converted to pyruvate. Measurement of NADH before and after addition of the enzyme allows the amount of lactate originally present to be determined. This is due to the absorbance of NAD (280 nm) differs from the absorbance of its reduced analog NADH (340 nm).

REAGENTS

The following were all purchased through Sigma Chemical (St. Louis, MO):

- A. Lactate dehydrogenase (# 826-6)
 - B. NAD (Sigma Chemical Co., cat. # N-6522; free acid; 98% purity; FW = 663.4); store in dessicator in freezer
1. Use 10 mg of NAD per 10 samples
 2. To make the working buffer, add 2.0 mL glycine buffer (see below) and 4.0 mL of water per 10 mg of NAD used.
 3. Working buffer is stable at room temperature for 4 hr, or for 24 hr at 2 - 6°C.

C. Lactic acid; 4.44 mmol/L; (#826-10)

1. To make stock standard solutions add the following:
 - a. 4.44 mmol/L → 1.000 mL lactate standard plus 0.0 mL water
 - b. 2.22 mmol/L → 0.500 mL lactate standard plus 0.500 mL water
 - c. 1.11 mmol/L → 0.250 mL lactate standard plus 0.250 mL water
 - d. 0.555 mmol/L → 0.125 mL lactate standard plus 0.875 mL water
 - e. 0.000 mmol/L → 0.00 mL lactate standard plus 1.00 mL water
2. To make working standards add 0.2 mL of each stock standard to 0.4 mL of the 8% perchloric acid. Vortex and store at 2-6°C. Discard after 3 d.

D. Glycine Buffer (#826-3)

1. Contains glycine (0.6 mol/L), and hydrazine (0.5 mol/L). pH = 9.2. Store at 2 – 6 °C and discard if turbidity is present.
2. Glycine buffer may also be made by adding 23 g of glycine to 20 mL of hydrazine hydrate (54% hydrazine; Sigma #H9507) and q.s. to 500 mL with distilled water. The pH of the solution should be approximately 9.

E. Perchloric acid (8% w/v). Not supplied with kit. Prepare by adding 140 mL of 70% (w/w) Perchloric acid to a 2 L volumetric flask. Add distilled water to volume.

SPECIMEN COLLECTION and STORAGE

- A.** After blood sample is obtained, pipet as soon as possible, 2.0 mL of blood into a centrifuge tube containing 4-mL of cold 8% perchloric acid, vortex.
- B.** Centrifuge mixture for 15 – 20 min at $1500 \times g$ at 4 °C.
- C.** Remove supernatant and store at -20°C until analyzed. Analysis should be conducted as soon as possible, usually within two weeks.

ANALYSIS

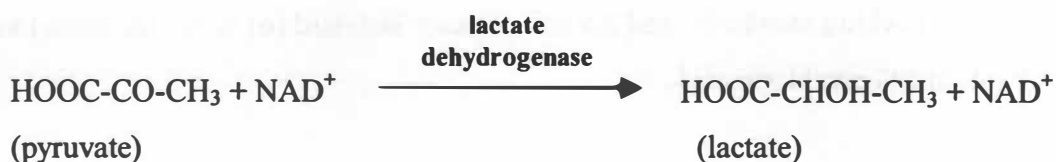
- A. Pipet 200 μL of NAD-glycine working buffer into each well of a 96-well microtiter plate. Add 20 μL of blank, standard, or sample, mix gently (set up so reader mixes before reading).
- B. Read absorbance (E_1) at 340 nm (Bio-Kinetics reader, model EL340, Bio-Tek Instruments, Winooski, VT).
- C. Add 10 μL of lactate dehydrogenase to each well, mix. Incubate plates for 15 min at 37°C (or 30 min at 25°C).
- D. Read absorbance (E_2) at 340 nm.
- E. Take difference between $E_2 - E_1$. Regress absorbance difference of standards against concentrations for calculation of samples.

APPENDIX 8

PYRUVATE ANALYSIS

PYRUVATE ANALYSIS

This procedure is adapted from that of the Sigma Pyruvate Kit (Sigma Chemical Co., St. Louis, MO, cat. # 726-UV). The standard calibrations and procedures have been modified for use on a microtiter plate reader. The principle of this procedure is based on the conversion of pyruvate to lactate by lactate dehydrogenase (E.C.# 1.1.1.27) in the presence of NADH:



When excess NADH is present, all pyruvate is converted to lactate. Measurement of NADH before and after addition of the enzyme allows the amount of pyruvate originally present to be determined. This results because the absorbance of NADH (340 nm) differs from the absorbance of its oxidized analog NAD (280 nm).

REAGENTS

The following were all purchased through Sigma Chemical (St. Louis, MO):

A. Trizma Base Solution (#726-4)

B. NADH (Sigma Chemical Co., cat. # N-8129; disodium salt; 98% purity; FW = 709.4)

1. Use 1 mg of NADH per 10 tubes
2. To make the working buffer, add 2.2 mL Trizma Base per 1.0 mg of NAD used.
3. Working buffer is stable at room temperature for 4 hr, or for 24 hr at 2 - 6°C.

C. Pyruvic Acid Standard Solution (# 726-10); 4 mg/dL (0.45 mmol/L).

1. To make stock standard solutions add the following:
 - a. 0.068 mmol/L → 0.2 mL pyruvate standard plus 3.8 mL water
 - b. 0.136 mmol/L → 0.4 mL pyruvate standard plus 3.6 mL water
 - c. 0.204 mmol/L → 0.6 mL pyruvate standard plus 3.4 mL water
 - d. 0.272 mmol/L → 0.8 mL pyruvate standard plus 3.2 mL water
 - e. 0.340 mmol/L → 1.0 mL pyruvate standard plus 3.0 mL water
2. To make working standards, add 2.0 mL of stock standard (or water for blank) to 4.0 mL of 8% perchloric acid.

D. Lactate dehydrogenase (#826-6)

F. Perchloric acid (8% w/v). Not supplied with kit. Prepare by adding 140 mL of 70% (w/w) Perchloric acid to a 2 L volumetric flask. Add distilled water to volume.

SPECIMEN COLLECTION and STORAGE

- A. After blood sample is obtained, pipet as soon as possible, 2.0 mL of blood into a centrifuge tube containing 4-mL of cold 8% perchloric acid, vortex.
- B. Centrifuge mixture for 15 – 20 min at $1500 \times g$ at 4 °C.
- C. Remove supernatant and store at 2-6°C until analyzed. Analysis should be conducted as soon as possible, usually within four weeks.

ANALYSIS

- A. Pipet 1 mL of blank, standard, and samples into 12 × 75 culture tubes.
- B. Add 250 µL of Trizma base to tubes, vortex.
- C. Add 250 µL of Trizma-NADH solution to tubes, vortex.
- D. Take E₁ reading at 340 nm.
- E. Quickly add 10 µL of lactate dehydrogenase, vortex.

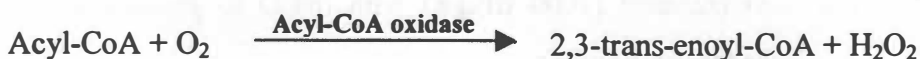
- F. After minimum 5 min incubation at room temperature read E_2 at 340 nm.
- G. Take difference between $E_2 - E_1$. Regress absorbance difference of standards against concentrations for calculation of samples.

APPENDIX 9

NON-ESTERIFIED FATTY ACID ANALYSIS

NON-ESTERIFIED FATTY ACID ANALYSIS

Plasma non-esterified fatty acids (NEFA) concentrations can be quantified using a WAKO NEFA C kit (Wako Chemicals USA, Inc., Richmond, VA). This kit utilizes an enzymatic colorimetric method that is based upon the following reactions:



MEHA = 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline

4-AAP = 4-aminoantipyrine

REAGENTS

- A. Color Reagent A: provided lyophilized in glass vials that consists of: acyl-CoA synthase, ascorbate oxidase, coenzyme A, adenosine triphosphate, 4-aminoantipyrine
- B. Solvent A: bottle containing 65 mL aqueous solution that consists of: 0.05 M phosphate buffer (pH = 6.9), 3.0 mM magnesium chloride, surfactant, and stabilizers
- C. Color Reagent B: provided lyophilized in glass vials that consists of: acyl-CoA oxidase and peroxidase
- D. Solvent B: bottle containing 130 mL aqueous solution that consists of: MEHA and surfactant
- E. NEFA Standard Solution: bottle containing 10 mL aqueous solution that consists of: 1.0 mmol/L Oleic acid, surfactant, and stabilizers

PREPARATION

A. Preparation of samples:

1. If plasma/serum samples were frozen, adequate thawing to room temperature and mixing of thawed sample are essential for precision.
2. If using plasma, be aware of the potential for fibrin clots to interfere with sample pipetting.

B. Preparation of standards:

1. Dilute provided standard (1000 $\mu\text{Eq/L}$) with dH_2O to provide 125, 250, 500 $\mu\text{Eq/L}$ standards (serial dilution).
2. Validation tests show distilled water as an acceptable diluent.

C. Preparation of Reagent A:

1. Mix Color Reagent A by adding 10 mL of the Solvent A to each vial.
2. Gently invert the vial until contents are completely dissolved.
3. Combine with 13.3 mL of dH_2O .
4. Mix again and record preparation date.

D. Preparation of Reagent B:

1. Mix Color Reagent B by adding 20 mL of Solvent B to each vial.
2. Gently invert until contents are completely dissolved.
3. Combine with 33.3 mL of dH_2O .
4. Mix again and record preparation date.

NOTE: After reconstitution, store Color Reagents A and B in amber bottles at 2 – 6°C for up to 5 days. One set of A and B vials is sufficient to analyze 66 tubes.

ANALYSIS

A. Assay blank, standards, and samples in duplicates.

B. Add 50 μL of serum, plasma, standard, or blank to each tube

C. Add 350 μ L of Color Reagent A mixture, vortex

NOTE: Can refrigerate for up to 60 min at 2 – 6°C if necessary.

D. Incubate tubes in waterbath at 37°C for exactly 20 min.

E. Following incubation, add 800 μ L of the Color Reagent B mixture to each tube, vortex.

F. Incubate a second time at 37°C for exactly 20 min.

G. Remove tubes from waterbath, allow to equilibrate at room temperature for 5 min.

H. Read at 550 nm using a blank prepared with reagents and should be completed within 60 min.

NOTES:

1. Do not mix reagents and supplies from test kits bearing different lot numbers.
2. This assay is sensitive to heparin and plasma samples using heparin as an anticoagulant are unsuitable.
3. Samples that are hemolyzed or overly icteric may yield inaccurate results.
4. Ascorbic acid interferes with the assay so ascorbate oxidase is automatically included in the Reagent A mixture.
5. Although not used in this experiment, a useful modification of the NEFA C kit for use with a 96-well plate was published by Johnson and Peters (1993. J. Anim. Sci. 71:753-756).

APPENDIX 10

PARA-AMINOHIPPURIC ACID ANALYSIS

PARA-AMINOHIPPURIC ACID ANALYSIS

Blood concentrations of para-aminohippuric acid (PAH) are measured colorimetrically. Standards used in the assay are dilutions of the PAH used in the corresponding sampling session.

REAGENTS FOR FILTRATE

- A. 20% Trichloroacetic acid (TCA; Cl_3CCOOH)
- B. Distilled H_2O

METHOD FOR FILTRATE

- A. Pipette 1.0 mL of whole blood or standard into a 15 mL polypropylene centrifuge tube containing 5 mL of dH_2O , vortex.
- B. Pipette 5.0 mL of the blood or standard and dH_2O mixture into a second 15 mL polypropylene centrifuge tube containing 5.0 mL of TCA, vortex.
- C. Let stand 60 min or overnight (can be left at this stage if refrigerated).
- D. Filter samples through Whatman #4 filter paper into 16 x 150 mm flint glass culture tubes.
- E. Add boiling chip to each culture tube and place a marble on top of each tube.
- F. Boil over low heat in a water bath for 30 min after fine bubbles appear in each tube.
- G. Remove from water bath and allow to cool to room temperature before removing marbles.
- H. Standards from 1.5% PAH infusion solution are made and processed following the same steps as outlined for samples (considered 10:10 dilutions).
 - 1. Make up a 1:500 dilution for sheep (or animals under 250 kg) from 1.5% infusion solution.
 - 2. Make up a 1:100 dilution for dogs from 0.25% infusion solution.
 - 3. Used a 1:1000 dilution from 1.5% infusion solution for calves (≤ 100 kg).

4. Blank consists of 5 mL of dH₂O and 5 mL of TCA.

NOTE: Be sure to run these dilutions through the same procedures as the blood samples, but do not have to boil

REAGENTS FOR ANALYSIS

- A. 1.2 N Hydrochloric Acid (HCl; Fisher Scientific Co., cat.# A-144-S): a 1:10 dilution of 12.4 N HCl considered close enough.
- B. Sodium Nitrite (NaNO₂; Sigma Chemical Co., cat. # S-2252): 100 mg of NaNO₂ brought up to 100 mL with dH₂O.
- C. Ammonium Sulfamate (NH₄OSO₂NH₂; Sigma Chemical Co., cat. # A-8670): 500 mg of ammonium sulfamate brought up to 100 mL with dH₂O.
- D. Coupling Reagent (N-1-Naphthylethylenediamine dihydrochloride; Sigma Chemical Co., cat. # N-5889) 100 mg of coupling reagent brought up to 100 mL with dH₂O.

NOTE: All reagents should be refrigerated in amber bottles and can be stored indefinitely or until turbidity or color develops.

ANALYSIS

- A. Pipette 1.0 mL of blank, standard, or filtrate into 12 x 75 mm borosilicate culture tubes in duplicate.
- B. Add 0.2 mL of 1.2 N HCl to all tubes, vortex.
- C. The following steps should be done with no more than 5 min between additions and tubes should be vortexed after each addition to all tubes:
 1. 0.1 mL sodium nitrite, vortex
 2. 0.1 mL ammonium sulfamate, vortex
 3. 0.1 mL coupling reagent, vortex

- D. Wait 10 min (minimum) for color development.
- E. Read absorbance on spectrophotometer at 540 nm.
- F. Calculations are made as a percentage of the standard.

PAH STOCK INFUSION SOLUTION (10%)

- A. Para-aminohippuric acid (Eastman Kodak Corp., cat. # 5704): **NOTE:** This product is no longer available; a substitute can be obtained from Sigma Chemical Co., cat.#A1422.
- B. Sodium Hydroxide (Fisher Scientific Co., cat. # S-318)
- C. 0.9% Physiological Saline Solution (PSS; Fisher Scientific Co., cat. # AB7138-09-19)
- D. Dissolve each reagent separately in PSS:
 - 1. 44.9 g of PAH
 - 2. 10.5 g of NaOH
- E. Add the NaOH solution to the PAH solution and stir (may have to heat gently to fully dissolve).
- F. Filter through Whatman #4 filter paper.
- G. Titrate filtrate to pH to 7.4 with 1 N HCl or 4 M NaOH.
- H. Bring final volume to 500 mL.
- I. Store 150 mL aliquots in sterile containers at -20°C.
- J. For 1.5% PAH infusion solution take a 150 mL aliquot and bring volume up to 1000 mL with PSS and filter through 0.22 micron filter into a sterile container (Nalgene MF75 Series filter units; Fisher Scientific Co., cat. # 09-740-2A).
- K. Store refrigerated (2-6°C) until used (be sure to allow infusate to warm to room temperature before infusion into animal).

APPENDIX 11

VOLATILE FATTY ACID ANALYSIS

VOLATILE FATTY ACID ANALYSIS

The principle of this method is chromatographic separation of volatile fatty acids based on the procedure of Reynolds et al. (1986; J. Anim. Sci. Suppl. 63(1):424) and modifications by Quigley and Heitmann (1991; J. Anim. Sci. 69:1178-1187) and Reynolds et al. (2002; J. Dairy Sci. 85:1804-1814). Sample preparation is performed using ion exchange chromatography to concentrate and separate VFA from other plasma compounds and sample analysis for VFA is performed using gas chromatography.

REAGENTS

NOTE: Always use the highest purity water when making up reagents.

A. H₂O/Triton-X mixture – 20 µL Triton X-100 (Sigma cat. # T9284) per 250 mL H₂O.

NOTE: triton X-100 is very viscous and difficult to accurately pipette.

B. 1 M NaOH – 40.0 g of NaOH per liter of water.

C. 10 mM NaOH – Dilute 5 mL of 1 M NaOH to 500 mL. **NOTE:** Make each day of use!

D. 1 M HCl – Dilute 83 mL of concentrated HCl to 1 liter.

E. 0.175 M (5%) Zinc Sulfate – 100g ZnSO₄ · 7H₂O (reagent grade; uneffervesced); dissolve in water to make 2 liters (can also use 0.3 N ZnSO₄; Sigma cat. # 14-4, 500 mL).

F. 0.15 M Barium Hydroxide – 95 g Ba(OH)₂ · 8H₂O; dissolve in recently boiled and cooled deionized H₂O to make 2 liters. Fairly stable kept in stoppered container and out of contact with air (can also use 0.3 N Ba(OH)₂; Sigma cat. #14-3, 500 mL).

Actual concentrations of the zinc and barium solutions are not so important, but they must exactly neutralize each other. To check this:

1. Pipette exactly 10 mL of ZnSO_4 solution to an Erlenmeyer flask containing approximately 40 mL of H_2O . Add 2 drops of phenolphthalein indicator (0.5 g/dL in 95% EtOH).
2. Slowly titrate with $\text{Ba}(\text{OH})_2$ solution, while under constant mixing (use a stirring bar in flask).
3. Titrate until 1 drop of $\text{Ba}(\text{OH})_2$ solution turns the titrated solution a faint pink that lasts approximately 1 minute.
4. The titration of 10.0 mL of ZnSO_4 should require 10.0 (± 0.05 mL) of $\text{Ba}(\text{OH})_2$ solution.
5. If solutions do not exactly neutralize, dilute or strengthen the ZnSO_4 solution to achieve neutrality. Do not attempt to alter the $\text{Ba}(\text{OH})_2$ solution.
6. Repeat titration ... if necessary.
7. Keep zinc and barium solutions tightly stoppered. $\text{Ba}(\text{OH})_2$ solution will have a white precipitate of BaCO_3 upon prolonged exposure to air.

G. 0.01 M Oxalic acid

H. Stock blood standard – The stock solution used is composed of:

Acid	MW	Approximate g/L	Approx. final conc. after dilution (mM)
Acetate	60.0	3.0	2.0
Propionate	74.0	0.9	0.5
Butyrate	88.1	0.8	0.4
Isobutyrate	88.1	0.4	0.2
Valerate	102.1	0.4	0.15
Isovalerate	102.1	0.4	0.15

- I. Working blood standard** – Add 4 mL of stock blood standard and 4 mL of stock internal standard to 100 mL volumetric and q.s. to volume with 0.01 M oxalic acid.

- J. Stock internal standard (6.56 mM) – Weigh 0.0762 g of 2-ethylbutyric acid (MW = 116.16) and q.s. to 100 mL with water.
- K. Working internal standard (WISTD; 1.31 mM) – Add 50 mL of stock internal standard to a 250 mL volumetric flask. *Quantum sufficit* to volume with water.

SAMPLE COLLECTION AND STORAGE

- A. Serum – Blood drawn into a tube which does not contain an anticoagulant and allowed to clot. Serum is separated from clot and may be stored refrigerated for several days (2 – 6°C) or frozen.
- B. Plasma – Add whole blood directly to a tube containing an anticoagulant. Most common anticoagulants may be used. May be stored frozen (-20°C).

ION EXCHANGE MATERIALS

A. Materials:

1. AG 50W-X8: 100-200 mesh cation resin; top column (Bio-Rad cat. # 142-1441).
2. AG 4-X4: free base form; 100-200 mesh anion resin; bottom column (Bio-Rad cat. #140-4341).
3. Polypropylene Econo-Column (Bio-Rad cat. # 731-1550)

B. Packing:

1. Pack columns by preparing a slurry of resin in water. Allow resin to settle and remove fines with a transfer pipette or by pouring off. Stir up and allow to settle two more times. Remove fines each time.
2. Agitate slurry by swirling with a transfer pipette and transfer into columns:
 - a. Cation column: 1.8 mL resin.
 - b. Anion column: 0.6 mL resin (may add slightly more, as resin will shrink).

C. Column Regeneration:

1. Cation (top column):
 - a. Fill reservoir (10 mL) with 1M NaOH. Stir up resin (with first filling)
 - b. Rinse twice with water
 - c. Fill reservoir with 1M HCl twice
 - d. Rinse twice with water, or until the effluent is neutral.
2. Anion (bottom column):
 - a. Fill reservoir with 1M NaOH twice. Stir up resin with first filling.
 - b. Rinse with water twice.

D. NOTES:

1. Always keep columns covered with water (when leaving overnight etc.). If allowed to sit overnight or longer, ALWAYS leave water over the resin bed and plug the outlet (Do not let the resins dry).
2. Prior to use, stir up resins with a transfer pipette. This will minimize the establishment of channels through the resins.
3. Resins have a very long lifespan. If cared for properly, > 100 samples can pass through resin without affecting performance. When resin has degraded beyond use the flow rate rapidly decreases to zero.

SAMPLE PREPARATION

A. Add the following to 50 mL centrifuge tubes:

1. 6 mL water/Triton-X.
2. 2 mL whole blood or plasma.
3. Vortex and let stand for 5 minutes.
4. Add 4 mL of barium hydroxide; vortex; and let stand for 5 minutes.
5. Add 4 mL of zinc sulfate; vortex; and let stand for 5 minutes.
6. Centrifuge for 15 minutes at 15,000 x g

B. To a second tube (50 mL – polypropylene), add the following:

1. 0.4 mL of WISTD.
2. Decant supernatant from above centrifugation step.
3. Vortex.

ASSAY PROCEDURE

- A. Regenerate columns as above; store resins in water until ready for use.
- B. Unplug the outlet of each column and resin should immediately be stirred with a Pasteur pipette. As mentioned above, this will prevent air bubbles and promote an even flow through the column. After water has completely drained through, place cation tubes on top of anion columns.
- C. Pour supernatant/WISTD mixture into top of cation column. Allow sample to drip through both columns simultaneously and completely.

NOTES:

1. All liquid will not go onto the column at once.
 2. Pour liquid slowly and deliberately. Try to disturb the top of the resin bed as little as possible. May be advantageous to add initial amounts in a drop-wise manner and then pour in remaining sample.
-
- D. Rinse empty tubes twice with 2 mL of water and pour into columns. Let each rinse drip through completely before adding the next. Do not collect effluent.
 - E. Rinse top (cation) column once with 2 mL of water (being careful to rinse down the sides of column and avoid stirring up resin bed). Allow to drip through. Do not collect effluent.
 - F. Remove the top column. Plug each column and fill with water.
 - G. Rinse bottom (anion) column once with 2 mL of water (being careful to rinse down the sides of column and avoid stirring up resin bed). Allow to drip through. Do not collect effluent.

H. Place storage vials beneath each anion column. Add 10 mL of 10 mM NaOH to each column and allow to drip through completely.

NOTE: The pH of the collected liquid should be above 11 to minimize the loss of VFA. Check a minimum of 1 sample per rack (if required adjust with 10 mM NaOH).

I. Place vials in convection oven until dry (overnight); temperature should not exceed 50°C.

NOTES:

1. Do not allow samples to remain in oven for extended periods of time.
2. Samples may also be lyophilized – collect in plastic storage vials, freeze vials, and then freeze dry overnight.

J. Reconstitute with 1 mL 0.01 M oxalic acid and mix.

K. Add 0.1 mL of regenerated cation resin to solution.

L. Allow sample to stand for one hour

M. Filter solution using a 0.45 µm syringe filter and transferred to a gas chromatograph vial and cap.

Alternative method: Transfer sample to microcentrifuge tube and centrifuge for 5 minutes. Pipette supernatant from tube (being careful not to disturb the resin “pellet”) into a gas chromatograph vial and cap.

GAS CHROMATOGRAPHY

A. Use an J&W Scientific HP-FFAP gas chromatography column 10 m x 0.53 mm x 1.0 µm capillary column (Agilent Technologies Inc.; Palo Alto, CA).

B. Carrier gas – helium at 100 mL/minute (head pressure at 5 psi); flame ionization detector - using a Hewlett Packard model 5890 gas chromatograph, with an automatic

sampler model 7673A and a HP 6890 Series integrator (Hewlett Packard, Wilmington, DE).

C. Injection volume = 1 μ L (splitless injection)

D. Oven temperature = 80°C; injector temperature = 180°C; detector temperature = 250°C

E. Run time is 30 minutes.

F. Sample acid concentrations corrected for recovery of internal standard.

VITA

James Levi Klotz, III was born in Bridgeport, West Virginia on April 24, 1974. He spent his first seven years growing up in Clarksburg, WV, then four years in Pendleton, New York, three years in Humacao, Puerto Rico, and four years in Columbia, Tennessee. He received his high school diploma from Columbia Central High in June, 1992. He then entered The University of Tennessee, Knoxville majoring in Animal Science. He received a Bachelor of Science degree, in Agriculture from The University of TN, Knoxville in May, 1996. He married Ursula Adell Stricklin of Savannah, TN on July, 19, 1997. Remaining at The University of TN, Knoxville, he began his graduate work under the tutelage of Dr. R.N. Heitmann in Ruminant Nutrition. He received his Master of Science degree with a major in Animal Science in December, 1999 and continued his graduate work with Dr. Heitmann as a doctoral student. He received his Doctor of Philosophy degree, with a major in Animal Science in May, 2004.