Nutrient Synchrony
in Preruminant Calves

Joost van den Borne

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Abstract

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In animal nutrition, the nutrient composition of the daily feed supply is composed to match the nutrient requirements for the desired performance. The time of nutrient availability within a day is usually considered not to affect the fate of nutrients. The aim of this thesis was to evaluate effects of the time of nutrient availability within a day (i.e. nutrient synchrony) on the protein and energy metabolism in preruminant calves. Two types of nutrient synchrony were studied: (1) synchrony between total nutrient supply and requirements within a day, and (2) synchrony between protein and carbohydrate availability. The studies were mainly conducted in heavy preruminant calves, because those animals have a very low efficiency of protein utilization for growth compared with other farm animals, such as pigs and lambs, allowing a large potential for improvement. Increasing the feeding frequency increased the efficiency of protein utilization in preruminant calves. This was however not detected when short-term measurements of amino acid metabolism (12 h urea production and oral leucine oxidation) were considered. Dietary carbohydrates were almost completely oxidized, unaffected by feeding level, in heavy preruminant calves. Glucose homeostasis improved with increasing feeding frequency. In pigs, an asynchronous availability of glucose and amino acids within a day reduced protein utilization but did not affect fat retention. In preruminant calves, however, an asynchronous availability of glucose and amino acids did not decrease the efficiency of protein utilization but substantially increased fat retention. Separating the intake of protein and lactose over meals inhibited postprandial plasma insulin responses, but increased glucose excretion in urine. Intramuscular fat content and oxidative enzyme activity increased with decreasing nutrient synchrony in an oxidative muscle in calves. Oxidative enzyme activity is not an appropriate indicator of whole-body heat production in growing calves.

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Chapter 1

General introduction

Feed evaluation and nutrient requirements

Animals and man require nutrients to maintain essential physiological processes and to deposit new body tissues. The use of macronutrients (carbohydrates, fat and protein) for maintenance and growth processes has become a well visited research area in many species. Requirements for energy and protein have been extensively studied and were defined for man (FAO/WHO/UNU, 2004), companion animals (NRC, 1985; NRC, 1986), laboratory animals (NRC, 1995) and farm animals (NRC, 1998; CVB, 2000; NRC, 2001). In addition to the nutrient requirements, the nutrient composition of dietary ingredients should be known to formulate balanced diets which accurately meet the daily needs for the desired performance. In animal nutrition, feedstuffs are classically characterized by their chemical composition (e.g. crude protein, crude fat, crude fibre and starch), and average values are adopted in feed evaluation tables (e.g. CVB, 2000; Beyer *et al.*, 2003). These tabulated values, or analyzed values of a specific batch of feedstuffs, are used to compose diets and to match dietary nutrient supply with nutrient requirements on a daily basis.

Nutrient dynamics

Despite supplying nutrients to the level of the average daily requirements, there may be periods within a day during which this harmony is disturbed by circadian variation in nutrient requirements and availability. The requirements for energy and protein depend on the physiological situation of the animal which is obviously not constant during a day. For example, physical activity in farm animals varies within a day and affects the energy requirements (e.g., Schrama *et al.*, 1996). Although some circadian variation in nutrient requirements can be expected, it is generally assumed that most metabolic processes, especially maintenance processes, occur continuously and are relatively constant throughout the day.

Nutrient absorption, on the other hand, can be rather peak-wise and may also differ between individual macronutrients. Fluctuations in nutrient availability are induced by the feeding pattern and by the characteristics of dietary ingredients. Firstly, the feeding pattern is often discontinuous and divided in a few meals. Farm animals such as sows and veal calves, but also companion animals, such as cats and dogs, and man are usually meal-fed. Circadian fluctuations in nutrient availability will therefore occur as a consequence of feeding these discrete meals. Also when feed was provided *ad libitum* to growing pigs (Bruininx *et al.*,

2002; Hyun & Ellis, 2002; Whittemore *et al.*, 2002) and veal calves (Senn *et al.*, 2000), as well as to grazing dairy cows (Taweel *et al.*, 2006) and sheep (Orr *et al.*, 1997), circadian patterns of feed intake were observed. It is evident, however, that feed intake is much more constant during the day in animals which have *ad libitum* access to feed than in meal-fed animals. Secondly, the characteristics of dietary ingredients determine the kinetics of nutrient availability after feed intake in non-ruminants. The rate of passage and/or digestion of specific ingredients impacts on the time of post-absorptive availability of nutrients within a day. The physical form of carbohydrates (Weurding, 2002; Englyst & Englyst, 2005) and proteins (Smith & Sissons, 1975; Minnekus, 1998) affect the rates of passage and digestion prior to nutrient absorption in monogastric animals. In ruminants, this effect is less clear, because nutrient absorption is delayed by fermentation in the rumen compartment. Studies on the time of macronutrient availability in ruminants have therefore mainly focused on matching the supply of nitrogen and energy yielding substrates to the ruminal microbes instead of on post-absorptive availability (Richardson *et al.*, 2003; Valkeners *et al.*, 2004).

In non-ruminants, however, it is not well known whether the time at which nutrients are absorbed within a day is critical for their utilization for maintenance and growth processes. Feed is ingested in a pulsatile manner that not only results in fluctuations in nutrient availability, but also affects circulating hormone concentrations (Dawson, 1999). Finally, the capacity of the animal's metabolism to utilize the available nutrients either directly or after a temporary storage will determine consequences of a fluctuating nutrient supply for nutrient utilization. In this thesis, the concept of matching the timing of absorption of nutrients and the metabolic demand of nutrients within a day is defined as nutrient synchrony.

Nutrient synchrony

Three types of nutrient synchrony are distinguished. The first type of nutrient synchrony concerns the matching of the supply of nutrients with the requirements for growth and maintenance processes within a day. As most metabolic processes are assumed to take place continuously, it can be expected that type 1 asynchrony occurs when animals are fed at a low frequency (Figure 1A). Especially for nutrients for which the animal has a limited capacity for deposition, like amino acids, there may be a temporal oversupply relative to the requirements, resulting in oxidative losses and thus a lower efficiency of nutrient utilization. Moreover, nutrients from quickly hydrolysed ingredients will amplify this asynchrony at a low feeding frequency.
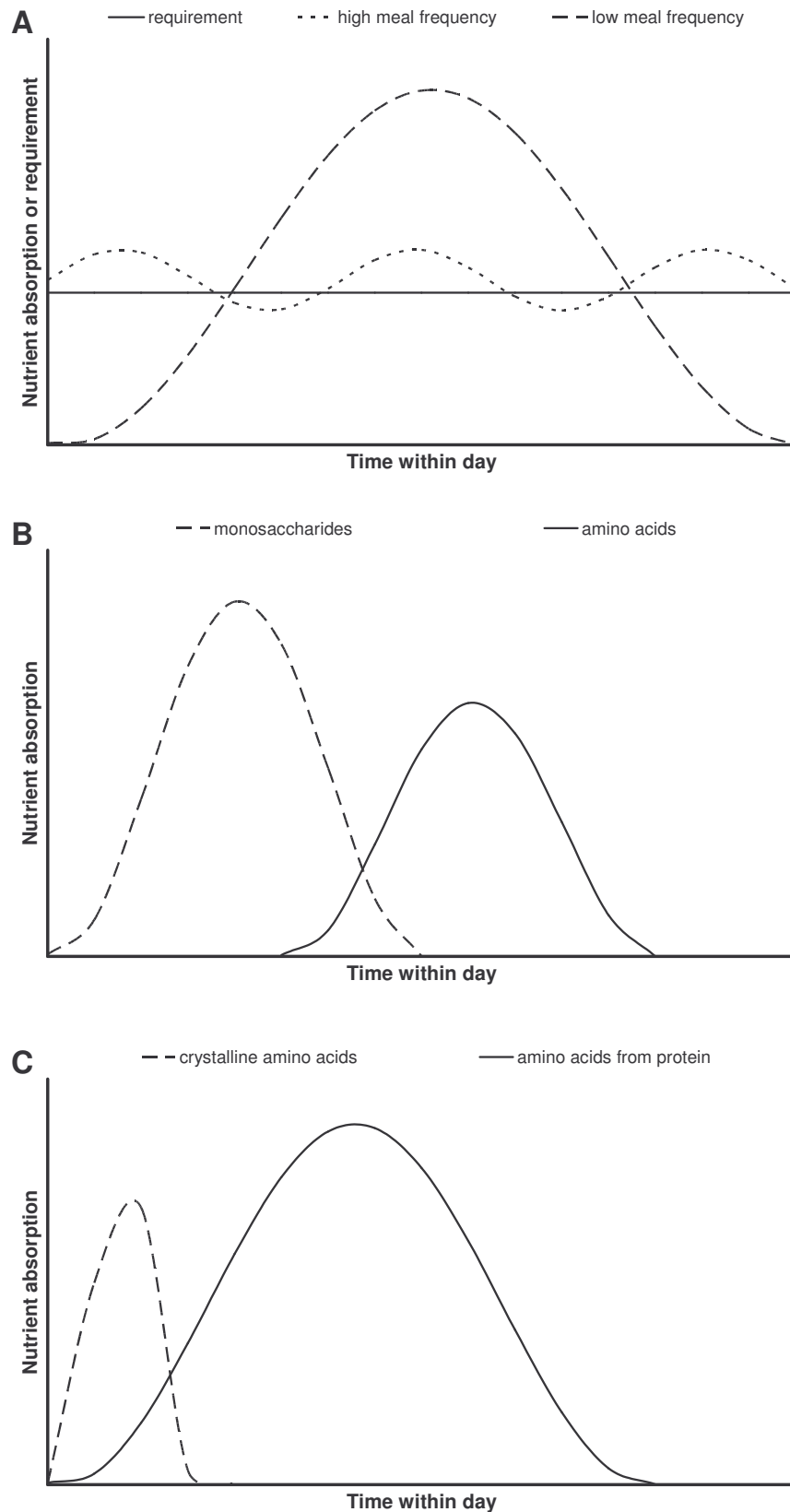
Figure 1. The three types of nutrient synchrony: synchrony of nutrient supply and requirements within a day (type 1; panel A), synchrony of the supply of individual nutrients, such as glucose and protein (type 2; panel B), and synchrony of the supply of constituents of individual nutrients, such as amino acids (type 3; panel C).

The second type of nutrient synchrony concerns the simultaneous availability of protein (i.e. amino acids) and glucose (Figure 1B). A separation in intake between macronutrients within a day or a difference in the kinetics of protein versus carbohydrate digestion and absorption can induce a type 2 asynchrony. When utilization of one macronutrient depends on the availability of the other, this type of asynchrony can affect the nutrient partitioning in growing animals. The third type of nutrient synchrony can be important when protein-bound and free, crystalline amino acids are simultaneously fed. To obtain an optimal amino acid composition for performance, animal diets are often supplemented with single, indispensable amino acids in crystalline form. These amino acids, however, do not have to be hydrolyzed from dietary protein, which may cause a time lag between the absorption of the crystalline and the originally protein-bound amino acids (Figure 1C). In this thesis, the first two types of nutrient synchrony are further explored.

Preruminant calf as a model

The heavy preruminant calf, i.e. exceeding 100 kg of body weight, was used as a model to study effects of nutrient synchrony on nutrient utilization in this thesis. In preruminant calves, milk bypasses the non-functional rumen by closure of the oesophageal groove. As a consequence, nutrients are not subjected to rumen fermentation. The main nutrients in a calf milk replacer (lactose, fat and protein) are digested by enzymatic pathways (pregastric enzymes, pancreatic enzymes and brush-border enzymes), and amino acids, peptides, long chain fatty acids, glucose and galactose are absorbed like in monogastric animals. Hence, unlike in ruminants, nutrient absorption in preruminants closely follows their intake pattern. Intestinal and colonic fermentation are limited in the preruminant calf. Milk replacer diets are devoid of non-starch polysaccharides, whereas diets for pigs and poultry usually contain more than 10% of non-starch polysaccharides. The preruminant calf was chosen as a model for two main reasons.

First, nutrient ingestion in preruminant calves occurs in a pulsatile manner. Calves are commonly fed twice daily and nutrient absorption can be expected to be relatively peak-wise (as described above). Moreover, non-clotting protein sources, like vegetable proteins or whey, have increasingly replaced skimmed milk protein in calf milk replacers during the past decades. The higher net portal amino acid flux after feeding non-clotting protein sources than after feeding clotting protein sources in preruminant calves (Verdonk *et al.*, 1999) indicates potential interactions between the protein source and frequency of feed supply. Diurnal

patterns of nutrient absorption are substantially affected by the ingredients incorporated in calf milk replacers. Absorption kinetics are more pronounced (viz. larger diurnal fluctuations) when calves are fed non-clotting protein sources than when protein sources are used which clot in the abomasum.

Second, heavy preruminant calves utilize dietary protein very inefficiently for protein gain. Gerrits et al. (1996) showed that approximately 70% of an increase in N intake was excreted in urine, allowing a large potential to increase protein utilization in heavy preruminant calves. The inefficiency in protein utilization in heavy preruminant calves is greater that that in several other farm animals at a similar stage of maturity (Chapter 2).

Developments in veal production

Young preruminant (male) calves from dairy farms are commonly transported to specialized veal farms where milk replacer diets are provided until slaughter weight is reached. The produced veal is characterized by its paleness and tenderness. During the past 25 years, the development of the Dutch veal industry did not follow that of the dairy industry. Although the number of dairy farms decreased almost threefold, from about 67.000 in 1980 to less than 24.000 in 2005, the number of veal farms increased from approximately 2.800 to more than 3.300 during the same period (Figure 2A; CBS, 2006). Similarly, the number of dairy cows decreased by almost 1 million to less than 1.5 million in 2005, whereas the number of calves increased from 582.000 to 829.000 (Figure 2B; CBS, 2006). It should be mentioned, however, that this counting of livestock was done at a single moment (April 2005), whereas almost two batches of calves can be finished in one year on each farm. This ultimately resulted in 1.4 million slaughtered calves in 2005 (of which 82% were veal calves; PVE, 2006), which almost equals the number of dairy cows present in The Netherlands. Therefore, the import of young calves from neighbouring countries, amounting to almost 650.000 in 2004 (CBS, 2006), has allowed the sector to develop independently of the dairy sector. Apart from the numeric size of the sector, also veal calf husbandry and nutrition have been subject to major changes throughout the years. Following EU guidelines, national legislation (Kalverbesluit, 1997) has defined a minimal surface area which has to be provided to each calf and obliged the intake of roughage in addition to milk feeding. From 2004, all calves older than 8 weeks are group-housed.
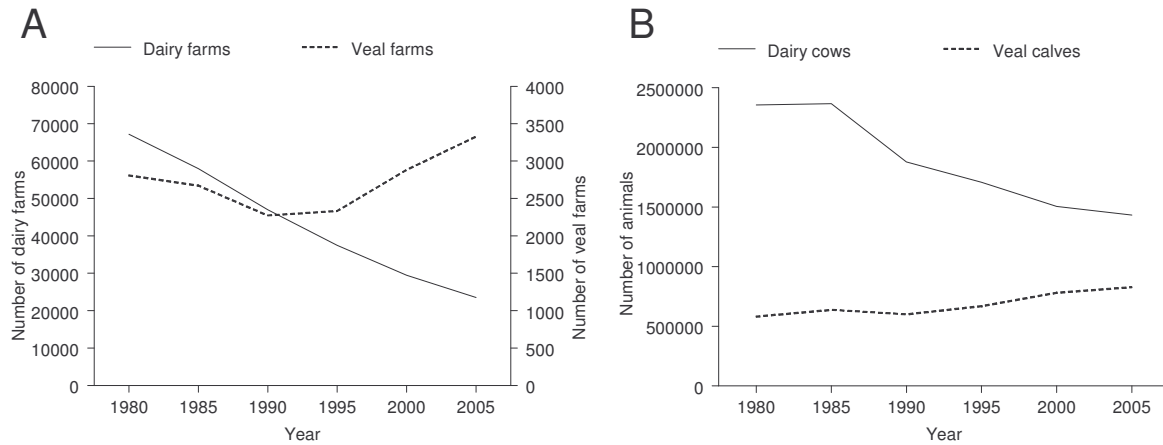
Figure 2. Development of the number of dairy and veal farms (panel A) and the number of dairy cows and veal calves (panel B) in The Netherlands during the last 25 years (CBS, 2006).

The group-housing of calves, as well as the advancing technical possibilities, have increased the use of computer-controlled milk feeding systems for dairy calves and for veal calves (Hepola, 2003; Jensen, 2006). As a result, within-day feeding patterns can be more easily manipulated and concepts of nutrient synchrony can be directly applied in practical calf nutrition. From a nutritional point of view, effects of replacing skimmed milk protein by vegetable proteins on digestive processes have been extensively studied. However, the use of non-milk proteins also results in different absorption kinetics and post-absorptive consequences of within-day absorption kinetics are not clear yet.

Aim of the research and outline of the thesis

The main objective of the work described in this thesis was to quantify the effects of nutrient synchrony on protein and fat deposition, using the preruminant calf as a model. First, the protein utilization in heavy preruminant calves is reviewed in Chapter 2. Then, effects of a type 1 nutrient synchrony (nutrient supply vs. nutrient requirements) on nitrogen and energy balance (Chapter 3) and within-day nutrient oxidation (Chapter 4) are described in a feeding frequency study. Effects of a type 2 nutrient synchrony (separation of individual nutrient availability in time) on protein and energy utilization are subsequently described for growing pigs (Chapter 5) and preruminant calves (Chapter 6). In Chapter 7, the influence of type 2 nutrient synchrony on muscle composition and muscle enzyme activities in preruminant calves is presented. Finally, the general discussion (Chapter 8) focuses on the consequences

of nutrient synchrony for glucose homeostasis in preruminant calves. The main hypotheses in this thesis were:

Type 1 nutrient synchrony

-   It was hypothesized that an increased feeding frequency would increase protein deposition in heavy preruminant calves when a non-clotting protein source is used. In addition, effects on protein utilization were expected to be more pronounced at a high than at a low feeding level (Chapter 3).

-   Urea production rates and oxidation of orally supplied L-[1-^{13}C]leucine were expected to mirror, inversely, the efficiency of protein utilization from the nitrogen balance measurements in Chapter 3 (Chapter 4).

-   It was hypothesized that substantial amounts of glucose were used for *de novo* fatty acid synthesis in heavy preruminant calves (Chapter 4).

-   Increasing the feeding frequency was expected to improve glucose homeostasis and decrease urinary glucose excretion (glucosuria) in heavy preruminant calves (Chapter 8).

Type 2 nutrient synchrony

-   Synchronizing protein and starch intake within a day was hypothesized to decrease protein retention in growing pigs. A decrease in protein retention will result in either in an increase in fat deposition or an increase in heat production (Chapter 5).

-   Synchronizing protein and lactose intake within a day was hypothesized to decrease protein retention in heavy preruminant calves. A decrease in protein retention will result in either in an increase in fat deposition or an increase in heat production (Chapter 6).

-   It was hypothesized that oxidative enzyme activities would decrease and intramuscular fat content would increase with decreasing nutrient synchrony in preruminant calves. Oxidative enzyme activities in skeletal muscle were expected to be correlated with average whole body heat production in heavy preruminant calves (Chapter 7).

-   Higher responses in plasma glucose and insulin concentrations were expected after a high lactose than after a high protein meal. It was hypothesized that synchronizing protein and lactose supply would improve glucose homeostasis and decrease glucose excretion in urine in preruminant calves (Chapter 8).

References

Beyer M, Chudy A, Hoffmann L, Jentsch W, Laube W, Nehring K & Schiemann R (2003) *Rostock feed evaluation system*. Miltenberg-Frankfurt: Plexus Verlag.

Bruininx EMAM, Heetkamp MJW, Van den Bogaart D, Van der Peet-Schwering CMC, Beynen AC, Everts H, Den Hartog LA & Schrama JW (2002) A prolonged photoperiod improves feed intake and energy metabolism of weanling pigs. *J Anim Sci* **80**, 1736-1745.

CBS (2006) Landbouwtelling.

CVB (2000) *Chemical composition, digestibility and feeding value of feedstuffs (in Dutch)*. Lelystad, The Netherlands: Centraal Veevoeder Bureau.

Dawson JM (1999) Variation in nutrient supply and effects on whole body anabolism. *In: Protein Metabolism and Nutrition Proceedings of the VIII*[th] *International Symposium on Protein Metabolism and Nutrition* [GE Lobley, A White and JC MacRae, editors]. Wageningen Pers, pp 101-126.

Englyst KN & Englyst HN (2005) Carbohydrate bioavailability. *Br J Nutr* **94**, 1-11.

FAO/WHO/UNU EC (2004) *Human energy requirements*. Rome, Italy: FAO.

Gerrits WJJ, Tolman GH, Schrama JW, Tamminga S, Bosch MW & Verstegen MWA (1996) Effect of protein and protein-free energy intake on protein and fat deposition rates in preruminant calves of 80 to 240 kg live weight. *J Anim Sci* **74**, 2129-2139.

Hepola H (2003) Milk feeding systems for dairy calves in groups: Effects on feed intake, growth and health. *Appl Anim Behav Sci* **80**, 233-243.

Hyun Y & Ellis M (2002) Effect of group size and feeder type on growth performance and feeding patterns in finishing pigs. *J Anim Sci* **80**, 568-574.

Jensen MB (2006) Computer-controlled milk feeding of group-housed calves: The effect of milk allowance and weaning type. *J Dairy Sci* **89**, 201-206.

Kalverbesluit (1997) Besluit van 22 september 1997, houdende wijziging van het Kalverbesluit. Staatsblad van het Koninkrijk der Nederlanden, 's Gravenhage. Besluit 478.

Minnekus M (1998) Development and validation of a dynamic model of the gastrointestinal tract. PhD thesis, Utrecht University.

NRC (1985) *Nutrient requirements of dogs. Revised edition*. Washington DC, USA: National Academy Press.

NRC (1986) *Nutrient requirements of cats. Revised edition*. Washington DC, USA: National Academy Press.

NRC (1995) *Nutrient requirements of laboratory animals. Fourth revised edition*. Washington D.C., USA: National Academy Press.

NRC (1998) *Nutrient requirements of swine. Tenth revised edition*. Washington DC, USA: National Academy Press.

NRC (2001) *Nutrient requirements of dairy cattle. Seventh revised edition*. Washington DC, USA: National Academy Press.

Orr RJ, Penning PD, Harvey A & Champion RA (1997) Diurnal patterns of intake rate by sheep grazing monocultures of ryegrass of white clover. *Appl Anim Behav Sci* **52**, 65-77.

PVE (2006) *Vee, vlees en eieren in Nederland 2005*. 's-Gravenzande: Van Deventer.

Richardson JM, Wilkinson RG & Sinclairs LA (2003) Synchrony of nutrient supply to the rumen and dietary energy source and their effects on the growth and metabolism of lambs. *J Anim Sci* **81**, 1332-1347.

Schrama JW, Verstegen MWA, Verboeket PHJ, Schutte JB & Haaksma J (1996) Energy metabolism in relation to physical activity in growing pigs as affected by type of dietary carbohydrate. *J Anim Sci* **74**, 2220-2225.

Senn M, Gross-Lüem S, Leuenberger H & Langhans W (2000) Meal patterns and meal-induced metabolic changes in calves fed milk ad lib. *Physiol Behav* **70**, 189-195.

Smith RH & Sissons JW (1975) The effect of different feeds, including those containing soya-bean products, on the passage of digesta from the abomasum of the preruminant calf. *Br J Nutr* **33**, 329-349.

Taweel HZ, Tas BM, Smit HJ, Tamminga S & Elgersma A (2006) A note on eating behaviour of dairy cows at different stocking systems - diurnal rhythm and effects of ambient temperature. *Appl Anim Behav Sci* **98**, 315-322.

Valkeners D, Théwis A, Piron F & Beckers Y (2004) Effect of imbalance between energy and nitrogen supplies on microbial protein synthesis and nitrogen metabolism in growing double-muscled Belgian Blue bulls. *J Anim Sci* **82**, 1818-1825.

Verdonk JMAJ, Gerrits WJJ, Beelen GM & Jansman AJM (1999) Effect of protein source on portal nutrient fluxes in pre-ruminant calves. *In: The VIIIth International Symposium on Protein Metabolism and Nutrition* [GE Lobley, A White and JC MacRae, editors]. Wageningen Pers, The Netherlands, pp 47 (Abstr).

Weurding RE (2002) Kinetics of starch digestion and performance of broiler chickens. PhD thesis, Wageningen University.

Whittemore EC, Kyriazakis I, Tolkamp BJ & Emmans GC (2002) The short-term feeding behavior of growing pigs fed foods differing in bulk content. *Physiol Behav* **76**, 131-141.

Chapter 2

Reviewing the low efficiency of protein utilization in heavy preruminant calves – a reductionist approach

JJGC van den Borne[1], JMAJ Verdonk[2], JW Schrama[3]
& WJJ Gerrits[1]

[1]*Animal Nutrition Group and* [3]*Aquaculture and Fisheries Group, Department of Animal*

Sciences, Wageningen University. P.O. Box 338, 6700 AH, Wageningen, The Netherlands

[2]*CCL Research, Department Nutrition and Feed Research, P.O. Box 107, 5460 AC, Veghel,*

The Netherlands

Abstract

The efficiency of protein utilization for growth in preruminant calves is decreasing with increasing body weight. In contrast to calves weighing less than 100 kg of body weight, heavy preruminant calves do not respond in protein retention to an increased intake of indispensable amino acids in dose-response studies. The marginal efficiency of protein utilization is low compared with pigs and milk-fed lambs at a similar stage of maturity. A reductionist approach was taken to perceive the potential mechanisms for the low protein utilization in preruminant calves. Neither an imbalance in the dietary protein to energy ratio nor a single limiting indispensable amino acid was responsible for the low efficiency. Also, amino acids were not specifically used to detoxify ammonia. Alternative hypotheses to explain the low efficiency are discussed and result in (i) a reduced post-absorptive supply of amino acids: e.g. by fermentation of milk in the (premature) rumen or preferential amino acid utilization by specific tissues; or (ii) a reduced post-absorptive amino acid utilization: e.g. by decreased insulin sensitivity, utilization of amino acids for gluconeogenesis or an asynchronous nutrient supply. In conclusion, several mechanisms for the low efficiency of protein utilization in heavy preruminant calves were excluded. Other physiological processes which are potentially involved remain to be studied, because the large potential for improving protein utilization in heavy preruminant calves asks for further exploration of their amino acid metabolism.

Introduction

In contrast to the comprehensive work on protein digestion in milk-fed calves (Toullec & Guilloteau, 1989; Lallès *et al.*, 1995; Le Huërou-Luron *et al.*, 1998; Montagne *et al.*, 2002; Verdonk *et al.*, 2002b), the post-absorptive utilization of amino acids has only been scarcely investigated. This lack of attention is rather surprising, since only about 10% of the ingested nitrogen is lost with faeces, and an additional 40–50% with urine. Furthermore, the efficiency of protein utilization decreases with increasing body weight (BW) in preruminant calves. Although this reduction is in accordance with the decreasing amino acid utilization for protein accretion with increasing stage of maturity (Grizard *et al.*, 1999), the efficiency in heavy preruminant calves is extremely low compared with other species. In pigs, for example, gross efficiencies of protein utilization of 60% up to as high as 81% were reported (Chung & Baker, 1992; Markert *et al.*, 1993; Gahl *et al.*, 1995; Mnilk *et al.*, 1996). For a sound comparison of efficiencies, however, different species should be compared at a similar stage of maturity, and marginal efficiencies, rather than gross efficiencies, should be compared. The marginal efficiency expresses the response of protein deposition rate to increased digestible protein intake, and consequently excludes digestion inefficiency and amino acid requirements for maintenance. It has the additional advantage of showing less between-experiment variation than the gross efficiency and is thus more suitable for comparison across literature sources.

Figure 1 shows the marginal efficiency with which digestible protein, or the first limiting amino acid, was used for protein gain in pigs, preruminant lambs and preruminant calves during (early) development. The protein mass, as the percentage of protein mass at maturity, is used to indicate the stage of maturity of each species. Assumptions for the mature protein mass were based on literature values for pigs (Knap, 2000), sheep (Freetly *et al.*, 1995; Freetly *et al.*, 2002), and Holstein-Friesian bulls (Calo *et al.*, 1973). Marginal efficiencies vary substantially between studies and even within species at a similar stage of maturity. It is, however, illustrated that the marginal efficiency of protein retention is remarkably low in milk-fed calves as compared to pigs and milk-fed lambs. The differences are more pronounced with increasing protein mass. An exception in the figure are the control pigs of Krick et al. (1993) that had a marginal efficiency of only 32%, but an energy constraint to protein deposition could not be excluded in that study.

This paper reviews the work, performed at the TNO Nutrition and Food Research Institute, ILOB, Wageningen, The Netherlands, over the last twenty years (in part unpublished), in which the low efficiency of protein utilization in heavy preruminant calves was established.

Following a reductionist approach and based on experimental evidence, various factors are excluded as reasons for the low efficiency. Alternative mechanisms are presented and discussed.

Figure 1. Marginal efficiency of protein retention with increasing maturity in pigs (●; [1]Hennig et al., 1982; [2]Campbell & Dunkin, 1983; [3]Noblet et al., 1987; [4]Batterham et al., 1990; [5]Krick et al., 1993; [6]Bikker, 1994; [7]Dourmad et al., 1996; [8]Mnilk et al., 1996; [9]Williams et al., 1997; [10]Möhn et al., 2000; [11]De Lange et al., 2001; [12]Nieto et al., 2002), preruminant lambs (Δ; [13]Walker & Faichney, 1964; [14]Black et al., 1973; [15]Phillips & Walker, 1980), and preruminant calves (■; [16]Donnelly & Hutton, 1976; [17]Gerrits et al., 1996; [18]Tolman & Beelen, 1996; [19]Blome et al., 2003).

The age dependency of protein utilization in preruminant calves

Although Donnelly & Hutton (1976) found a marginal efficiency of protein utilization of only 45% in young calves (40-70 kg BW), other studies reported substantially higher marginal efficiencies of 66-69% (Tolman & Beelen, 1996; Blome *et al.*, 2003). Furthermore, from various sources (Ternouth *et al.*, 1985; Van Weerden & Huisman, 1985; Keusenhoff, 1992), gross efficiencies of digestible protein utilization of 60-68% could be calculated for preruminant calves of similar BW.

The clear response of protein gain to increased protein intake in young calves was confirmed in amino acid requirement studies (Tolman & Wiebenga, 1991; Tolman *et al.*, 1991). The

amino acids studied were lysine, threonine and methionine+cysteine, and all experiments were performed with preruminant, male Holstein-Friesian × Dutch-Friesian crossbreeds, measuring nitrogen balance as the response variable to increased intakes of the amino acid of interest. Skimmed milk protein and crystalline amino acids were used as the only protein sources. The dietary crude protein content depended on the BW range of the calves and decreased from 220 g/kg for calves between 50 and 70 kg BW to 180 g/kg for the calves between 220 and 250 kg BW. Skimmed milk protein provided up to 160 g/kg of the diet for calves between 50 and 70 kg, and up to 120 g/kg for the calves between 220 and 250 kg. The remaining protein (60 g/kg) was provided as crystalline amino acids, except for the amino acid of study which was added in graded levels. In total, crystalline amino acids provided maximally 70 g/kg of the diet (a mixture of indispensable and dispensable amino acids). A quadratic response of nitrogen balance to increased intakes of lysine and methionine+cysteine was obtained for preruminant calves between 50 and 70 kg BW ($P < 0.01$) (Tolman, 1996). Similar studies were performed in heavy preruminant calves between 120 and 260 kg BW (Tolman & Wiebenga, 1991; Tolman *et al.*, 1991). Interestingly, responses of nitrogen balance to increased intakes of lysine and methionine+cysteine in heavy preruminant calves (120-260 kg BW) were absent (Figure 2). Nitrogen balance only responded (slightly) to lysine intake in the range of 120-140 kg BW ($P < 0.05$). Possible explanations for the lack of response in heavy preruminant calves to an increasing amino acid intake include the following: (1) the use of large quantities of dispensable crystalline amino acids in these studies, and (2) a protein-energy imbalance in these amino acid requirement studies. Crystalline amino acids were included because skimmed milk powder was used as the main protein source, complicating a design of diets limited in specific indispensable amino acids. The possibility of an imbalanced protein to energy ratio was subsequently studied in serial slaughter experiments (Gerrits *et al.*, 1996).

Protein to energy imbalance

In pigs, much more than in preruminant calves, research efforts have concentrated on the interaction between dietary protein and dietary energy (Campbell *et al.*, 1985; Black *et al.*, 1986; Dunkin *et al.*, 1986). At low protein intakes, growing pigs will preferentially use absorbed amino acids with a high efficiency for deposition in the body. With increasing intakes, protein retention will increase almost linearly up to the point at which other factors than protein intake limit protein retention. When maximum protein deposition has not been

reached, energy intake is the limiting factor at this breakpoint in the curve. Any further increase in protein intake would not result in additional protein retention.
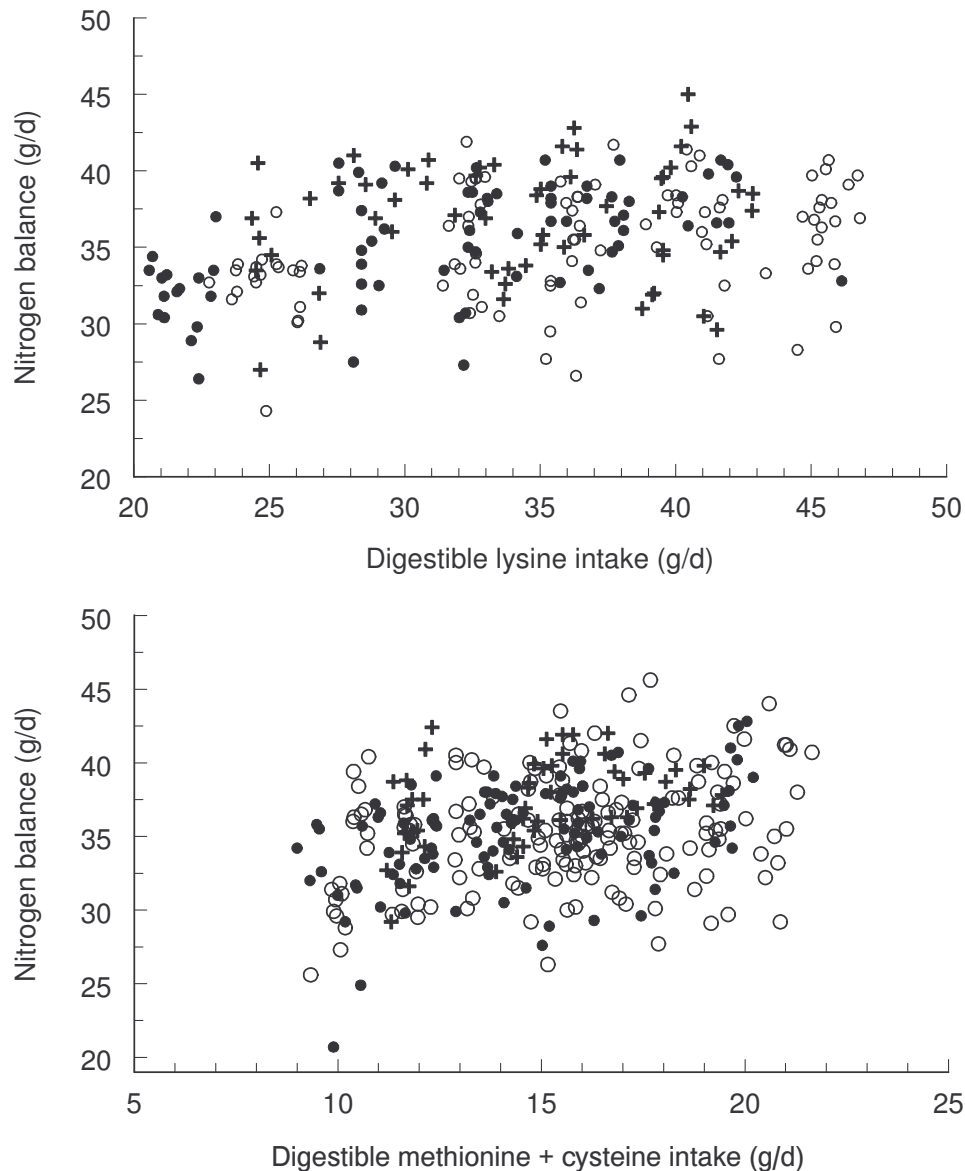
Figure 2. The effect of increased intake of lysine (top graph) and methionine + cysteine (bottom graph) on the N-balance of preruminant calves between 120 and 140 kg BW (●), 170 and 190 kg BW (○) and 240 and 260 kg BW (+). The top graph represents pooled data of nine trials (*n* = 226), the bottom graph pooled data of 13 trials (*n* = 330). Every symbol represents one observation, i.e. a 5-days' nitrogen balance of one calf. Data from Tolman & Wiebenga, 1991 and Tolman *et al.*, 1991. Nitrogen balance only responded to lysine intake in the range of 120-140 kg BW ($P < 0.05$).

Following the same approach for preruminant calves, two slaughter experiments were conducted to quantify the responses of protein and fat deposition rates to changes in protein and protein-free energy intake in preruminant calves from 80 to 240 kg BW (Gerrits *et al.*,

1996). Preruminant calves (90 male Holstein-Friesian × Dutch-Friesian crossbreeds) were fed a wide range of protein intakes (milk proteins only) at each of two protein-free energy intake levels.

Extra protein-free energy intake mainly resulted in extra fat deposition, but also increased protein deposition rate, even at low protein intakes (Gerrits *et al.*, 1996). This is agreement with a study in ruminating steers receiving an abomasal infusion of amino acids and energy-yielding nutrients (Schroeder *et al.*, 2004). An increased protein intake resulted in extra fat and protein deposition (Gerrits *et al.*, 1996), which corresponds with results in young preruminant calves (40-70 kg BW; Donnelly & Hutton, 1976). Briefly, the results showed the absence of clearly distinguishable protein and energy dependent phases for protein deposition in calves, unlike the linear-plateau concept which is used for pigs. In addition, the efficiency of utilization of extra dietary protein was found to be very low: for every 100 g increase in protein intake, protein deposition increased only with about 30 g (Gerrits *et al.*, 1996). It was concluded that the response of preruminant calves to increased nutrient intakes clearly differs from that of pigs, and that the lack of response of nitrogen balance in the amino acid requirement studies (Tolman & Wiebenga, 1991; Tolman *et al.*, 1991) was not primarily caused by a limiting dietary energy supply.

Amino acid imbalance

The possibility of a limiting indispensable amino acid causing both the low efficiency of nitrogen utilization in the slaughter trials (Gerrits *et al.*, 1996) and the lack of response in the amino acid requirement studies (Tolman & Wiebenga, 1991; Tolman *et al.*, 1991) still existed.

Gerrits et al. (1998) presented the amino acid profiles in different body fractions and in the whole body of preruminant calves in the range of 80 to 240 kg BW as affected by protein and energy intake. For the detection of a possible dietary amino acid imbalance, they hypothesized that the marginal efficiency of utilization of at least one indispensable amino acid would be substantially higher than the reported marginal efficiency of 30% for total protein (Gerrits *et al.*, 1998). The marginal efficiencies found, however, were very low and within the rather narrow range of 11 to 29% for all indispensable amino acids (Gerrits *et al.*, 1998). Amongst the conditionally dispensable amino acids, arginine showed an efficiency of utilization of about 90%. The quantity of arginine ingested exceeded the quantity of arginine retained (Gerrits *et al.*, 1998). When compared with weaned pigs, which require at least 40% of the

arginine requirement to be supplied by the diet, it seemed therefore unlikely that arginine was limiting protein deposition (Gerrits *et al.*, 1998). However, more recent studies suggest that arginine metabolism in neonatal, milk-fed animals may differ from that in weaned animals (Fligger *et al.*, 1997; Hüsler & Blum, 2002; Kim *et al.*, 2004) (see paragraph 6.2), but it is not known if arginine supplementation affects protein deposition in heavy preruminant calves. The efficiency of utilization of cysteine was also high, on average 74%, while the efficiency of methionine was only about 27%. Utilization of methionine for the production of cysteine can not be excluded, but the relative increase in cysteine intake with increasing protein intake was low compared with other amino acids, which may have complicated the estimation of a marginal efficiency of cysteine (Gerrits *et al.*, 1998). In conclusion, not one of the indispensable amino acids limited the rate of protein deposition. This means that other reasons are responsible for the low efficiency of protein utilization in preruminant calves.

Amino acids for ammonia detoxification

A temporary high portal flux of ammonia potentially stimulates amino acid utilization for ureagenesis (Milano & Lobley, 2001). In ureagenesis, mitochondrial ammonia and cytosolic aspartate are precursors for the ornithine cycle. Amino acids can be used as predominant N-donors for aspartate, which incurs a penalty on amino acids available for protein synthesis (Lobley & Milano, 1997). Degradation of amino acids to provide precursors for the ornithine cycle could (partly) explain an inefficient utilization of amino acids for protein gain. This was shown in short-term (2-3 h) studies in sheep (Orzechowsky *et al.*, 1988) and dairy cows (Symonds *et al.*, 1981), although other studies indicate that ammonia-N may provide more than the theoretically expected 50% of the N-atoms in urea (Luo *et al.*, 1995; Lobley *et al.*, 1996; Milano *et al.*, 2000).

In preruminant calves, feeding diets based on either skimmed milk protein or a mixture of soy and wheat gluten (50/50) resulted in high portal ammonia fluxes, representing 9 to 19% of the dietary nitrogen intake (or 6 and 13 g ammonia-N/d) respectively (Verdonk *et al.*, 2002a). Nutrient absorption and consequently portal nutrient and ammonia fluxes in preruminant calves were peak-wise (Verdonk *et al.*, 1999), which indicates that high fluxes of ammonia are to be processed by the liver within a relatively short time-span. The ammonia may originate from intestinal amino acid deamination, but also from protein fermentation in the gastrointestinal tract. To quantify the contribution of amino acids to the ammonia detoxification process, Gerrits et al. (1999; 2001) infused ammonia, as NH_4HCO_3, in the

colon of preruminant calves with an average BW of 165 kg, at each of two protein intake levels (58 and 85 g N/d; n = 12 for each treatment), and measured the increase of nitrogen losses compared with infusion of $NaHCO_3$. Infusion rates were 10 g ammonia-N per day. Also, a tracer dose of $^{15}NH_4Cl$ was infused to estimate the kinetics and recovery of the infusate by analysis of ^{15}N enrichment in urine and faeces. Faeces and urine were separately and quantitatively collected during a 6-d balance period after an adaptation period to the dietary protein intake level (17 days) and to infusion into the colon (7 days). Calculated from the total nitrogen excretion, on average only 75% (variation between animals: 0-180%) of the infused ammonia-N was recovered in urine and no interaction with protein intake was found (Table 1).

Table 1. Least square means of faecal and urinary nitrogen output and recovery of ^{15}N in preruminant calves at two levels of protein intake (58 vs. 83 g N/d) and at two levels of ammonia infusion in the colon (0 vs. 10 g N/d) with $^{15}NH_4Cl$ added as tracer to the ammonia infusion. Data from Gerrits *et al.*, 2001 (n = 12 for each treatment).

	Main effects[1]			
	Low	High	SEM	*P*-value
Faecal N-output, g N/d				
Protein intake	6.8	7.6	0.38	0.15
Ammonia infusion	6.9	7.4	0.23	0.18
Recovery ^{15}N in faeces, %				
Protein intake	6.2	8.4	0.70	0.05
Urinary N-output, g N/d				
Protein intake	27.1	42.5	0.95	< 0.001
Ammonia infusion	31.1	38.6	0.78	< 0.001
Recovery ^{15}N in urine, %				
Protein intake	48.5	53.9	1.97	0.07

[1]Two-way interactions were not significant (P > 0.05)

Calculated from the tracer infusion, on average 51% (variation between animals: 36-63%) of the infused $^{15}NH_4Cl$ was recovered in urine. This implies that infused ammonia was only partially recovered in urine (from tracer calculations), and that the low net recovery of ammonia-N (collected up to 48 h after the end of infusion) could indicate a metabolic role of ammonia. The net recovery of infused ammonia-N was expected to exceed 100%. This would

indicate extra amino acid catabolism. The low net recovery suggests, however, that no additional amino acids were catabolized to provide aspartate for the ornithine cycle. This could be due to the flexibility of the enzyme systems involved, i.e. glutamate dehydrogenase and carbamoyl-phosphate synthase 1 / ornithine transcarbamylase, to supply aspartate and citrulline for urea synthesis respectively. In conclusion, it is unlikely that ureagenesis itself contributes to the inefficiency of nitrogen utilization in preruminant calves.

Alternative mechanisms

From the work described above, it was concluded that marginal efficiencies of 30 – 35% are normal for preruminant calves > 100 kg BW, using milk proteins as the sole protein source in the diet. Following a reductionist approach, it was shown that neither a protein-energy imbalance, an imbalanced amino acid profile nor the ureagenesis are responsible for the low marginal efficiency in heavy preruminant calves. Alternative hypotheses are presented and discussed below. Although individual mechanisms are described, the reason for a low efficiency of protein utilization in heavy preruminant calves may be multi-factorial in nature. Interactions between factors may be important to explain the decreased protein utilization. To identify main effects and for the sake of simplicity, mechanisms are discussed separately.

Fermentation of milk in the rumen

The estimation of the marginal efficiency of protein utilization may be complicated by ruminal drinking, certainly depending on the feeding method, but potentially also on feeding level and nutrient composition. Several studies described the etiology and pathology of ruminal drinking (Breukink *et al.*, 1988; Rademacher *et al.*, 2003; Gentile *et al.*, 2004), but studies reporting quantitative measurements of milk leakage into the rumen are scarce. Guilhermet et al. (1975) and Wise et al. (1984) used preruminant calves equipped with a rumen canula to estimate leakage of milk. Generally, both studies showed a large variation in the amount of milk recovered in the rumen between individual calves, which averaged ~7% (Guilhermet *et al.*, 1975) and 20% (Wise *et al.*, 1984) of the milk ingested. Leakage considerably increases with age and is higher when animals are drinking from a bucket (~40%) than when sucking a nipple (< 1%; Guilhermet *et al.*, 1975). Tadeu dos Santos et al. (1986), not mentioning the feeding method, reported 3% of spillage into the rumen in calves with a good appetite and 57% in calves with reduced appetite. At our research station, Van

Leeuwen (1978) collected duodenal chyme after an oral dose of synthetic amino acids in milk and found a recovery of 84% in the duodenum. When amino acids were dissolved in water, only 10% was recovered in the duodenum (Van Leeuwen, 1977), illustrating a poor closure of the oesophageal groove after drinking of water. Since the quantity of milk appearing in the rumen and nitrogen losses during (protein) fermentation are unknown, it is difficult to estimate the quantitative impact of ruminal drinking on the estimated marginal efficiency of protein utilization. There are no clear indications that increasing protein intake increases ruminal drinking, and therefore have a significant impact on marginal protein efficiency. It is, however, clear that ruminal drinking can be quantitatively important, and an important source of between-animal variation.

Preferential utilization by particular tissues

Following digestion and absorption, amino acids are available for protein synthesis or oxidation. High rates of protein turnover of specific tissues, like the gut, liver, pancreas and kidney, could have affected the marginal efficiency of single amino acids as calculated by Gerrits et al. (1998). The contribution of tissues with a high turnover rate may be higher than can be expected based on their contribution to empty body protein, because high rates of protein turnover may be associated with increased oxidative losses (Liu *et al.*, 1995). Also, a specific preference of particular tissues to utilize indispensable amino acids can lead to an imbalance of amino acids at the site of deposition. The digestive tract, for example, can metabolize dietary indispensable amino acids in monogastrics (Burrin & Stoll, 2003) and ruminants (MacRae *et al.*, 1997). We performed a small-scale study in which portal amino acid fluxes were measured in four preruminant calves (161 kg BW) fed clotting and non-clotting protein sources (Verdonk *et al.*, 1999; Verdonk *et al.*, 2002a). The calves were surgically fitted with catheters in the portal vein, the carotid artery and the mesenteric vein and were assigned to a diet (20% crude protein) containing either skimmed milk protein or soy and wheat gluten. In a cross-over design with two collection periods of two days each, portal and arterial blood samples were taken after feeding of 1040 g milk replacer.

The portal availability, as percentage of intake, of the indispensable amino acids was not affected by protein source ($P > 0.05$). Average values and comparable values for other farm animals from literature are presented in Table 2. A flawless comparison between species is hampered by different experimental conditions in conducted studies. The highly negative

portal cysteine flux in the dairy cow (Table 2), for example, was likely due to a methionine deficiency in that study.

Table 2. Net portal fluxes of amino acids as percentage of amino acid intake (milk-fed calf and milk-fed pig) or apparent ileal digestible amino acids (pig, dairy cow and sheep) for several farm animals.

Amino acid	Milk-fed calf[1]	Milk-fed pig[2]	Pig[3]	Dairy cow[4]	Sheep[5]
Threonine	86	50	64	43	72
Tryptophan	ND	ND	92	ND	ND
Methionine	86	59	80	67	85
Cysteine	ND	17	93	-162	-3
Valine	89	67	74	51	68
Isoleucine	85	74	83	62	75
Leucine	80	66	75	62	70
Lysine	91	52	87	55	80
Histidine	93	70	103	95	96
Phenylalanine	96	57	83	76	84
Tyrosine	98	132	88	ND	97
Arginine	150	142	102	63	89
Aspartic acid	50	28	48	9	49
Serine	101	71	98	75	117
Glutamic acid	3	-2	-132	9	-51
Glycine	87	61	75	42	134
Alanine	180	190	215	80	107
Proline	65	87	48	9	95

[1] Verdonk *et al.*, 2002b

[2] Stoll *et al.*, 1997; Stoll *et al.*, 1998a; Stoll *et al.*, 1998b; Bos *et al.*, 2005

[3] Lenis *et al.*, 1996; Van der Meulen *et al.*, 1997; Reverter *et al.*, 2000

[4] Berthiaume *et al.*, 2001

[5] MacRae *et al.*, 1997; Rémond *et al.*, 2003

It does, however, provide some interesting leads. Firstly, the relatively high recovery of dietary indispensable amino acids in the portal vein of the milk-fed calf suggests that there is less amino acid sequestration in the portal drained viscera in preruminant calves than in the other species shown. This is presumably affected by the relatively high feeding level in the calf study. In pigs and beef steers, increasing the feeding level does not proportionally increase the irreversible loss rate over the portal drained viscera, which implies that portal amino acid recoveries increase with feed intake (Lapierre *et al.*, 1999; Van Goudoever *et al.*,

2000). Secondly, the absence of one poorly recovered indispensable amino acid in milk-fed calves indicates that the amino acid balance is not drastically disturbed by passing the portal drained viscera.

Finally, portal arginine availability is high in milk-fed animals, both in calves and pigs. A major contribution of total arginine supply has to originate from endogenous synthesis in milk-fed animals, because milk supplies only about 60% of the arginine an animal needs for maximal growth (Williams & Hewitt, 1979; Kim *et al.*, 2004). In neonatal pigs, the small intestine is the major site of endogenous arginine synthesis, but during development it becomes the major site of citrulline production, which is converted to arginine by the kidney (Wu & Morris, 1998). Studies on arginine metabolism have mainly been performed in milk-fed pigs during the first 21 d of life (Kim *et al.*, 2004). Arginine supplementation has been shown to increase growth in young calves (Fligger *et al.*, 1997; Hüsler & Blum, 2002), but mechanisms involved have not yet been studied. From this point of view, the high marginal efficiencies for arginine in heavy preruminant calves (97% for 80-160 kg BW; 89% for 160-240 kg BW) (Gerrits *et al.*, 1998) may have some biological significance. Conversely, the low marginal efficiency of proline (~35%), which is a precursor for endogenous arginine synthesis, did not suggest any limitation of arginine availability. Arginine kinetics have not been studied in preruminant calves and quantification of arginine synthesis and the interaction with the ornithine cycle activity and identification of the tissue(s) involved would be interesting.

The liver is the second tissue which can be individually studied. Although several studies report hepatic nutrient fluxes in preruminant calves (Houlier *et al.*, 1990; Houlier *et al.*, 1991; Ortigues *et al.*, 1995; Ortigues *et al.*, 1996), the fate of individual amino acids is not described and amino acid catabolism by the liver can not be quantified. In conclusion, the few data available do not directly suggest preferential utilization of amino acids by specific tissues in preruminant calves.

Recycling of urea nitrogen

Recycling of urea by microbes in the gastrointestinal tract of dairy cows (67% of urea synthesis) and sheep (61% of urea synthesis) contributes to a large extent to the nitrogen supply in ruminant animals and can even exceed the apparent digestible nitrogen intake (Lapierre & Lobley, 2001). In milk-fed calves, this partial reuse of urea is expected to be negligible due to the absence of non-starch polysaccharides provoking microbial activity in

the gastrointestinal tract. The recovery of an intravenous pulse dose of ^{13}C-urea was about 80% in 48 h urine of milk-fed calves (Gerrits *et al.*, 2001), which indicates that urea recycling is substantially lower than in ruminants (Lapierre & Lobley, 2001). Provision of roughage (viz. potential carbon precursors) will definitely increase urea recycling and therefore the utilization of digestible protein for growth, provided that ruminal drinking is not promoted by the provision of roughage.

Utilization of amino acids for gluconeogenesis

Donkin and colleagues (1994; 1995; 1997) have intensively studied gluconeogenesis in hepatocyte monolayers of milk-fed calves as affected by insulin and glucagon. They showed that the hepatic capacity for gluconeogenesis from lactate in milk-fed calves is much higher than in ruminant calves. Also, Ortigues et al. (1995) suggested that the Cori cycle can be of greater importance in preruminants than in ruminants. For propionate, the gluconeogenic capacity was shown to be at least as high as in ruminant calves (Donkin & Armentano, 1995). This substantial capacity for gluconeogenesis in preruminant calves seems to be redundant, because large amounts of monosaccharides are supplied by the diet. On the contrary, ruminating animals have an obligate need for gluconeogenesis, and it has been suggested that gluconeogenesis from amino acids is quantitatively important, even if the dietary glucose supply is abundant (Lobley, 1992). If genetically predisposed, preruminants potentially catabolize amino acids for gluconeogenesis, causing a low marginal efficiency of protein utilization.

In vivo quantification of gluconeogenesis and especially the contribution of different precursors (e.g. lactate, glycerol and amino acids), in preruminant calves is lacking. Therefore, despite the considerable gluconeogenic capacity and enzyme activity in milk-fed calves, the quantity of amino acids used for gluconeogenesis is not known.

Decreased insulin sensitivity

Heavy preruminant calves often develop a certain level of insulin resistance, indicated by postprandial hyperglycemia, hyperinsulinemia and glucosuria (Doppenberg & Palmquist, 1991; Hostettler-Allen *et al.*, 1994). The etiology of insulin resistance in preruminant calves is likely to be multi-factorial, as reviewed by Blum and Hammon (1999). A high level of circulating insulin down-regulates its own receptors, which may result in altered cellular

glucose and amino acid transport and compromised protein synthesis. Feeding large amounts of milk (especially lactose) to ontogenically ruminant animals, may contribute to the development of insulin resistance (Hostettler-Allen *et al.*, 1994; Blum & Hammon, 1999). In addition, increasing protein intake decreased insulin resistance, as indicated by urinary glucose excretion and plasma concentrations of glucose and insulin, in preruminant calves (Gerrits & Blum, 1998). Mechanistic explanations for the effect of protein intake on insulin resistance are not yet clarified. If insulin resistance coincides with reduced protein utilization, this effect will increase with increasing protein intake, thus contributing to a low marginal efficiency of protein utilization.

The consequences of insulin resistance for the utilization of amino acids in preruminant calves are, however, unknown. The effects of non insulin-dependent diabetes on protein utilization in human subjects are generally absent (Biolo *et al.*, 1992; Luzi *et al.*, 1993), although Gougeon et al. (1994) reported increased rates of protein breakdown and a more negative nitrogen balance in diabetic subjects. The low feeding level (non-growing man) and post-absorptive measurements complicate the extrapolation of these results to quickly growing milk-fed calves. Moreover, endocrine regulation of protein metabolism in the heavy preruminant calf is not unambiguous, because the responsiveness to insulin is apparently different in ruminants and non-ruminants (Oddy *et al.*, 1987; Garlick & Grant, 1988; Wester *et al.*, 2004). Insufficient insight in the regulation of protein metabolism in preruminant calves raises the question if insulin resistance contributes to the low marginal efficiency of protein utilization.

Post-absorptive nutrient asynchrony

Although all macronutrients are provided simultaneously to preruminant calves, an asynchronous post-absorptive availability of individual nutrients may occur. Separation of amino acid and glucose availability in time, for example, can be expected when skimmed milk protein is fed to calves. Casein (~80% of milk protein) clots in the calf's abomasum and leads to a slow release of protein into the intestinal tract (Houlier *et al.*, 1991; Longenbach & Heinrichs, 1997; Verdonk *et al.*, 2002b), while lactose does not have clotting abilities and is absorbed relatively quickly compared with dietary protein (Grizard *et al.*, 1982; Verdonk *et al.*, 2002b) (Figure 3). An asynchronous availability of glucose and amino acids within a day was shown to substantially decrease protein utilization in adult man (reviewed by Munro, 1951) and growing rats (Geiger, 1948).
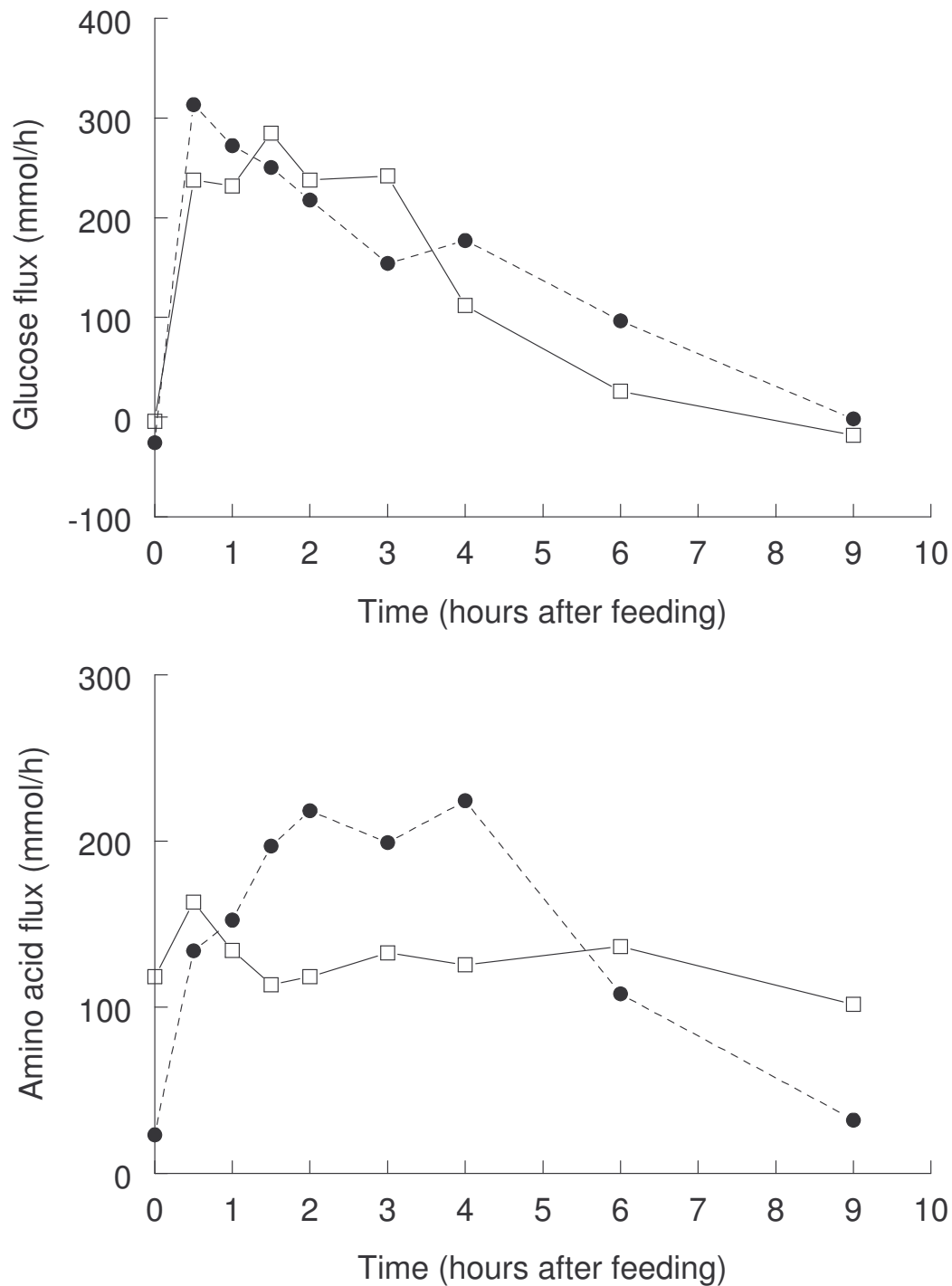
Figure 2. Net portal fluxes of plasma free α-amino acids and glucose in preruminant calves (160 kg BW; *n* = 4), fed diets based on either skimmed milk protein (□, ——) or vegetable proteins (●, - - - -). Data from Verdonk et al. (1999; 2002a).

Apart from an asynchrony between individual nutrients, also an asynchrony between total nutrient supply and total nutrient requirements within a day may occur. Preruminant calves are usually fed twice daily, resulting in two peaks of metabolically available nutrients, while

in ruminants, these patterns are absent because of eating patterns and gradual passage of feed through the rumen compartment. It can be hypothesized that in milk-fed calves, the inefficiency of protein utilization can to some extent be the consequence of a temporary excessive amino acid availability. In man, for example, it has been shown that protein retention increased with a slower digestion rate of dietary protein (Boirie *et al.*, 1997; Dangin *et al.*, 2001). The peak-wise glucose availability, on the other hand, requires flexibility of metabolic pathways to clear glucose from the systemic circulation. A disturbed glucose metabolism may indirectly also affect protein utilization (see previous paragraph).

Finally, asynchrony occurs when individual amino acids are not available at the same moment. In calf milk replacers, vegetable protein sources (mainly soy, wheat, potato) have partly replaced skimmed milk protein during the past decades, with subsequent addition of crystalline amino acids to reach an ideal amino acid pattern for growth. Supplementation of the limiting amino acids as crystalline amino acids to the feed compensates this limitation and improves growth performance. Absorption kinetics of the supplemented free amino acids and the protein-bound amino acids, however, differ (Metges *et al.*, 2000; Yen *et al.*, 2004) and may lead to an amino acid imbalance at the site of deposition.

Skimmed milk protein and respectively synthetic amino acids (Tolman & Wiebenga, 1991; Tolman *et al.*, 1991) or caseinates (Gerrits *et al.*, 1996) were used as protein sources in the studies which demonstrated the low efficiency of protein utilization in preruminant calves. Therefore, the contribution of each type of nutrient asynchrony to the low marginal efficiency may have varied between studies. In general, interpretation of studies about nutrient asynchrony is complicated, since the three types of nutrient asynchrony are often confounded. The use of a slowly digestible protein source (Boirie *et al.*, 1997), for example, results in a more gradual supply of amino acids during the day, but also in an increased asynchrony between amino acid and glucose absorption. Separate effects of different types of nutrient asynchrony can counteract, as shown by the interaction between protein source and feeding frequency on amino acid oxidation in pigs (Batterham & Bayley, 1989).

Conclusions and implications

It was determined that utilization of extra dietary protein for protein gain is lower in preruminant calves than in several other species of farm animals. Especially preruminant calves above 100 kg BW showed a low marginal efficiency of protein utilization (< 30%). The inefficient utilization could not be explained by either a protein to energy imbalance or an

imbalanced amino acid profile in the diet. Also the utilization of amino acids for ammonia detoxification did not contribute to the low efficiency. Alternative mechanisms result in either a reduced post-absorptive supply of amino acids or any indispensable amino acid (e.g. fermentation of milk or preferential utilization by tissues), or a reduced utilization of the post-absorptive available amino acids (e.g. insulin resistance or nutrient asynchrony). Nonetheless, the possibility of multiple factors being involved should not be excluded.

The low efficiency of protein utilization illustrates that there is a large potential for improvement of the efficiency of growth in heavy preruminant calves. More insight in the amino acid metabolism of preruminant calves is needed to decrease nitrogen losses. Furthermore, mechanisms of amino acid utilization in preruminant calves could also be relevant for ruminants in which similar studies are hampered by rumen fermentation processes.

References

Batterham ES, Andersen LM, Baignent DR & White E (1990) Utilization of ileal digestible amino acids by growing pigs: effect of dietary lysine concentration on efficiency of lysine retention. *Br J Nutr* **64**, 81-94.

Batterham ES & Bayley HS (1989) Effect of frequency of feeding of diets containing free or protein-bound lysine on the oxidation of [^{14}C]lysine or [^{14}C]phenylalanine by growing pigs. *Br J Nutr* **62**, 647-655.

Berthiaume R, Dubreuil P, Stevenson M, McBride BW & Lapierre H (2001) Intestinal disappearance and mesenteric and portal appearance of amino acids in dairy cows fed ruminally protected methionine. *J Dairy Sci* **84**, 194-203.

Bikker P (1994) Protein and lipid accretion in body components of growing pigs. PhD thesis, Wageningen Agricultural University.

Biolo G, Tessari P, Inchiostro S, Bruttomesso D, Sabadin L, Fongher C, Panebianco G, Fratton MG & Tiengo A (1992) Fasting and postmeal phenylalanine metabolism in mild type 2 diabetes. *Am J Physiol* **263**, E877-E883.

Black JL, Campbell RG, Williams IH, James KJ & Davies GT (1986) Simulation of energy and amino acid utilisation in the pig. *Res Dev Agric* **3**, 121-145.

Black JL, Pearce GR & Tribe DE (1973) Protein requirements of growing lambs. *Br J Nutr* **30**, 45-60.

Blome RM, Drackley JK, McKeith FK, Hutjens MF & McCoy GC (2003) Growth, nutrient utilization, and body composition of dairy calves fed milk replacers containing different amounts of protein. *J Anim Sci* **81**, 1641-1655.

Blum JW & Hammon HM (1999) Endocrine and metabolic aspects in milk-fed calves. *Domest Anim Endocrinol* **17**, 219-230.

Boirie Y, Dangin M, Gachon P, Vasson MP, Maubois JL & Beaufrère B (1997) Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proc Natl Acad Sci* **94**, 14930-14935.

Bos C, Stoll B, Fouillet H, Gaudichon C, Guan X, Grusak MA, Reeds PJ, Burrin DG & Tomé D (2005) Postprandial intestinal and whole body nitrogen kinetics and distribution in piglets fed a single meal. *Am J Physiol* **288**, E436-E446.

Breukink HJ, Wensing T, Van Weeren-Keverling Buisman A, Van Bruinessen-Kapsenberg EG & De Visser NA (1988) Consequences of failure of the reticular groove reflex in veal calves fed milk replacer. *Vet Quart* **10**, 126-135.

Burrin DG & Stoll B (2003) Intestinal nutrient requirements in weanling pigs. *In: Weaning the pigs - Concepts and consequences*, [JR Pluske, J Le Dividich and MWA Verstegen, editors]. Wageningen, The Netherlands. Wageningen Academic Publishers, pp. 301-335.

Calo LL, McDowell RE, Van Vleck LD & Miller PD (1973) Parameters of growth of Holstein-Friesian bulls. *J Anim Sci* **37**, 417-422.

Campbell RG & Dunkin AC (1983) The effects of energy intake and dietary protein on nitrogen retention, growth performance, body composition and some aspects of energy metabolism of baby pigs. *Br J Nutr* **49**, 221-230.

Campbell RG, Taverner MR & Curic DM (1985) Effects of sex and energy intake between 48 and 90 kg live weight on protein deposition in growing pigs. *Anim Prod* **40**, 497-503.

Chung TK & Baker DH (1992) Methionine requirement of pigs between 5 and 20 kilograms body weight. *J Anim Sci* **70**, 1857-1863.

Dangin M, Boirie Y, Garcia-Rodenas C, Gachon P, Fauquant J, Callier P, Ballèvre O & Beaufrère B (2001) The digestion rate of protein is an independent regulating factor of postprandial protein retention. *Am J Physiol* **280**, E340-E348.

De Lange CFM, Gillis AM & Simpson GJ (2001) Influence of threonine intake on whole-body protein deposition and threonine utilization in growing pigs fed purified diets. *J Anim Sci* **79**, 3087-3095.

Donkin SS & Armentano LE (1994) Regulation of gluconeogenesis by insulin and glucagon in the neonatal bovine. *Am J Physiol* **266**, R1229-R1237.

Donkin SS & Armentano LE (1995) Insulin and glucagon regulation of gluconeogenesis in preruminating and ruminating bovine. *J Anim Sci* **73**, 546-551.

Donkin SS, Bertics SJ & Armentano LE (1997) Chronic and transitional regulation of gluconeogenesis and glyconeogenesis by insulin and glucagon in neonatal calf hepatocytes. *J Anim Sci* **75**, 3082-3087.

Donnelly PE & Hutton JB (1976) Effects of dietary protein and energy on the growth of Friesian bull calves, I. Food intake, growth, and protein requirements. *N Z J Agric Res* **19**, 289-297.

Doppenberg J & Palmquist DL (1991) Effect of dietary fat level on feed intake, growth, plasma metabolites and hormones of calves fed dry or liquid diets. *Livest Prod Sci* **29**, 151-158.

Dourmad JY, Guillou D, Sève B & Henry Y (1996) Response to dietary lysine supply during the finishing period in pigs. *Livest Prod Sci* **45**, 179-186.

Dunkin AC, Black JL & James KJ (1986) Relation between energy intake and nitrogen retention in entire male pigs weighing 75 kg. *Br J Nutr* **55**, 201-207.

Fligger JM, Gibson CA, Sordillo LM & Baumrucker CR (1997) Arginine supplementation increases weight gain, depresses antibody production, and alters circulating leukocyte profiles in preruminant calves without affecting plasma growth hormone concentrations. *J Anim Sci* **75**, 3019-3025.

Freetly HC, Nienaber JA & Brown-Brandl T (2002) Relationships among heat production, body weight, and age in Finnsheep and Rambouillet ewes. *J Anim Sci* **80**, 825-832.

Freetly HC, Nienaber JA, Leymaster KA & Jenkins TG (1995) Relationships among heat production, body weight, and age in Suffolk and Texel ewes. *J Anim Sci* **73**, 1030-1037.

Gahl MJ, Crenshaw TD & Benevenga NJ (1995) Diminishing returns in weight, nitrogen, and lysine gain of pigs fed six levels of lysine from three supplemental sources. *J Anim Sci* **73**, 3177-3187.

Garlick PJ & Grant I (1988) Amino acid infusion increased the sensitivity of muscle protein synthesis in vivo to insulin: effect of branch chain amino acids. *Biochem J* **254**, 579-584.

Geiger E (1948) The importance of the time element in feeding of growing rats. Experiments with delayed supplementation of protein. *Science* **108**, 42-43.

Gentile A, Sconza S, Lorenz I, Otranto G, Rademacher G, Famigli-Bergamini P & Klee W (2004) d-Lactic acidosis in calves as a consequence of experimentally induced ruminal acidosis. *J Vet Med Series A* **51**, 64-70.

Gerrits WJJ, Beelen GM, Dijkstra J & Verdonk JMAJ (2001) Ammonia infusion and starch fermentation in preruminant calves (150-180 kg) [in Dutch]. In *Internal report nr V99001, ID TNO Animal Nutrition*, pp. 30. Wageningen, The Netherlands: TNO Nutrition.

Gerrits WJJ & Blum JW (1998) A role of protein intake in the development of insulin resistance in preruminant calves. *In: Symposium on Growth in Ruminants: Basic Aspects, Theory and Practice for the Future*, [JW Blum, T Elsasser and P Guilloteau, editors]. Berne, Switzerland. pp. 310 (Abstr).

Gerrits WJJ, Dijkstra J, Verdonk JMAJ, Beelen GM & Boer H (1999) Effects of ammonia and starch infusion in the colon of preruminant calves. *In: The VIIIth International Symposium on Protein Metabolism and Nutrition*, [GE Lobley, A White and JC MacRae, editors]. Aberdeen, UK. pp. 55 (Abstr).

Gerrits WJJ, Schrama JW & Tamminga S (1998) The marginal efficiency of utilization of all ileal digestible indispensible amino acids for protein gain is lower than 30% in preruminant calves between 80 and 240 kg live weight. *J Nutr* **128**, 1774-1785.

Gerrits WJJ, Tolman GH, Schrama JW, Tamminga S, Bosch MW & Verstegen MWA (1996) Effect of protein and protein-free energy intake on protein and fat deposition rates in preruminant calves of 80 to 240 kg live weight. *J Anim Sci* **74**, 2129-2139.

Gougeon R, Pencharz PB & Marliss EB (1994) Effect of NIDDM on the kinetics of whole-body protein metabolism. *Diabetes* **43**, 318-328.

Grizard J, Dardevet D, Papet I, Mosoni L, Patureau-Mirand P, Attaix D, Tauveron I, Bonin D & Arnal M (1999) Nutrient regulation of skeletal muscle protein metabolism in animals. The involvement of hormones and substrates. *Nutr Res Rev* **39**, 61-74.

Grizard J, Toullec R, Guilloteau P & Patureau-Mirand P (1982) Effect of the kinetics of gastric emptying of food on blood insulin levels in the preruminant calf [in French]. *Reprod Nutr Dev* **22**, 475-484.

Guilhermet R, Mathieu CM & Toullec R (1975) Transit des aliments liquides au niveau de la gouttière œsophagienne chez le veau préruminant et ruminant [in French]. *Ann Zootech (Paris)* **24**, 69-79.

Hennig U, Wünsche J, Meinl M, Borgmann E & Kreienbring F (1982) The influence of graded protein supply at a high energy level on the fattening performance and the retention and utilisation of feed energy, protein and amino acids by female fattening pigs. 3. N retention and N and lysine metabolism determined by means of N balance and the analysis of the carcasses. *Arch Anim Nutr* **32**, 637-649.

Hostettler-Allen RL, Tappy L & Blum JW (1994) Insulin resistance, hyperglycemia, and glucosuria in intensively milk-fed calves. *J Anim Sci* **75**, 160-173.

Houlier ML, Patureau-Mirand P, Durand D, Bauchart D, Bayle G & Lefaivre J (1990) Influence de la vitesse d'absorption des acides aminés sur leur bilan hépatique chez le veau préruminant [in French]. *Reprod Nutr Dev* **30**, 135 (Abstr).

Houlier ML, Patureau-Mirand P, Durand D, Bauchart D, Lefaivre J & Bayle G (1991) Transport des acides aminés dans l'aire splanchnique par le plasme sanguin et le sang chez le veau préruminant [in French]. *Reprod Nutr Dev* **31**, 399-410.

Hüsler BR & Blum JW (2002) Metabolic and endocrine changes in response to endotoxin administration with or without oral arginine supplementation. *J Dairy Sci* **85**, 1927-1935.

Keusenhoff R (1992) Einflußfaktoren auf die N-Ausscheidungen beim Kalb [in German]. *Arch Tierz* **35**, 571-579.

Kim SW, McPherson RL & Wu G (2004) Dietary arginine supplementation enhances the growth of milk-fed young pigs. *J Nutr* **134**, 625-630.

Knap PW (2000) Time trends of Gompertz growth parameters in "meat-type" pigs. *Anim Sci* **70**, 39-49.

Krick BJ, Boyd RD, Roneker KR, Beermann DH, Bauman DE, Ross DA & Maisinger DJ (1993) Porcine somatotropin affects the dietary lysine requirement and net lysine utilization for growing pigs. *J Nutr* **123**, 1913-1922.

Lallès JP, Toullec R, Bouchez P & Roger L (1995) Antigenicity and digestive utilization of four soya products by the preruminant calf. *Livest Prod Sci* **41**, 29-38.

Lapierre H, Bernier JF, Dubreuil P, Reynolds CK, Farmer C, Oeullet DR & Lobley GE (1999) The effect of intake on protein metabolism across splanchnic tissues in growing beef steers. *Br J Nutr* **81**, 457-466.

Lapierre H & Lobley GE (2001) Nitrogen cycling in the ruminant: a review. *J Dairy Sci* **84, Suppl E**, E223-E236.

Le Huërou-Luron I, Gestin M, Le Dréan G, Romé V, Bernard C, Chayvialle JA & Guilloteau P (1998) Source of dietary protein influences kinetics of plasma gut regulatory peptide concentration in response to feeding in preruminant calves. *Comp Biochem Physiol A* **119**, 817-824.

Lenis NP, Bikker P, Van der Meulen J, Van Diepen JTM, Bakker JGM & Jongbloed AW (1996) Effect of dietary neutral detergent fiber on ileal digestibility and portal flux of nitrogen and amino acids and on nitrogen utilization in growing pigs. *J Anim Sci* **74**, 2687-2699.

Liu SM, Lobley GE, Macleod NA, Kyle DJ, Chen XB & Orskov ER (1995) Effects of long-term protein excess or deficiency on whole-body protein turnover in sheep nourished by intragastric infusion of nutrients. *Br J Nutr* **73**, 829-839.

Lobley GE (1992) Control of the metabolic fate of amino acids in ruminants: a review. *J Anim Sci* **70**, 3264-3275.

Lobley GE & Milano GD (1997) Regulation of hepatic nitrogen metabolism in ruminants. *Proc Nutr Soc* **56**, 547-563.

Lobley GE, Weijs PJM, Connell A, Calder AG, Brown DS & Milne E (1996) The fate of absorbed and exogenous ammonia as influenced by forage or forage-concentrate diets in growing sheep. *Br J Nutr* **76**, 231-248.

Longenbach JI & Heinrichs AJ (1997) A review of the importance and physiological role of curd formation in the abomasum of young calves. *Anim Feed Sci Technol* **73**, 85-97.

Luo QJ, Maltby SA, Lobley GE, Calder AG & Lomax MA (1995) The effect of amino acids on the metabolic fate of $^{15}NH_4Cl$ in isolated sheep hepatocytes. *Eur J Biochem* **228**, 912-917.

Luzi L, Petrides AS & De Fronzo RA (1993) Different sensitivity of glucose and amino acid metabolism to insulin in NIDDM. *Diabetes* **42**, 1868-1877.

MacRae JC, Bruce LA, Brown DS, Farningham DAH & Franklin M (1997) Absorption of amino acids from the intestine and their net flux across the mesenteric- and portal-drained viscera of lambs. *J Anim Sci* **75**, 3307-3314.

Markert W, Kirchgessner M & Roth FX (1993) Bilanzstudien zur Reduzierung der N-Ausscheidung von Mastschweinen [in German]. *J Anim Physiol Anim Nutr* **70**, 159-171.

Metges CC, El-Khoury AE, Selvaraj AB, Tsay RH, Atkinson A, Regan MM, Bequette BJ & Young VR (2000) Kinetics of L-[1-^{13}C]leucine when ingested with free amino acids, unlabeled or intrinsically labelled casein. *Am J Physiol* **278**, E1000-E1009.

Milano GD, Hotston-Moore A & Lobley GE (2000) Influence of hepatic ammonia removal on ureagenesis, amino acid utilization and energy metabolism in the ovine liver. *Br J Nutr* **83**, 307-315.

Milano GD & Lobley GE (2001) Liver nitrogen movements during short-term infusion of high levels of ammonia into the mesenteric vein of sheep. *Br J Nutr* **86**, 507-513.

Mnilk B, Harris CI & Fuller MF (1996) Lysine utilization by growing pigs: simultaneous measurement of protein accretion and lysine oxidation. *Br J Nutr* **75**, 57-67.

Möhn S, Gillis AM, Moughan PJ & De Lange CFM (2000) Influence of dietary lysine and energy intakes on body protein deposition and lysine utilization in the growing pig. *J Anim Sci* **78**, 1510-1519.

Montagne L, Salgado P, Toullec R & Lallès JP (2002) Enzymes of the small intestine of the calf: Effect of dietary protein source on the activities of some enzymes in the small intestinal mucosa and digesta. *J Sci Food Agric* **82**, 1772-1779.

Munro HN (1951) Carbohydrate and fat as factors in protein utilization and metabolism. *Physiol Rev* **31**, 449-488.

Nieto R, Miranda A, García MA & Aguilera JF (2002) The effect of dietary protein content and feeding level on the rate of protein deposition and energy utilization in growing Iberian pigs from 15 to 50 kg body weight. *J Nutr* **88**, 39-49.

Noblet J, Henry Y & Dubois S (1987) Effect of protein and lysine levels in the diet on body gain composition and energy utilization. *J Anim Sci* **65**, 717-726.

Oddy VH, Lindsay DB, Barker PJ & Northrop AJ (1987) Effect of insulin on hind limb and whole-body leucine and protein metabolism in fed and fasted lambs. *Br J Nutr* **58**, 143-154.

Ortigues I, Martin C & Durand D (1996) Circadian changes in net nutrient fluxes across the portal-drained viscera, the liver, and the hindquarters in preruminant calves. *J Anim Sci* **74**, 895-907.

Ortigues I, Martin C, Durand D & Vermorel M (1995) Circadian changes in energy expenditure in the preruminant calf: whole animal and tissue level. *J Anim Sci* **73**, 552-564.

Orzechowsky A, Pierzynowski S, Motyl T & Barej W (1988) Net hepatic metabolism of ammonia, propionate and lactate in sheep in relation to gluconeogenesis and ureagenesis. *J Anim Physiol Anim Nutr* **59**, 113-122.

Phillips DD & Walker DM (1980) Milk replacers containing isolated groundnut protein for preruminant lambs: the effect of protein concentration and energy intake on the requirement for lysine. *Aust J Agric Sci* **31**, 133-145.

Rademacher G, Korn N & Friedrich A (2003) The ruminal drinker as patient in practice [in German]. *Tierärztl Umschau* **58**, 115-125.

Rémond D, Bernard L, Chauveau B, Nozière P & Poncet C (2003) Digestion and nutrient net fluxes across the rumen, and the mesenteric- and portal-drained viscera in sheep fed with fresh forage twice daily: Net balance and dynamic aspects. *Br J Nutr* **89**, 649-666.

Reverter M, Lundh T, Gonda HL & Lindberg JE (2000) Portal net appearance of amino acids in growing pigs fed a barley-based diet with inclusion of three different forage meals. *Br J Nutr* **84**, 483-494.

Schroeder GF, Titgemeyer EC, Awawdeh MS & Gnad DP (2004) Effects of energy supply on methionine utilization by growing steers. *J Dairy Sci* **87, Suppl 1**, 115 (Abstr).

Stoll B, Burrin DG, Henry J, Jahoor F & Reeds PJ (1997) Phenylalanine utilization by the gut and liver measured with intravenous and intragastric tracers in pigs. *Am J Physiol* **273**, G1208-G1217.

Stoll B, Burrin DG, Henry J, Yu H, Jahoor F & Reeds PJ (1998a) Dietary amino acids are the preferential source of hepatic protein synthesis in piglets. *J Nutr* **128**, 1517-1524.

Stoll B, Henry J, Reeds PJ, Yu H, Jahoor F & Burrin DG (1998b) Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets. *J Nutr* **128**, 606-614.

Symonds HW, Mather DL & Collis KA (1981) The maximum capacity of the liver of the adult dairy cow to metabolize ammonia. *Br J Nutr* **46**, 481-486.

Tadeu dos Santos G, Toullec R, Roger R, De la Grange H & Guilloteau P (1986) Caractéristiques digestive des veaux de boucherie s'adaptant mal en atelier d'engraissement [in French]. *Reprod Nutr Dev* **26**, 1217.

Ternouth JH, Stobo JF & Roy JHB (1985) The effect of milk substitute concentration upon the intake, digestion and growth of calves. *Anim Prod* **41**, 151-159.

Tolman GH (1996) The lysine, methionine+cystine and threonine requirement and utilization of non-ruminating veal calves of 50-70 kg. *In: Protein Metabolism and Nutrition, EAAP publ no 81*, [AF Nunes, AV Portugal, JP Costa and JR Ribeiro, editors]. Vale de Santarém, Portugal. pp. 273-274.

Tolman GH & Beelen GM (1996) Endogenous nitrogen and amino acid flow in the terminal ileum of veal calves and the true digestibility of skim milk, soluble wheat and soya isolate proteins. *In: In: Veal, Perspectives to the Year 2000, Proceedings of an International Symposium*. Le Mans, France. Fédération de la Vitellerie Française, pp. 191-207.

Tolman GH & Wiebenga J (1991) The lysine and methionine+cystine requirement of Friesian veal calves in various weight ranges [in Dutch]. In *Internal report nr I 91-3752A, TNO Nutrition and Food Research Institute, Dept of Animal Nutrition and Meat Technology*. Wageningen, The Netherlands.

Tolman GH, Wiebenga J & Beelen GM (1991) The lysine and methionine+cystine requirement of Friesian veal calves (220-250 kg) [in Dutch]. In *Internal report nr I 91-3740, TNO Nutrition and Food Research Institute, Dept of Animal Nutrition and Meat Technology*. Wageningen, The Netherlands.

Toullec R & Guilloteau P (1989) Research into the digestive physiology of the milk-fed calf. *In: Nutrition and digestive physiology of monogastric farm animals*, [EJ Van Weerden and J Huisman, editors]. Wageningen. Pudoc, pp. 37-55.

Van der Meulen J, Bakker JGM, Smits B & De Visser H (1997) Effect of source of starch on net portal flux of glucose, lactate, volatile fatty acids and amino acids in the pig. *Br J Nutr* **78**, 533-544.

Van Goudoever JB, Stoll B, Henry JF, Burrin DG & Reeds PJ (2000) Adaptive regulation of intestinal lysine metabolism. *Proc Natl Acad Sci* **97**, 11620-11625.

Van Leeuwen P (1977) Duodenal passage of synthetic amino acids, dissolved in water, when supplied three hours preprandial [in Dutch]. In *Internal report nr 8120, TNO Nutrition and Food*

Research Institute, Dept of Animal Nutrition and Meat Technology. Wageningen, The Netherlands.

Van Leeuwen P (1978) Duodenal passage of synthetic amino acids, dissolved in milk, when supplied six hours preprandial [in Dutch]. In *Internal report nr 8120A, TNO Nutrition and Food Research Institute, Dept of Animal Nutrition and Meat Technology*. Wageningen, The Netherlands.

Van Weerden EJ & Huisman J (1985) Amino acid requirement of the young veal calf. *J Anim Physiol Anim Nutr* **53**, 232-244.

Verdonk JMAJ, Gerrits WJJ & Beelen GM (2002a) Effect of protein source on portal nutrient fluxes in preruminant calves [in Dutch]. In *Internal report nr V99030, ID TNO Animal Nutrition*. Wageningen, The Netherlands: ID TNO Animal Nutrition.

Verdonk JMAJ, Gerrits WJJ, Beelen GM & Jansman AJM (1999) Effect of protein source on portal nutrient fluxes in preruminant calves. *In: The VIIIth International Symposium on Protein Metabolism and Nutrition*, [GE Lobley, A White and JC MacRae, editors]. Aberdeen, UK. Wageningen Pers, The Netherlands, pp. 47 (Abstr).

Verdonk JMAJ, Gerrits WJJ & Beynen AC (2002b) Replacement of milk protein by vegetable protein in milk replacer diets for veal calves: digestion in relation to intestinal health. *In: Nutrition and health of the gastrointestinal tract*, [MC Blok, HA Vahl, L De Lange, AE Van de Braak, G Hemke and M Hessing, editors]. Wageningen. Wageningen Academic Publishers, pp. 183-198.

Walker DM & Faichney GJ (1964) Nitrogen balance studies with the milk-fed lamb. 3. Effect of different nitrogen intakes on growth and nitrogen balance. *Br J Nutr* **18**, 295-306.

Wester TJ, Lobley GE, Birnie LM, Crompton LA, Brown DS, Buchan V, Calder AG, Milne E & Lomax MA (2004) Effect of plasma insulin and branched-chain amino acids on skeletal muscle protein synthesis in fasted lambs. *Br J Nutr* **92**, 401-409.

Williams AP & Hewitt D (1979) The amino acid requirements of the preruminant calf. *Br J Nutr* **41**, 311-319.

Williams NH, Stahly TS & Zimmerman DR (1997) Effect of chronic immune system activation on body nitrogen retention, partial efficiency of lysine utilization, and lysine needs of pigs. *J Anim Sci* **75**, 2472-2480.

Wise GH, Anderson GW & Linnerud AC (1984) Relationship of milk intake by sucking and by drinking to reticular-groove reactions and ingestion behavior in calves. *J Dairy Sci* **67**, 1983-1992.

Wu G & Morris SM (1998) Arginine metabolism: nitric oxide and beyond. *Biochem J* **336**, 1-17.

Yen JT, Kerr BJ, Easter RA & Parkhurst AM (2004) Difference in rates of net portal absorption between crystalline and protein-bound lysine and threonine in growing pigs fed once daily. *J Anim Sci* **82**, 1079-1090.

Chapter 3

Effects of feeding frequency and feeding level on nutrient utilization in heavy preruminant calves

JJGC van den Borne, MWA Verstegen, SJJ Alferink, RMM Giebels
& WJJ Gerrits

Animal Nutrition Group, Department of Animal Sciences, Wageningen University, 6700 AH Wageningen, The Netherlands

Abstract

The objective of this study was to determine the effects of feeding frequency (FF) and feeding level (FL) on protein and energy metabolism in adapted, heavy preruminant calves. It was hypothesized that an increased FF would increase protein utilization by an improved synchrony between the supply of and requirements for protein during the day when a quickly hydrolysable protein source was used. Eighteen Holstein Friesian calves of 136 ± 3 kg body weight were assigned to FF (1, 2, or 4 meals daily) at 2 FL (1.5 or 2.5 times the metabolizable energy requirements for maintenance), except for calves fed once daily (only at a low FL). Calves were individually housed in respiration chambers during 2 experimental periods of 10 d. Whey protein was the only protein source in the diet. Neither FL nor FF affected apparent faecal nutrient digestibility. Increasing FF increased the efficiency of digestible protein utilization in calves. The increase was greater at a high FL (+11% from 2 to 4 meals/d) than at a low FL (+5% from 2 to 4 meals/d), but no significant interaction occurred between FL and FF. An increased FF and a higher FL enhanced fat deposition. Heat production was not affected by FF, but its circadian rhythm differed considerably between FF. Activity related heat production was not affected by FF or FL. Thus, increasing FF improved the efficiency with which protein and energy were utilized in heavy preruminant calves when a quickly hydrolysable protein source was used.

Abbreviations: AA, amino acid; BW, body weight; DM, dry matter; ER, energy retention; FF, feeding frequency; FL, feeding level; HP, heat production; HP_{act}, activity related heat production; HP_{cor}, heat production corrected for activity-related heat production; ME_m, metabolizable energy requirements for maintenance.

Introduction

The growth rate and body composition of milk-fed calves can be manipulated by varying the quantity and composition of the daily feed supply. Relationships have been described for young (Diaz *et al.*, 2001; Blome *et al.*, 2003) and older preruminant calves (Gerrits *et al.*, 1996). In addition to the average daily nutrient intake, the distribution of nutrient availability within a day also can be altered. Protein utilization for growth may be affected by the variation in availability of AA with time. Williams et al. (1986) found that raising the feeding frequency (FF) did not improve protein utilization in young milk-fed calves. The relatively low feeding level (FL), that is, 1 to 2 times the metabolizable energy requirements for maintenance (ME_m), may have concealed the potential effect of FF. Furthermore, the use of a clotting protein source (skimmed milk powder) may have resulted in a slow release and absorption of AA during the day and avoided high AA absorption peaks in that study. In practice, more rapidly hydrolysable protein sources, such as vegetable and whey protein, are increasingly used in milk replacer diets and result in a more rapid and peak-wise appearance of portal AA than when skimmed milk protein is fed (Verdonk *et al.*, 1999).

Studies about the effect of FF at different FL and using a rapidly hydrolysable protein source in milk-fed calves are lacking. Heavy preruminant calves utilize extra ingested protein with an extremely low efficiency (~ 30%) compared with young calves (66%; Gerrits *et al.*, 1996; Blome *et al.*, 2003). This considerable margin for improvement makes the heavy milk-fed calf a useful model for studying the effects of FF on protein utilization. Moreover, the mechanisms involved may be interesting for post-absorptive AA metabolism in growing ruminants, because similar protein-energy relationships exist (Gerrits *et al.*, 1996; Schroeder *et al.*, 2004) and technical difficulties restrict the assessment of digestible AA utilization in ruminant cattle (Titgemeyer, 2003).

The effects of FF on fat deposition are expected to be less pronounced than those on protein deposition, because the capacity for and flexibility of fat deposition are substantial. An increasing FF can be expected to increase physical activity and consequently activity-related heat production (HP_{act}). Therefore, energy retention (ER) as fat may decrease with increasing FF.

We hypothesized that increasing FF would lead to increased protein utilization in heavy preruminant calves. The effects were expected to be more pronounced at a high FL than at a low FL. Increasing the FF was expected to increase physical activity and decrease ER. The

aim of the study was to assess the effects of 3 FF at 2 FL on protein and energy utilization in adapted, heavy preruminant calves.

Materials and methods

Animals and housing

Eighteen male, Holstein Friesian calves were used in 9 trials of 2 calves of similar age (18 wk at start). Each trial consisted of 2 experimental periods. Both experimental periods were preceded by an adaptation period of 4 wk, which allowed the organ mass and metabolism of the calves to adapt fully to the experimental treatments.

The effects of FF (1, 2, or 4 meals/d) were studied at 2 FL ($1.5 \times ME_m$ and $2.5 \times ME_m$). Trials were assigned to FF. The same FF was used for both calves within a trial, because visual and (limited) auditory contact between the individually housed calves affected their physical activity and cephalic phase reflexes. Within trials, a low FL was adopted in period 1 ($1.5 \times ME_m$) and a high FL in period 2 ($2.5 \times ME_m$). In the first period, a low FL was used for all calves to permit comparison of different FF in a similar experimental period and at similar BW. As designed, animals at FF 1 were not fed at a high FL, because it is not feasible to feed calves at a high level in only one daily meal (Table 1). Calves at FF 1 served as controls for the effect of the experimental period, because FL was confounded with period in this study.

Table 1. Experimental design; number of observations per treatment

	Treatment				
	FF[1] 1	FF 2		FF 4	
Item	FL[2] low	FL low	FL high	FL low	FL high
Period 1	6	6	-	6	-
Period 2	6	-	6	-	6

[1]FF = feeding frequency

[2]FL = feeding level

The adaptation period allowed calves to adapt to the experimental treatments and housing conditions. Harnesses for the faecal collection bags were attached 5 d before the start of the experiment. At the start of each experimental period, calves were housed individually in one of 2 identical, size-adjustable climatic respiration chambers set to $2.5 \times 1.5 \times 2.0$ m (length × width × height). Within the chambers, calves were housed in metabolic cages (1.85×0.75 m).

Calves in the two separate chambers could see each other. Temperature was maintained at 18°C, relative humidity was 65%, and air velocity was <0.2 m/s. Calves were exposed to 13.5 h of lightness (0000 to 0030 h and 0530 to 1830 h; 50 lux) and 10.5 h of darkness (6 lux). The experiment was approved by the Ethical Committee of Wageningen University.

Diets and feeding

Calves were fed according to their metabolic BW ($kg^{0.75}$), adjusted daily for a projected average daily gain of 500 g at the low FL and 1500 g at the high FL. The ME_m was assumed to be 460 $kJ/(kg^{0.75} \cdot d)$, based on estimations for ME_m in heavy preruminant calves by Gerrits et al. (1996), and Van Es et al. (1967). The ingredient and analyzed nutrient composition of the experimental milk replacer is shown in Table 2.

Table 2. Ingredient composition and analyzed nutrient composition of the experimental diet

Ingredient, g/kg		Nutrient[2], g/kg DM	
Whey	372.9	Dry matter (g/kg)	978.0
Whey protein concentrate	360.0	Crude ash	73.1
Delactosed whey	70.0	Crude protein (N × 6.25)	190.5
Soy oil	144.0	Crude fat	195.8
Coconut oil	36.0	Lactose	470.3
DL-methionine	1.32	Gross energy (MJ/kg)	20.6
Mono potassium phosphate	2.32	Lysine	16.2
Calcium carbonate	6.60	Methionine	5.0
Magnesium oxide	0.84	Cystine	5.0
Vitamin and mineral premix[1]	6.00	Threonine	13.2
		Tryptophan[3]	3.1
		Isoleucine[3]	11.4

[1] Provided per kg of the experimental diet: calcium 7.8 g; phosphorus 6.5 g; magnesium 1.4 g; vitamin A 25,026 IU; vitamin D_3 2,000 IU; vitamin E 80 mg; vitamin C 130 mg; zinc 84 mg; copper 9 mg; iron 48 mg; manganese 15 mg; selenium 0.1 mg; cobalt 1.9 mg.

[2] Analyzed content, unless indicated otherwise.

[3] Calculated content.

Whey was used as only protein source, because it is a rapidly hydrolysable protein source that can be included at high levels in milk replacer diets. Milk replacer was reconstituted with water (140 g/L) and supplied in a bucket at a temperature of about 40°C. Roughage was not supplied. Feeding times were 0000 (FF 2 and FF 4), 0600 (FF 4), 1200 (FF 1, FF 2 and FF 4),

and 1800 h (FF 4). Additionally, calves at FF 1 were supplied with 3 L of warm water (40°C) at 0600 and 1800 h to prevent dehydration. Calves were allowed 15 min to consume the meal.

Measurements

Gas exchange was measured in 6-min intervals by measuring the exchange of oxygen, carbon dioxide, and methane as described by Verstegen et al. (1987). The posture of calves was measured every minute by infrared beam interruption and expressed as lying (i.e., lying during the complete 6-min interval) or non-lying (i.e., standing during at least 1 min of the 6-min interval). Physical activity was recorded with a radar Doppler device according to the method described by Wenk and Van Es (1976).

Calves were weighed before and after each balance period. Faeces were collected quantitatively in plastic bags which were harnessed to the calves. They were collected twice daily and stored at -20°C pending analyses. Urine was collected in a pit containing 500 mL (low FL) or 750 mL (high FL) of 4.5 M sulfuric acid. Aerial NH_3 and NH_4^+ in water that condensed on the heat exchanger were also collected quantitatively (Verstegen *et al.*, 1987). Feed was sampled during each experimental period. Feed refusals were collected 15 min after feeding and stored at -20°C pending analyses.

For determination of the DM content, feed refusals and fresh faeces were freeze-dried, feed samples were vacuum-dried at 80ºC, and air-dry faeces were dried in a forced air oven at 103ºC. All samples were dried to a constant weight according to ISO Standard 6496 (1998b). Following freeze-drying, faeces were ground to pass a 1-mm screen and kept for analyses. Nitrogen content was measured in fresh feed, feed refusals, faeces, urine, aerial NH_3 and water that condensed on the heat exchanger according to ISO Standard 5983 (1997). Crude fat content was determined after acid hydrolysis in feed and in freeze-dried faeces according to ISO Standard 6496 (1999). Crude ash content was determined in feed and in freeze-dried faeces. Samples were carefully incinerated in a muffle furnace by slowly increasing the temperature from 20ºC to 550ºC to prevent foaming, and subsequent incineration took place according to ISO Standard 5984 (2002). The lactose content was analyzed enzymatically in feed and in freeze-dried faeces (Enzytec; Diffchamb Biocontrol, Nieuwerkerk aan den IJssel, The Netherlands). Gross energy content was analyzed in feed, freeze-dried faeces and urine using adiabatic bomb calorimetry (model IKA-calorimeter C7000; IKA Werke GmbH & Co. KG, Staufen, Germany) according to ISO Standard 9831 (1998a). All analyses were carried out in duplicate, except the nitrogen content in urine which was determined in triplicate.

Calculations

For each balance period, intake of ME per chamber was calculated as the difference between digestible energy intake and the sum of urinary energy losses and methane production. From the gaseous exchanges, heat production (HP) was calculated according to the formula of Brouwer (1965). Energy retention was calculated by subtracting HP from ME intake. Retention of nitrogen was calculated from N in feed and excreta, aerial NH_3 and NH_4^+ in water that condensed on the heat exchanger. Energy retained as protein was derived from retained nitrogen, assuming 23.6 kJ/g of protein. Energy retention as fat was calculated by subtracting energy retained as protein from ER. For each calf within a balance period, the energy costs per unit of physical activity were estimated by regression of physical activity against heat production, using Equation 1:

$$HP_{ij} = \mu + P_i + \beta \times X_j + e_{ij} \qquad [1]$$

where HP is heat production during posture i and the 6-min period j; μ is the overall mean; P_i is the fixed effect of posture i (i = lying, nonlying); β = regression coefficient of heat production on activity counts; X_j is activity counts during the 6-min period j; and e_{ij} is an error term. Posture was included as a fixed effect in Equation 1, because the regression between HP and activity counts appeared to depend on posture, probably related to the distance of the animal to the radar meters. The extra activity costs of non-lying vs. lying were calculated for each calf for each balance period by subtracting the estimated intercept at zero activity of position lying from non-lying (HP_{nl-l}) calculated from Equation 1. Subsequently, for each calf within a balance period, HP_{act} was calculated as described in Equation 2:

$$HP_{act:j} = HP_{nl-l} + b_k \times X_j \qquad [2]$$

where $HP_{act:j}$ is activity-related heat production during the 6-min period j; HP_{nl-l} is the calculated extra activity costs of non-lying vs. lying; b_k is the regression coefficient, calculated using Equation 1 during posture k; and X_j is activity counts during the 6-min period j. Balance period and hourly means were calculated for HP, HP_{act}, and HP_{cor}. By subtracting HP_{act} from HP, the heat production not related to physical activity (HP_{cor}) was derived.

Statistical analysis

Apparent faecal digestibility and energy and nitrogen balance variables were analyzed for the effects of FF, FL, for the interaction between FF and FL, and for period by ANOVA in 2 ways. First, 3 separate models were used to test 1) the effect of FF within the low FL, 2) the effect of period for FF 1, and 3) the effects of FF and FL for FF 2 and 4. For all 3 models, repeated measurements within calves were used (PROC GLM in SAS; SAS Institute, Inc., Cary, NC). Second, the effects of FF, FL, and period were tested in one mixed model with repeated measurements within calves, using PROC MIXED in SAS (SAS Institute, Inc.). The mixed model included fixed effects of FF, FL, and period, and a random effect of each calf (Eq. 3).

$$Y_{ijk} = \mu + FF_i + FL_k + (FF \times FL)_{ik} + P_l + \varepsilon_{ijkl} \qquad [3]$$

where Y_{ijkl} is the dependent variable; μ is the average intercept; FF_i is the effect of feeding frequency i (i = 1, 2, 4); FL_k is the effect of feeding level k (k = 1, 2); P_l is the effect of period l (l = 1, 2), and ε_{ijkl} is an error term, which represents the random effect of calf within feeding frequency (j = 1,…, 6). Treatment effects were studied by pairwise comparisons using the Tukey method. The SAS software package version 9.1 (SAS Institute, Inc.) was used in all statistical evaluations.

Because the *P*-values and model predictions of all effects of the 3 models described above were identical to those obtained with the mixed model (Eq. 3), only the results of the mixed model are presented.

Results

General

Two animals were excluded from the experiment because of illness and feed refusals. Another calf was excluded from one of the experimental periods. The results were not affected (*P* > 0.10) by the experimental period. Therefore, the effect of experimental period was not included in the results.

Table 3. Influence of feeding frequency (FF; 1, 2 or 4 meals/d) and feeding level (FL; high vs. low) on mean BW, feed intake, average daily gain, and apparent faecal nutrient digestibility in preruminant calves. Values are means ± SEM.

	FF 1	FF 2		FF 4			*P*-value		
Item	FL low	FL low	FL high	FL low	FL high	SEM	FF	FL	FF × FL
No. of observations	8	5	6	6	6		-	-	-
Average BW, kg	130	143	175	133	173	5.9	0.362	< 0.001	0.043
Feed intake, g/d	1350	1437	2792	1393	2755	68.5	0.612	< 0.001	0.918
Feed intake, g/(kg$^{0.75}$·d)	35.1	34.6	57.0	35.2	56.7	0.49	0.888	< 0.001	0.257
ADG[1], g/d	515	561	1835	652	1645	125	0.711	< 0.001	0.164
Apparent faecal digestibility, in %									
Dry matter	94.2	95.9	95.2	95.5	95.5	0.87	0.274	0.785	0.556
Energy	95.3	95.5	94.2	95.4	95.2	0.83	0.753	0.475	0.360
Ash	85.1	84.6	85.5	85.7	88.1	1.27	0.260	0.662	0.068
Crude fat	93.6	95.4	92.5	94.7	93.9	1.77	0.721	0.480	0.442
Crude protein	92.2	91.8	90.9	92.6	93.1	0.79	0.172	0.855	0.148
Lactose[2]	100.0	100.0	99.9	99.9	100.0	0.03	-	-	-

[1] ADG = average daily gain.

[2] Errors for lactose digestibility were not normally distributed.

Nutrient digestibility

The effects of FL and FF on performance and apparent faecal nutrient digestibility are shown in Table 3. Realized feed intakes were similar to the preplanned intakes. Also, digestible nutrient intake (data not shown) did not differ ($P > 0.10$) between animals at a similar FL. The apparent faecal digestibility of DM, energy, protein, and fat exceeded 90%. Treatment effects were not observed. Ash digestibility was about 85%, and an increase of FF at a high FL tended to raise ash digestibility compared with an increased FF at a low FL (FF × FL, $P < 0.10$). Lactose digestibility was virtually complete at the faecal level.

Energy partitioning and protein utilization

Data on protein and energy utilization and effects of FL and FF are shown in Table 4.

Feeding level. Intakes of gross and metabolizable energy were, as expected, markedly higher ($P < 0.001$) at the high FL than at the low FL. Metabolizability of the digestible energy did not differ ($P > 0.10$) between FL. Heat production increased ($P < 0.001$) substantially with FL (146 kJ/(kg$^{0.75}$·d)), but HP_{act} was not affected ($P > 0.10$) by FL. Energy retention (both as protein and as fat) was positively affected ($P < 0.001$) by FL. The efficiency of nitrogen utilization for protein retention, expressed as percentage of digestible nitrogen intake, was not affected ($P > 0.10$) by FL.

Feeding frequency. At a similar FL, gross and metabolizable energy intakes did not differ ($P > 0.10$) between FF. Metabolizability of the digestible energy did not differ ($P > 0.10$) between FF and exceeded 95% for all treatments. The metabolizability of digestible energy tended to increase when 4 instead of 2 meals were fed at a high FL compared with a low FL (FF × FL, $P < 0.10$). Heat production was not affected by FF, although HP_{cor} tended to decrease ($P < 0.10$) with increasing FF. Energy retention, both as protein and as fat, increased ($P < 0.001$) with increasing FF. The efficiency of nitrogen utilization for protein retention, expressed either as the percentage of dietary nitrogen (data not shown) or as the percentage of digested nitrogen, increased 11% ($P < 0.05$), from 49.1% at FF 2 to 54.4% at FF 4 at the high FL.

Circadian rhythms

Circadian rhythms of HP and HP_{cor} are shown in Figure 1. Consistently, meal ingestion was followed by an HP peak (Figures 1a, 1c). When corrected for HP_{act}, whose circadian rhythm did not differ between treatments, a regular pattern of HP_{cor} was observed (Figures 1b, 1d).

Table 4. Influence of feeding frequency (FF; 1, 2 or 4 meals/d) and feeding level (FL; high vs. low) on energy and protein balance. Values are means ± SEM and expressed in $kJ/(kg^{0.75} \cdot d)$ unless mentioned otherwise.

Item	FF 1 FL low	FF 2 FL low	FF 2 FL high	FF 4 FL low	FF 4 FL high	SEM	FF	FL	FF × FL
Gross energy intake	754	744	1233	756	1228	10.4	-	-	-
Digestible energy intake	717[b]	711[b]	1161[a]	723[b]	1171[a]	9.4	0.671	< 0.001	0.954
Methane	2.5	3.2	4.6	4.7	5.4	0.93	0.214	0.270	0.484
Urinary energy	30[c]	27[c]	46[a]	28[bc]	36[b]	4.6	0.448	0.032	0.087
Metabolizable energy intake	683[b]	677[b]	1109[a]	688[b]	1130[a]	13.4	0.307	< 0.001	0.619
ME:DE[1], in %	95.3[b]	95.4[ab]	95.6[ab]	95.4[ab]	96.5[a]	0.42	0.518	0.227	0.089
Heat production									
Total	550[b]	556[b]	701[a]	528[b]	675[a]	15.6	0.194	< 0.001	0.912
Activity related	70	75	75	65	69	10.8	0.768	0.873	0.805
Resting	480[b]	483[b]	626[a]	463[b]	607[a]	9.1	0.055	< 0.001	0.957
Energy retention									
Total	133[cd]	119[d]	408[b]	159[c]	453[a]	15.2	< 0.001	< 0.001	0.812
As protein	74[c]	75[c]	120[b]	81[c]	136[a]	4.7	< 0.001	< 0.001	0.153
As fat	59[bc]	45[c]	288[a]	79[b]	317[a]	14.1	< 0.001	< 0.001	0.809
Nitrogen retention									
Efficiency, % of dN[2] intake	48.4[b]	50.9[ab]	49.1[b]	53.4[a]	54.5[a]	2.09	0.039	0.896	0.315

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

[1] ME:DE = metabolizable energy intake / digestible energy intake × 100.

[2] dN intake = digestible nitrogen intake.

Feeding level. An increased FL increased the minimal HP (+111 and +97 kJ/(kg$^{0.75}$·d)) and HP$_{cor}$ (+90 and +92 kJ/(kg$^{0.75}$·d)) for FF 2 and 4, respectively. At a higher FL, the amplitude of HP$_{cor}$ increased by 63% and 100% for FF 2 and 4, respectively. For both FF, a higher FL resulted in a delay of the HP$_{cor}$ peaks.

Feeding frequency. Although daily HP was not affected by FF (Table 4), the circadian rhythm of HP clearly differed between FF (Figures 1a, 1c). The minimal HP and HP$_{cor}$ decreased with decreasing FF. The amplitude of HP and HP$_{cor}$ increased with decreasing FF. Furthermore, the increase of the amplitude for H$_{cor}$ with decreasing FF was greater at a high FL (+100 kJ/(kg$^{0.75}$·d)) than at a low FL (+72 kJ/(kg$^{0.75}$·d)). A decreased FF delayed the time of the maximal H$_{cor}$. Following a meal at a low FL, for example, the maximal H$_{cor}$ was reached after 6 h for FF 1 and after about 1 h for FF 4.

Discussion

This study was conducted to assess the effects of FF (i.e., 1, 2, or 4 meals/d) at two FL (1.5 or 2.5 × ME$_m$) on apparent faecal digestibility and energy and protein metabolism in heavy preruminant calves. In the 4 wk prior to the start of the study, calves were well adapted to the experimental treatment. Feeding level was confounded with experimental period in this study. The effect of period could be tested only for calves fed once daily, and an effect appeared to be absent. However, an interaction between FL and period could not be excluded for calves at FF 2 and FF 4. Our main interest was in examining the effects of FF on nutrient utilization when a quickly hydrolysable protein source (i.e., whey protein) was used.

Nutrient digestibility

Apparent faecal digestibility was generally high and comparable to values in other studies (Donnelly & Hutton, 1976; Gerrits *et al.*, 1996; Diaz *et al.*, 2001). Nutrient digestibility was not affected by FL or FF. The lack of effect of FL is in accordance with findings in milk-fed calves of 60 kg BW (Donnelly & Hutton, 1976). In pigs, an increased FL resulted in a higher rate of passage of digesta, which may cause a reduction in nutrient digestibility (Roth & Kirchgessner, 1985). The analogy between a higher FL and a decreased FF (i.e., an increased meal size) may indicate that similar mechanisms are involved. Indeed, distribution of the daily milk supply over 2 (Ternouth *et al.*, 1977), 2 or 4 (Keusenhoff & Piatkowski, 1983), or 6 meals (J. Huisman, unpublished data) instead of 1 meal increased the rate of gastric emptying substantially, but did not affect apparent faecal nutrient digestibility in preruminant calves

(Keusenhoff & Piatkowski, 1983). The effect of rate of passage can be expected to be more pronounced when slowly and less-digestible feed ingredients are used. In the study of Williams et al. (1986), for example, apparent fat digestibility was very low (only 67%) for calves fed once daily and increased to 85% for animals fed 6 meals per day.

Energy partitioning and protein utilization

Feeding level. Metabolizability of the digestible energy was on average 95.6% which corresponds with other values found in preruminant calves (Gerrits *et al.*, 1996; Diaz *et al.*, 2001), whereas the metabolizability can be markedly lower in very young, unadapted calves (76%, Schrama *et al.*, 1992). Activity-related heat production was unaffected by FL in this study, whereas a higher FL was reported to decrease physical activity in pigs (Terlouw & Lawrence, 1993). As a percentage of HP, HP_{act} was numerically higher ($P = 0.159$) at a low FL (12.9%) than at a high FL (10.5%).

An increased FL resulted in a higher ER and also in a higher efficiency of utilization of ME for ER. This efficiency increased from 18-23% at a low FL to 36-40% at a high FL because of a decreased contribution of ME_m to total energy expenditure. The marginal efficiency of utilization of ME (i.e., excluding ME_m) could be calculated by regression of ER on the intake of ME for animals fed at both FL. The marginal efficiency was 72% and did not differ between animals fed 2 and 4 meals daily. Similar efficiencies, 68, 69% and 69%, respectively, were reported in calves up to 150 kg of BW (Van Es *et al.*, 1967; Van Es, 1970; Meulenbroeks *et al.*, 1986). Gerrits et al. (1996) found a slightly lower marginal efficiency of utilization of ME for ER (on average 60%) in calves of 80 to 160 kg BW but a similar efficiency (on average 71%) in calves of 160 to 240 kg of BW. The higher efficiency of utilization for heavy animals in their study coincided with lower estimates of ME_m.

The efficiency with which digestible protein was utilized was not affected by FL in this study. Dilution of the protein requirements for maintenance and independent additional effects of increased energy and protein intake on protein deposition (Gerrits *et al.*, 1996) could be expected to result in a higher efficiency of utilization of digestible protein for growth. On the other hand, increased temporal AA availability at a high FL may have exceeded the capacity for protein retention and possibly resulted in increased AA oxidation. The lack of effect of FL on protein utilization is in agreement with previous studies in milk-fed calves (Meulenbroeks *et al.*, 1986) and growing pigs (Bikker, 1994), but not with the study of Gerrits et al. (1996) in calves.
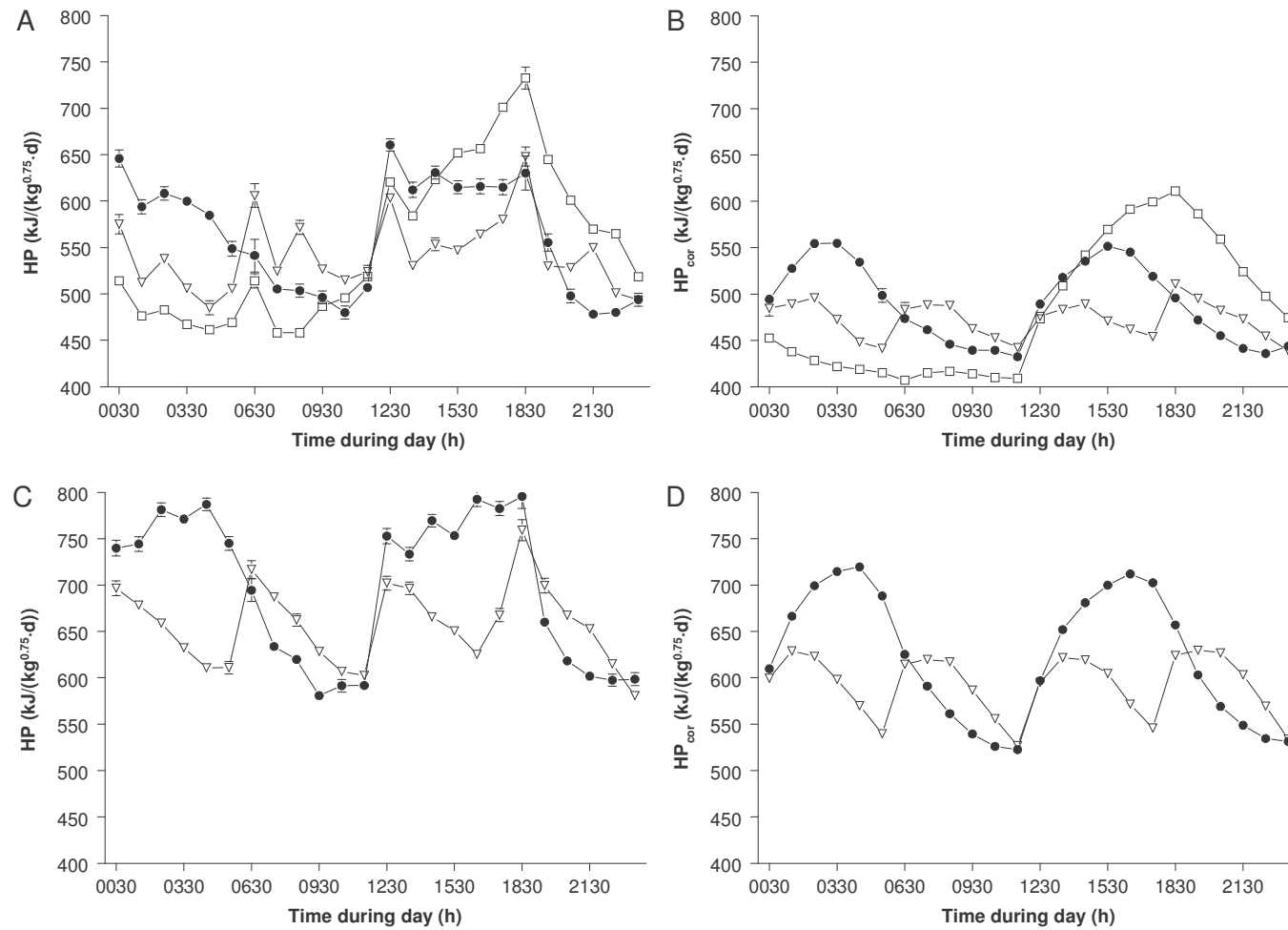
Figure 1. Influence of feeding frequency (FF) 1 (□), 2 (●) and 4 (▽) at a low (a and c) and a high feeding level (FL; b and d) on the circadian patterns of heat production (HP; a and b) and heat production corrected for activity (HP$_{cor}$; c and d) in heavy preruminant calves. Values are means ± SEM, n = 8 (□), n = 5 (●, in a and c), n = 6 (●, in b and d), or n = 6 (▽). The low FL was 690 kJ ME/(kg$^{0.75}$·d) and the high FL was 1150 kJ ME/(kg$^{0.75}$·d). Feeding times were 0000 (FF 2 and FF 4), 0600 (FF 4), 1200 (FF 1, FF 2 and FF 4) and 1800 h (FF 4).

Feeding frequency. Altogether, the numerically increased ME intake and decreased HP with increasing FF resulted in a significantly increased ER, both as protein and as fat. The realized ME intake did not differ between FF at the same FL and amounted to 1.48 and 2.43 $\times$ ME_m for the low and high FL, respectively. At both FL, HP was not affected by FF, but calves fed twice daily produced numerically slightly more heat than those fed 1 or 4 times daily (+1% and +5%, respectively) at a low FL.

The increased fat deposition in frequently fed calves contrasts with studies in humans and monogastric animals in which eating more frequently either decreased (especially in epidemiological observations) or did not affect body adiposity (Dawson, 1999; Bellisle, 2004). The increased energy expenditure for gastrointestinal tissues may partly explain the lower fat deposition when calves were fed less frequently. Early studies have shown that feeding animals less frequently can result in increased weights of the gastrointestinal tissues (pigs: Allee *et al.*, 1972; rats: Pocknee & Heaton, 1976) to cope with large meal sizes. Also, Walker et al. (1967) showed that the weight of the abomasum increased 41% when lambs were fed a similar amount of cow's milk in 2 instead of in 6 meals. Gastrointestinal hypertrophy with decreasing FF contributes to increased energy requirements, because portal-drained viscera account for a disproportionately large amount of HP. Although they represent only 6% of total BW in milk-fed calves, these tissues are responsible for 17% of HP, and even for 33 to 54% of the postprandial increase of HP (Ortigues *et al.*, 1995). An improved synchrony between energy supply and requirements may also have increased ER at a higher FF. Finally, an improved endocrine profile could have limited energy losses with increasing FF in the present study. Several studies (Doppenberg & Palmquist, 1991; Hostettler-Allen *et al.*, 1994) indicate that the capability of milk-fed calves to process large amounts of nutrients is limited, which can result in characteristic metabolic and endocrine changes related to hyperglycemia and insulin resistance. Feeding the daily milk supply in more than 5 meals avoided hyperglycemia and resulted in an endocrine pattern which was potentially more favorable for anabolism (Kaufhold *et al.*, 2000).

There was no effect of FF on physical activity, although feeding calves more frequently was anticipated to result in an increased HP_{act}. Also, when expressed as percentage of HP, HP_{act} was not affected ($P > 0.10$) by FF. The absence of an effect on physical activity could be due to the individual housing of animals, which has been shown to reduce activity in milk-fed calves (Veissier *et al.*, 1998). Furthermore, the cephalic phase may have induced relatively more physical activity in the more frequently fed calves, because certain intensive behaviors (e.g. manipulation and licking objects) were frequently observed during the preprandial period

(JJGC van den Borne, SJFM van der Heijden, EAM Bokkers, JE Bolhuis, and WJJ Gerrits, unpublished). Calves at FF 4 experienced the preprandial period 4 times a day instead of once daily for calves at FF 1. Similarly, LeBlanc and Diamond (1986) found in dogs that the amplitude of HP for 4 small meals (125 g) was twice as large as that for 1 large meal (500 g). They ascribed this effect to the heat produced during the cephalic phase for each meal. In dogs, heat production for a single meal was comparable to feeding four meals when the meal was preceded by three simulated meals (sham feeding), which suggests that sensory stimulation may interfere with the results. In the present study, calves could not see, smell, or hear the milk preparation. The fixed feeding pattern and long adaptation period, however, may have enabled them to anticipate the times of feeding. Finally, the provision of water and entrance into the respiration chambers to collect samples twice daily may have stimulated physical activity in calves at FF 1.

Increasing FF resulted in a more efficient utilization of digestible protein for protein gain. The relative increase in efficiency was 10.3% when the FF increased from 1 to 4 at a low FL and 11.0% when the FF increased from 2 to 4 at a high FL. We speculated that AA availability was temporarily less abundant at a higher FF, which may avoid temporal AA excesses and a concomitant increase in AA oxidation. Measurement of AA oxidation could provide further insight into the underlying mechanism that results in an increased AA utilization in calves. The effects of FF on protein utilization in human and rat studies are inconsistent (as reviewed by Dawson, 1999), and the results from FF experiments in pigs are also confusing (Partridge *et al.*, 1985; Mroz *et al.*, 1994). The effects of FF may depend on the kinetics of protein digestion and absorption. In pigs, for example, AA oxidation decreased with increasing FF when synthetic lysine was included in the diet, but it was not affected by FF when protein-bound lysine was used (Batterham & Bayley, 1989). This may explain why protein retention in (heavy) calves was affected by FF in the present study when whey protein was used, but not in (young) calves when skimmed milk protein was fed (Williams *et al.*, 1986).

We hypothesized that the increase in protein utilization with FF would be more pronounced at a higher FL. However, there was no significant interaction between FL and FF ($P = 0.315$), although protein utilization increased by 4.9% at a low FL and 11.0% at a high FL when the FF was increased from 2 to 4 meals daily.

Marginal efficiencies of protein utilization were calculated for animals fed at both FL. The marginal efficiency for calves at FF 2 (48.3%) was numerically, but not statistically ($P = 0.12$), lower than for calves at FF 4 (56.3%). Both values are considerably higher than values previously found in heavy preruminant calves (between 25 and 30%; Gerrits et al., 1996). The

different protein sources (whey vs. skimmed milk) may have contributed to the increased marginal efficiency in the present study.

Circadian rhythms

Surprisingly, the considerable differences in circadian fluctuations of HP did not result in a different HP for the 3 FF. The circadian rhythm of HP can be compared with the results in young calves of 60 kg BW (Ortigues *et al.*, 1995). Those researchers fed calves twice daily within an 8-h interval at a FL of 2.3 × ME_m. The amplitude of HP could be expected to be higher in their study than in the present study, based on the dissimilar interval (8 h) between the 2 meals in their study. Nonetheless, the amplitude was slightly lower in the study of Ortigues et al. (1995) (171 kJ/($kg^{0.75}$·d)) than in the present study at FF 2 and a high FL (198 kJ/($kg^{0.75}$·d)), although the minimal HP was substantially lower (91 kJ/($kg^{0.75}$·d)) in their study. The difference can be explained by the difference in FL and assuming an inefficiency of energy utilization of 28% (as found in this study). Also the use of skimmed milk powder in the study of Ortigues et al. (1995) may have caused a slow absorption of fatty acids and AA and consequently leveled off the HP peaks. The different protein source could also explain the 70% higher amplitude for HP_{cor} in calves of 175 kg BW (189 kJ/($kg^{0.75}$·d)) than in calves of 60 kg BW (112 kJ/($kg^{0.75}$·d)). Finally, the interval until maximum HP was more than 4 h for calves of 175 kg BW in the present study, while calves of 60 kg BW (Ortigues *et al.*, 1995) reached maximum HP within 1-2 h postprandial. This discrepancy could result from the use of a clotting protein source in the study of Ortigues et al. (1995), but may also indicate the previously mentioned insensitivity to insulin and metabolic difficulties of heavy milk-fed calves to handle glucose.

Conclusions

This experiment showed that increasing the frequency of feeding a rapidly hydrolysable protein source (i.e. whey) increased the efficiency of protein utilization in heavy preruminant calves. Feeding 4 instead of 2 daily meals at a high FL increased the efficiency of digestible protein utilization by 11%, from respectively 49.1 to 54.5%. The hypothesis that the efficiency of protein utilization would be more affected by FF at a high than at a low FL was not confirmed. Fat deposition increased with increasing FF. An increased FL resulted, as expected, in an increased fat deposition (+245 kJ/($kg^{0.75}$·d)) and a higher efficiency of energy

utilization (+18%). We concluded that the nutrient utilization in heavy preruminant calves increases with increasing FF of a rapidly hydrolysable protein source. This can be relevant for practical calf nutrition, because skimmed milk protein has generally been replaced by whey and vegetable protein sources in calf milk replacers.

Acknowledgements

References

Allee GL, Romsos DR, Leveille GA & Baker DH (1972) Metabolic adaptation induced by meal-eating in the pig. *J Nutr* **102**, 1115-1122.

Batterham ES & Bayley HS (1989) Effect of frequency of feeding of diets containing free or protein-bound lysine on the oxidation of [^{14}C]lysine or [^{14}C]phenylalanine by growing pigs. *Br J Nutr* **62**, 647-655.

Bellisle F (2004) Impact of the daily meal pattern on energy balance. *Scand J Nutr* **48**, 114-118.

Bikker P (1994) Protein and lipid accretion in body components of growing pigs. PhD thesis, Wageningen Agricultural University.

Blome RM, Drackley JK, McKeith FK, Hutjens MF & McCoy GC (2003) Growth, nutrient utilization, and body composition of dairy calves fed milk replacers containing different amounts of protein. *J Anim Sci* **81**, 1641-1655.

Brouwer E (1965) Report of sub-committee on constants and factors. *In: Energy Metabolism*, [KL Blaxter, editor]. London, UK. Academic Press, pp. 441-443.

Dawson JM (1999) Variation in nutrient supply and effects on whole body anabolism. *In: Proceedings of the VIIIth International Symposium on Protein Metabolism and Nutrition, Aberdeen, UK.* [GE Lobley, A White and JC MacRae, editors]. Wageningen Pers, pp. 101-126.

Diaz MC, Van Amburgh ME, Smith JM, Kelsey JM & Hutten EL (2001) Composition of growth of Holstein calves fed milk replacer from birth to 105-kilogram body weight. *J Dairy Sci* **84**, 830-842.

Donnelly PE & Hutton JB (1976) Effects of dietary protein and energy on the growth of Friesian bull calves, I. Food intake, growth, and protein requirements. *N Z J Agric Res* **19**, 289-297.

Doppenberg J & Palmquist DL (1991) Effect of dietary fat level on feed intake, growth, plasma metabolites and hormones of calves fed dry or liquid diets. *Livest Prod Sci* **29**, 151-158.

Gerrits WJJ, Tolman GH, Schrama JW, Tamminga S, Bosch MW & Verstegen MWA (1996) Effect of protein and protein-free energy intake on protein and fat deposition rates in preruminant calves of 80 to 240 kg live weight. *J Anim Sci* **74**, 2129-2139.

Hostettler-Allen RL, Tappy L & Blum JW (1994) Insulin resistance, hyperglycemia, and glucosuria in intensively milk-fed calves. *J Anim Sci* **75**, 160-173.

ISO (1997) Animal feeding stuffs. Determination of nitrogen content and calculation of crude protein content. Kjeldahl method. ISO 5983. International Organization for Standardization.

ISO (1998a) Animal feeding stuffs, animal products, and faeces or urine. Determination of gross calorific value. ISO 9831. International Organization for Standardization.

ISO (1998b) Animal feeding stuffs. Determination of moisture and other volatile matter content. ISO 6496. International Organization for Standardization.

ISO (1999) Animal feeding stuffs. Determination of fat content. ISO 6496. International Organization for Standardization.

ISO (2002) Animal feeding stuffs. Determination of crude ash. ISO 5984. International Organization for Standardization.

Kaufhold JN, Hammon HM, Bruckmaier RM, Breier BH & Blum JW (2000) Postprandial metabolism and endocrine status in veal calves fed at different fequencies. *J Dairy Sci* **83**, 2480-2490.

Keusenhoff R & Piatkowski B (1983) Der Einfluß verschiedener Tränkfrequenzen und Milchbehandlungen auf den Digestafluß am Duodenum des Kalbes. *Arch Tierernahr* **33**, 179-187.

LeBlanc J & Diamond P (1986) Effect of meal size and frequency on postprandial thermogenesis in dogs. *Am J Physiol* **250**, E144-E147.

Meulenbroeks J, Verstegen MWA, Van der Hel W, Korver S & Kleinhout G (1986) The effect of genotype and metabolizable energy intake on protein and fat gain in veal calves. *Anim Prod* **43**, 195-200.

Mroz Z, Jongbloed AW & Kemme PA (1994) Apparent digestibility and retention of nutrients bound to phytate complexes as influenced by microbial phytase and feeding regimen in pigs. *J Anim Sci* **72**, 126-132.

Ortigues I, Martin C, Durand D & Vermorel M (1995) Circadian changes in energy expenditure in the preruminant calf: whole animal and tissue level. *J Anim Sci* **73**, 552-564.

Partridge IG, Low AG & Keal HD (1985) A note on the effect of feeding frequency on nitrogen use in growing boars given diets with varying levels of free lysine. *Anim Prod* **40**, 375-377.

Pocknee RC & Heaton FW (1976) The effect of feeding frequency on the growth and composition of individual organs in the rat. *Br J Nutr* **35**, 97-104.

Roth FX & Kirchgessner M (1985) Verdaulichkeit und intestinale Passagerate beim Schwein in Abhängigkeit vam Fütterungsniveau und Rohfasergehalt des Futters. *Z Tierphysiol Tierernahr Futtermittelkd* **53**, 254-264.

Schrama JW, Van der Hel W, Arieli A & Verstegen MWA (1992) Alteration of energy metabolism of calves fed below maintenance during 6 to 14 days of age. *J Anim Sci* **70**, 2527-2532.

Schroeder GF, Titgemeyer EC, Awawdeh MS & Gnad DP (2004) Effects of energy supply on methionine utilization by growing steers. *J Dairy Sci* **87, Suppl. 1**, 115. (Abstr.).

Terlouw EMC & Lawrence AB (1993) Long-term effects of food allowance and housing on development of stereotypies in pigs. *Appl Anim Behav Sci* **38**, 103-126.

Ternouth JH, Roy JHB, Stobo IJF, Shotton SM & Gillies CM (1977) Concurrent studies of the flow of digesta in the duodenum and of exocrine pancreatic secretion in calves, 5. The effect of giving milk once and twice daily, and of weaning. *Br J Nutr* **37**, 237-249.

Titgemeyer EC (2003) Amino acid utilization by growing and finishing ruminants. *In: Amino acids in animal nutrition*, [JPF D'Mello, editor]. Wallingford, UK. CAB International, pp. 329-346.

Van Es AJH (1970) Simulation of the growth of veal calves fed liquid milk replacers. *In: Energy Metabolism of Farm Animals, Zurich, Switzerland*. [A Schürch and C Wenk, editors]. Juris Druck Verlag Zurich, pp. 97-100.

Van Es AJH, Nijkamp HJ, Van Weerden EJ & Van Hellemond KK (1967) Energy, carbon and nitrogen balance experiments with veal calves. *In: Energy metabolism of farm animals, Newcastle-upon-Tyne, UK*. [KL Blaxter, J Kielanowski and G Thorbek, editors]. Oriel Press, pp. 197-201.

Veissier I, Ramirez de la Fe AR & Pradel P (1998) Nonnutritive oral activities and stress responses of veal calves in relation to feeding and housing conditions. *Appl Anim Behav Sci* **57**, 35-49.

Verdonk JMAJ, Gerrits WJJ, Beelen GM & Jansman AJM (1999) Effect of protein source on portal nutrient fluxes in pre-ruminant calves. *In: The VIIIth International Symposium on Protein Metabolism and Nutrition, Aberdeen, UK*. [GE Lobley, A White and JC MacRae, editors]. Wageningen Pers, The Netherlands, pp. 47 (Abstr.).

Verstegen MWA, Van der Hel W, Brandsma H, Henken AM & Bransen AM (1987) The Wageningen respiration unit for animal production research: a description of the equipment and its possibilities. *In: Energy Metabolism in Farm Animals: Effects of Housing, Stress and Disease*, [MWA Verstegen and AM Henken, editors]. Dordrecht, The Netherlands. Martinus Nijhoff Publishers, pp. 21-48.

Walker DM, Cook LJ & Jagusch KT (1967) Nitrogen balance studies with the milk-fed lamb 5. Effect of frequency of feeding. *Br J Nutr* **21**, 275-287.

Wenk C & Van Es AJH (1976) Eine Methode zur Bestimmung des Energieauswandes für die körperlich Aktivität von wachsenden Küken. *Schweiz Landwirtsch Monatsh* **54**, 232.

Williams PEV, Fallon RJ, Brockway JM, Innes GM & Brewer AC (1986) The effect of frequency of feeding milk replacer to pre-ruminant calves on respiratory quotient and the efficiency of food utilization. *Anim Prod* **43**, 367-375.

Chapter 4

Effects of feeding frequency and feeding level on urea production and substrate oxidation in heavy preruminant calves

JJGC van den Borne[1], GE Lobley[2], MWA Verstegen[1], JM Muijlaert[1], SJJ Alferink[1] & WJJ Gerrits[1]

[1]*Animal Nutrition Group, Department of Animal Sciences, Wageningen University, 6700 AH Wageningen, The Netherlands*

[2]*Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB, United Kingdom*

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Abstract

The effect of feeding frequency (FF) on urea production and glucose and fatty acid oxidation was examined in heavy preruminant calves at each of two feeding levels (FL). In addition, fractional oxidation of orally provided 1-[1-^{13}C]leucine, [U-^{13}C]glucose and [2-^{13}C]glucose was studied to assess the pattern of dietary substrate oxidation and to estimate *de novo* fatty acid synthesis from glucose. Eighteen Holstein Friesian calves of 136 ± 3 kg body weight were assigned to FF (1, 2, or 4 meals daily) at 2 FL (1.5 or 2.5 times the metabolizable energy requirements for maintenance), except for calves fed once daily (only at a low FL). $^{13}CO_2$ excretion in breath was measured after oral supply of the stable isotopes, and gas exchange was measured by indirect calorimetry during 2 experimental periods of 10 d each in respiration chambers. After each period, urea production was measured by dilution of a primed, continuous infusion of [^{13}C]urea. Whey protein was the only protein source in the diet. Diurnal kinetics of urea production, but not total urea production, were affected by FF in heavy preruminant calves. Postprandial urea production increased with increasing FL, but 12 h production rates did not differ between treatments. Daily production rates correlated well with urinary N excretion ($r = 0.98$; $P < 0.001$). The $^{13}CO_2$ recovery from orally provided L-[1-^{13}C]leucine increased with increasing FF at a low FL, and decreased with increasing FF at a high FL. It was not correlated with amino acid oxidation as percentage of protein intake (calculated from urinary N excretion). Dietary glucose was almost completely oxidized (80% from [^{13}C]glucose and 94% from indirect calorimetry), regardless of the FL. In addition, because recoveries of [U-^{13}C]glucose and [2-^{13}C]glucose as CO_2 did not differ, *de novo* fatty acid synthesis from glucose was considered negligible in preruminant calves. In conclusion, urea production and oxidation of orally provided L-[1-^{13}C]leucine did not (significantly) decrease with increasing FF, which was not in line with the observed increase in protein deposition from N-balance measurements. The increase in fat deposition with increased feed intake almost exclusively originated from a reduced oxidation of fatty acids, whereas glucose is virtually completely oxidized regardless the feeding level.

Abbreviations: AUC, area under the curve; FF, feeding frequency; FL, feeding level; ME, metabolizable energy; ME_m, metabolizable energy requirements for maintenance; MPE, mean prediction error; MSPE, mean square prediction error; OXCHO, glucose oxidation; OXF, fatty acid oxidation; OXP, amino acid oxidation; RQ, respiratory quotient.

Introduction

The quantity and amino acid composition of dietary protein affects protein gain in growing animals and has been included in appropriate feed evaluation systems (NRC, 1998; CVB, 2000). Usually, the within-day distribution of protein intake is assumed not to influence the efficiency of protein utilization for growth. In preruminant calves, increasing the feeding frequency (FF) did not result in an improved protein utilization when skimmed milk protein was used as the sole protein source (Williams et al., 1986). Nonetheless, replacing the skimmed milk protein by non-clotting protein sources, such as wheat, soy or whey, resulted in a considerably faster but more transient portal appearance of amino acids (Verdonk et al., 1999). Recently, we studied the effect of FF using a milk replacer that contained the non-clotting whey protein in a nitrogen and energy balance study with heavy preruminant calves and found greater protein and fat deposition with increasing FF (Van den Borne et al., 2006b). As the digestible nutrient intakes were identical for all calves within FL, this suggested that the within day pattern of amino acid availability and consequently the amino acid oxidation was affected by FF.

In addition, FF will undoubtedly induce within-day variation in glucose metabolism. Apart from effects on glucose homeostasis, this is also expected to exert influence on protein metabolism. First, analysis of hormone and metabolite profiles in blood samples taken from the calves in the FF study of Van den Borne et al (2006b) showed large fluctuations in glucose and insulin concentrations within a day, with decreased postprandial insulin to glucose ratios with increasing FF (T Vicari, JJGC van den Borne, WJJ Gerrits, Y Zbinden and JW Blum, unpublished). Consequently, urinary glucose excretion decreased with increasing FF. Similar effects of meal frequency on glucose homeostasis and insulin concentrations were observed previously in man (Jenkins, 1997) and in rats (Romsos & Leveille, 1974) and may be associated with diurnal patterns of glucose oxidation and non-oxidative glucose disposal (Yki-Järvinen *et al.*, 1987; Groop & Ferrannini, 1993). Second, if not oxidized (or excreted in urine) glucose is stored as glycogen or converted to fatty acids *de novo*. Ruminants usually do not need high activities of the enzymes citrate lyase (to convert citrate to acetyl CoA and oxaloacetate) and malate dehydrogenase (to convert oxaloacetate to malate and NAD). In preruminant calves, however, the activity of ATP citrate lyase in liver and adipose tissue was substantially higher than in ruminating calves (Roehrig *et al.*, 1988) which indicates *de novo* fatty acid synthesis from glucose. Third, the increased protein deposition in heavy preruminant calves could be the result of a decrease in glucose oxidation by increasing the

protein-free energy availability. Gerrits et al (1996) showed that increasing the intake of fat and lactose increased protein deposition independent from protein intake.

To understand which of these options might operate in heavy preruminant calves the effects of FF were studied at a low and a high feeding level (FL). It was hypothesized that i) an increased FF would result in a decreased urea production and lower L-[1-^{13}C]leucine oxidation postprandially; ii) the increased protein deposition with increasing FF (Van den Borne et al., 2006b) would be associated with a decreased glucose oxidation; iii) substantial amounts of glucose are used for *de novo* fatty acid synthesis in heavy preruminant calves.

Materials and methods

Experimental design

The experimental design has been described in detail by Van den Borne et al (2006b) and is summarized in Table 1. Briefly, eighteen male Holstein Friesian calves were used in nine trials each of two calves of similar age. Each trial consisted of two experimental periods, each preceded by an adaptation period of four weeks. The experiment had a 2×3 factorial arrangement of the factors FL ($1.5 \times ME_m$ and $2.5 \times ME_m$) and FF (1, 2 or 4 meals/d). Within each trial one particular FF was applied, because visual and (limited) auditory contact between the individual calves could affect physical activity and cephalic phase reflexes. Within trials, a low FL was adopted in period 1 ($1.5 \times ME_m$) and a high FL in period 2 ($2.5 \times ME_m$). This order was used so that different FF could be compared across a similar experimental period and at similar BW. Animals at FF 1 were not offered the high FL because it was not feasible to feed calves at such a high level in only one meal (Table 1). Calves at FF 1 served as controls for the effect of experimental period, because FL was confounded with period in this study.

Table 1. Experimental design; number of observations per treatment

| | FF[1] 1 | FF 2 | | FF 4 | |
| | Treatment | | | | |
Item	FL[2] low	FL low	FL high	FL low	FL high
Period 1	6	6	-	6	-
Period 2	6	-	6	-	6

[1]FF = feeding frequency

[2]FL = feeding level

Diets and feeding

Calves were fed according to their metabolic BW ($kg^{0.75}$). Feed supply was adjusted daily for a projected average daily gain of 500 g at the low FL and 1500 g at the high FL. The metabolizable energy requirements for maintenance (ME_m) was assumed to be 460 $kJ/(kg^{0.75}{\cdot}d)$. Whey was used as the only protein source in the milk replacer, because it is a rapidly hydrolysable protein source which can be included at high levels in diets for preruminant calves. The detailed ingredient and analyzed nutrient composition of the experimental milk replacer has been presented by Van den Borne et al (2006b). Milk replacer was reconstituted with water (140 g/L) and supplied at a temperature of about 40°C in a bucket. Roughage was not supplied. Feeding times were 0000 (FF 2 and 4), 0600 (FF 4), 1200 (FF 1, 2 and 4) and 1800 h (FF 4). In addition, calves at FF 1 were supplied with 3 L of warm water (40°C) at 0600 and 1800 h to prevent dehydration. Calves were allowed 15 min to consume the meal. Average intake energy and protein intakes were 753 (SEM 3.4) $kJ/(kg^{0.75}{\cdot}d)$ and 6.9 (SEM 0.04) $g/(kg^{0.75}{\cdot}d)$ for calves at the low FL and 1228 (SEM 6.4) $kJ/(kg^{0.75}{\cdot}d)$ and 11.4 (SEM 0.07) $g/(kg^{0.75}{\cdot}d)$ at the high FL.

Measurements

The experiment consisted of a 10-d period during which calves were housed in respiration chambers and a 4-d period during which calves were housed on a metabolic cage in a climate controlled stable. During the first 10 days, the exchange of oxygen, carbon dioxide, and methane was measured over 6-min intervals as described by Verstegen et al (1987). Nitrogen and energy balances were measured in the respiration chambers (Van den Borne et al., 2006b). In addition, $^{13}CO_2$ production was measured during 6-min intervals by non-dispersive infrared spectrometry (Advance Optima Uras 14, ABB, Frankfurt, Germany) as described by Alferink et al (2003).

Sequestration of bicarbonate was measured on day 3, leucine oxidation on day 6 and glucose oxidation (of $[U-^{13}C]$glucose and $[2-^{13}C]$glucose) on days 4 and 8 respectively of the 10-d period in the respiration chambers. Bicarbonate sequestration was determined by measuring $^{13}CO_2$ excretion after injecting a pulse dose of 4.7 mmol $[^{13}C]$sodium bicarbonate (99.1 atom%; Mass Trace Inc., Woburn, MA, USA) into the ear vein of each calf. The infusate was prepared in 10 mL sterile 0.15 M NaCl and was injected within 2 min at 1400 h, i.e. 2 h after feeding for each treatment. Oxidation of leucine was quantified from $^{13}CO_2$ excretion after an oral bolus dose of $L-[1-^{13}C]$leucine (99.0 atom%; Cambridge Isotope Laboratories, Andover,

MA, USA). L-[1-^{13}C]leucine was added to the milk replacer (25.2 µmol/g dietary protein) offered at 1200 h. Oxidation of glucose was quantified from $^{13}CO_2$ excretion after an oral bolus dose of [U-^{13}C]glucose (99.0 atom%; Cambridge Isotope Laboratories, Andover, MA, USA) added at 2.5 µmol/g dietary lactose to the milk replacer at 1200 h. The [2-^{13}C]glucose (99.0 atom%; Cambridge Isotope Laboratories, Andover, MA, USA) was used to measure fractional oxidation of an oral dose (added as 15 µmol/g dietary lactose). It was assumed that the recoveries of [U-^{13}C]glucose and [2-^{13}C]glucose would be similar when glucose is completely oxidized but if *de novo* fatty acid synthesis from glucose occurs, then oxidation of the two tracers would differ (i.e. a higher recovery of $^{13}CO_2$ from [U-^{13}C]glucose than from [2-^{13}C]glucose due to generation of $^{13}CO_2$ as pyruvate is converted to acetate by the action of pyruvate dehydrogenase. The labelled C-atom from [2-^{13}C]glucose is however not released if acetate is formed and used for fatty acid synthesis (Berg et al., 2002).

After the 10-d period in a respiration chamber, the calves were prepared with a central venous catheter (16G/1.70 × 1.60 mm; Secalon T, Becton Dickinson, Alphen aan den Rijn, The Netherlands) in each jugular vein. The catheters were attached to the skin using Vicryl suturing (Ethicon, Sumerville, NJ, USA) and were extended with a 200 cm 3-layer Lectroflex extension tube (Vygon, Valkenswaard, The Netherlands) and a 25 cm Lectro-spiral extension tube (Vygon). Tape (Fermoflex and Elastoplast, Instruvet, Cuijk, The Netherlands) was used to cover the catheter and to maintain the extensions in their position. From day 11 at 1500 h, a solution of [^{13}C]urea (99.0 atom%; Cambridge Isotope Laboratories, Andover, MA, USA; 1 mg/g dissolved in sterile 0.15 M NaCl) was continuously infused at 0.11 mmol/h into the right jugular vein for 52 h preceded by a priming dose to enrich the body urea pool to 0.60 or 0.30 atom% excess for the low and the high FL respectively. On day 13, blood samples were taken from the right jugular vein between 0700 and 1900 h at 30-min intervals. Blood was immediately transferred into lithium heparin tubes (Vacutainer, Becton Dickinson, Alphen aan de Rijn, The Netherlands), stored on ice until plasma was collected after centrifugation at $1500 \times g$ for 10 min. Plasma samples were stored at -20°C pending analyses.

Urea concentration was measured spectrophotometrically with urease and glutamate dehydrogenase (Cobas Integra 800; Roche Diagnostics, Basel, Switzerland). The enrichment of ^{13}C in urea was measured as CO_2 released by urease. Briefly, plasma was first deproteinized by adding 5 mL methanol, mixing and storing of the tubes at -20°C for 2 h. After centrifugation at $1500 \times g$, the supernatant was transferred into a 10 ml Exetainer tube (Labco, High Wycombe, UK) and 50 µL of 12 M HCl added and mixed. After evaporation to dryness in a water bath at 40°C under a gentle stream of nitrogen 1 mL of (boiled) water and

300 µL of 1 M HCl were added, mixed and evaporated to dryness again. Then, 1.5 mL of a cold buffer solution (27 g KH_2PO_4 and 53.5 g Na_2HPO_4 per L; pH = 7.0) containing urease (50 U/mL; Sigma Chemical Co., St. Louis, MO, USA) was added on ice. The tubes were covered with rubber lids, flushed with argon for 10 sec to replace the air in the headspace and then incubated at 37ºC in a gently shaking water bath for 45 min. The reaction was stopped by injection of 0.5 mL 7 M phosphoric acid and the ^{13}C enrichment in CO_2 measured in the headspace using a Finnigan Delta C continuous-flow isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany). The enrichment was expressed as atom percent (atom %) $^{13}CO_2$.

Calculations

A Mitscherlich-exponential nonlinear model, as proposed by Rook et al (1993) for lactation curves was used. The model was fit to the 30 min means of $^{13}CO_2$ excretion in breath (corrected for background enrichment) after ingestion of L-[1-^{13}C]leucine, [U-^{13}C]glucose and [2-^{13}C]glucose and after infusion of [^{13}C]sodium bicarbonate:

$$y = (b_0 \cdot c \cdot t^{(-c-1)} \cdot b_1^c) / (1 + (b_1 / t)^c)^2 \qquad [1]$$

where y = $^{13}CO_2$ production (in µmol/min) at time t (min); b_0, b_1 and c (all>0) are parameters that determine the scale and shape of the curve.

The nonlinear least squares regression procedure (PROC NLIN, SAS Inst. Inc., Cary, NC, USA) was used for curve fitting. Recovery of the ingested marker was calculated by analytical integration of the area under the $^{13}CO_2$ excretion curve over a period of 24 h after administration of the isotope, $b_0 / (1 + b_1^c \cdot 1440^{-c})$, and expressed relative to the amount of ingested or infused isotope by dividing the area under the curve by the dose of isotope corrected for enrichment and chemical purity. The time to peak was calculated as $t_{max} = (b_1^c \cdot (1-c) / (-c-1))^{(1/c)}$. The maximum $^{13}CO_2$ excretion (mmol/h) was calculated as $y_{max} = b_0 \cdot c \cdot t_{max}^{(-c-1)} \cdot b_1^c / (1 + (b_1 / t_{max})^c)^2$ and was expressed as percentage of the dose. Recoveries and maxima for the $^{13}CO_2$ excretion after the ingestion of leucine and glucose tracers were corrected for bicarbonate sequestration.

Goodness of fit of all curve-fits were judged by comparing the predicted $^{13}CO_2$ excretion values from fitted curve with the observed values using the mean squared prediction error (MSPE) (Bibby & Toutenburg, 1977). The MSPE and mean were calculated over the time

until 90% of the total recovery was achieved. This was performed, because the values beyond this point (i.e. when the curve is close to baseline for many hours) have a disproportionately high contribution to the calculation of the mean. The root MSPE was scaled to the observed mean (mean prediction error, MPE) and decomposed into errors due to overall bias, errors due to deviation of the regression slope from unity and errors due to random variation. Also the correlation between predicted and observed values was calculated.

The urea production was calculated in 30-min intervals by analytical integration:

$$\frac{\mathrm{d}e}{\mathrm{d}t} = \frac{\mathrm{I} - \mathrm{e} \cdot \mathrm{P}}{\mathrm{V} \cdot \mathrm{C}}$$

where t = time (h), e = [^{13}C]urea enrichment (as a fraction of total urea C), I = rate of infusion of [`^{13}C]urea (mmol/h), P = rate of urea production (mmol/h), V = volume of distribution of urea (L), and C = concentration of urea in V (mmol/L). For calculating urea production rates, distribution volume was assumed to be body water mass, estimated as 60% of BW, and urea was assumed to be equally distributed in total body water (Harmeyer et al., 1973; Hammond et al., 1990). The urea concentration in this pool was calculated from the plasma water urea concentration. Plasma urea enrichment was assumed to represent urea enrichment in the total urea pool. Urea production was presented as hourly means from 0700 until 1900 h. For comparison with daily urinary N excretion, urea-N production rates were extrapolated to a complete 24-h period.

Glucose and fatty acid oxidation rates were calculated hourly from gas exchange rates and by correction for urea-N production rates. The constants of Brouwer (1965) were used to calculate hourly rates of glucose (OXCHO) and fatty acid oxidation (OXF) (see Chwalibog et al., 1992):

OXCHO (in g/(kg$^{0.75}$ · h)) =   -2.968 · O$_2$ (in L/(kg$^{0.75}$ · h)) + 4.174 · CO$_2$ (in L/(kg$^{0.75}$ · h)) – 2.446 · urea-N (in g/(kg$^{0.75}$ · h))

OXF (g/(kg$^{0.75}$ · h)) = 1.719 · O$_2$ (in L/(kg$^{0.75}$ · h)) – 1.719 · CO$_2$ (in L/(kg$^{0.75}$ · h)) – 1.963 · urea-N (in g/(kg$^{0.75}$ · h))

The same formulas were used for calculation of the average daily OXCHO and OXF, but daily urinary N excretion values, instead of urea-N production rates, and daily gas exchange rates were used. The daily oxidation rate of amino acids (OXP) was calculated as:

OXP (in $g/(kg^{0.75} \cdot d)$) =   urinary N (in $g/(kg^{0.75} \cdot d)$) $\cdot$ 6.25

Responses of OXP, OXCHO and OXF to a meal were calculated from hourly values as areas under the curve over a 6 h period after feed intake ($AUC_{12\text{-}18}$). Similarly, the responses in excess of preprandial values (i.e. 2 h before feeding) were calculated ($\Delta AUC_{12\text{-}18}$).

Statistical analysis

All data were analyzed for the effect of FF, FL, the interaction between FL and FF, and period by ANOVA using PROC MIXED in SAS. The factorial design was analyzed by a mixed model for the fixed effects of FF, FL and period, and the random effect of each calf (Eq. [3]).

$$Y_{ijkl} = \mu + FF_i + FL_j + (FF \times FL)_{ij} + P_k + \varepsilon_{ijkl} \qquad [2]$$

where Y_{ijkl} = dependent variable; μ = average intercept; FF_i = effect of feeding frequency i (i = 1, 2, 4); FL_j = effect of feeding level j (j = 1, 2); P_k = effect of period k (k = 1, 2), and ε_{ijkl} = error term, which represents the random effect of calf within feeding frequency (l = 1,…, 6). If main effects were significant, post-hoc t-tests were used to compare individual treatments. Recovery of ^{13}C, the maximum $^{13}CO_2$ excretion and the time of the maximum $^{13}CO_2$ excretion were compared between treatments for each isotope tested. In addition, the ^{13}C recoveries of $[U\text{-}^{13}C]$glucose and $[2\text{-}^{13}C]$glucose were compared. Hourly means and 12 h rates of urea production, and hourly means, $\Delta AUC_{12\text{-}18}$ and daily means of OXP, OXCHO and OXF were compared between treatments. Pearson correlation coefficients were calculated for relationships between urinary N excretion and urea-N excretion for FF 2 and 4, and between average daily OXP and $L\text{-}[1\text{-}^{13}C]$leucine oxidation for all treatments. The SAS software package version 9.1 (SAS Inst. Inc., Cary, NC, USA) was used for all statistical evaluations.

Results

Urea production

Hourly urea production rates were generally unaffected by FF, but an increased FF increased ($P < 0.01$) and decreased ($P < 0.05$) urea production rates at 1130 and 1630 h respectively (Figure 1). The increase in FL increased ($P < 0.05$; at 0830, 0930, 1130, 1330, 1430 and 1530

h) and tended to increase ($P < 0.10$; at 1730 and 1830) hourly urea production rates. The 12 h urea production rate was not affected by FF ($P = 0.586$), but increased ($P < 0.01$) with increasing FL from 13.1 mmol/kg$^{0.75}$ at the low FL to 20.9 mmol/kg$^{0.75}$ at the high FL. The AUC$_{12-18}$ for urea production tended to decrease ($P = 0.088$) with increasing FF and increased ($P < 0.001$) with increased FL (Table 2), whereas the ΔAUC$_{12-18}$ decreased with FF ($P = 0.017$) but was not affected by FL.
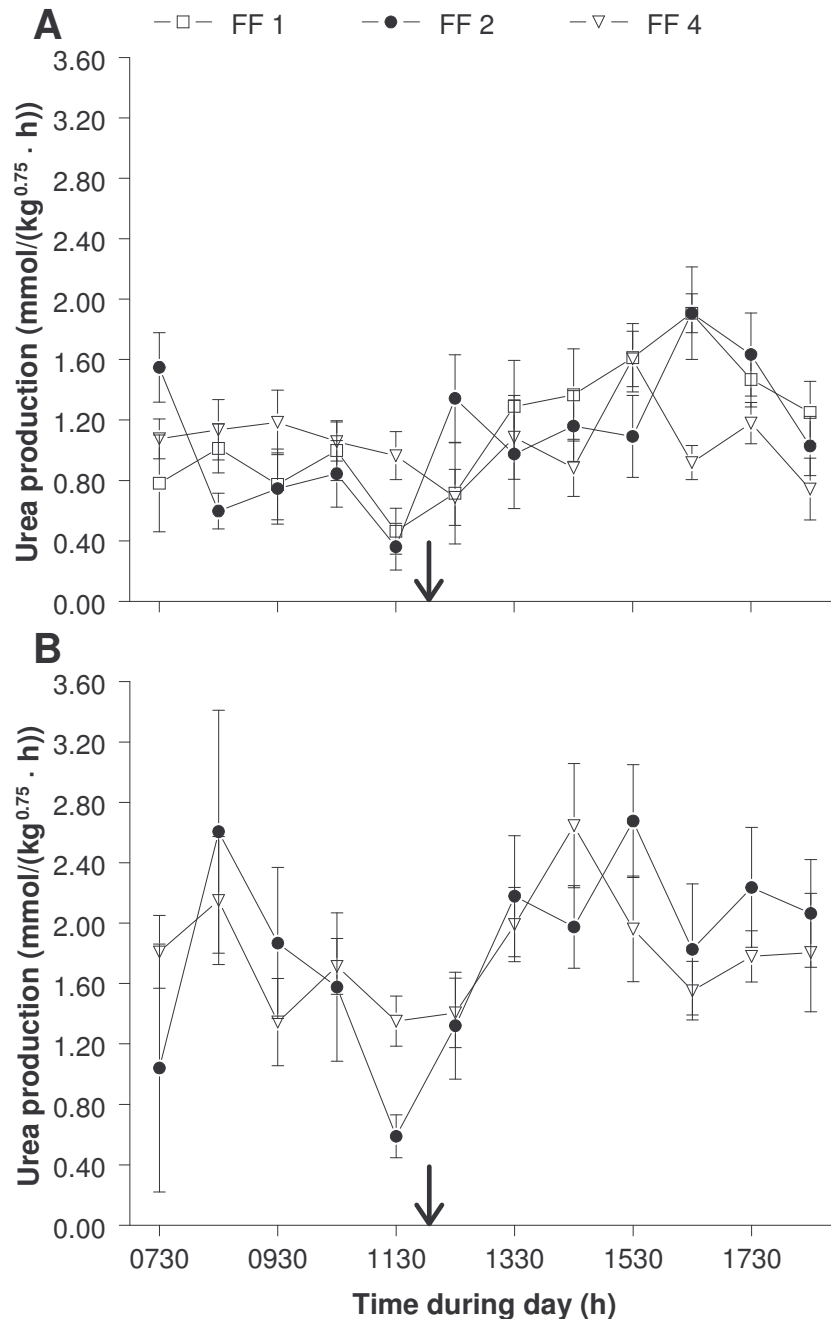
Figure 1. Influence of feeding frequency (FF) 1 ($\square$), 2 ($\bullet$) and 4 ($\triangledown$) at a low (690 kJ ME/(kg$^{0.75} \cdot$ d); panel A) and at a high feeding level (1150 kJ ME/(kg$^{0.75} \cdot$ d); panel B) on the circadian pattern of urea production in heavy preruminant calves. Values are means $\pm$ SEM, n = 8 ($\square$), n = 5 ($\bullet$, in panel A), n = 6 ($\bullet$, in panel B), or n = 6 ($\triangledown$). All calves were fed at 1200 h ($\downarrow$).
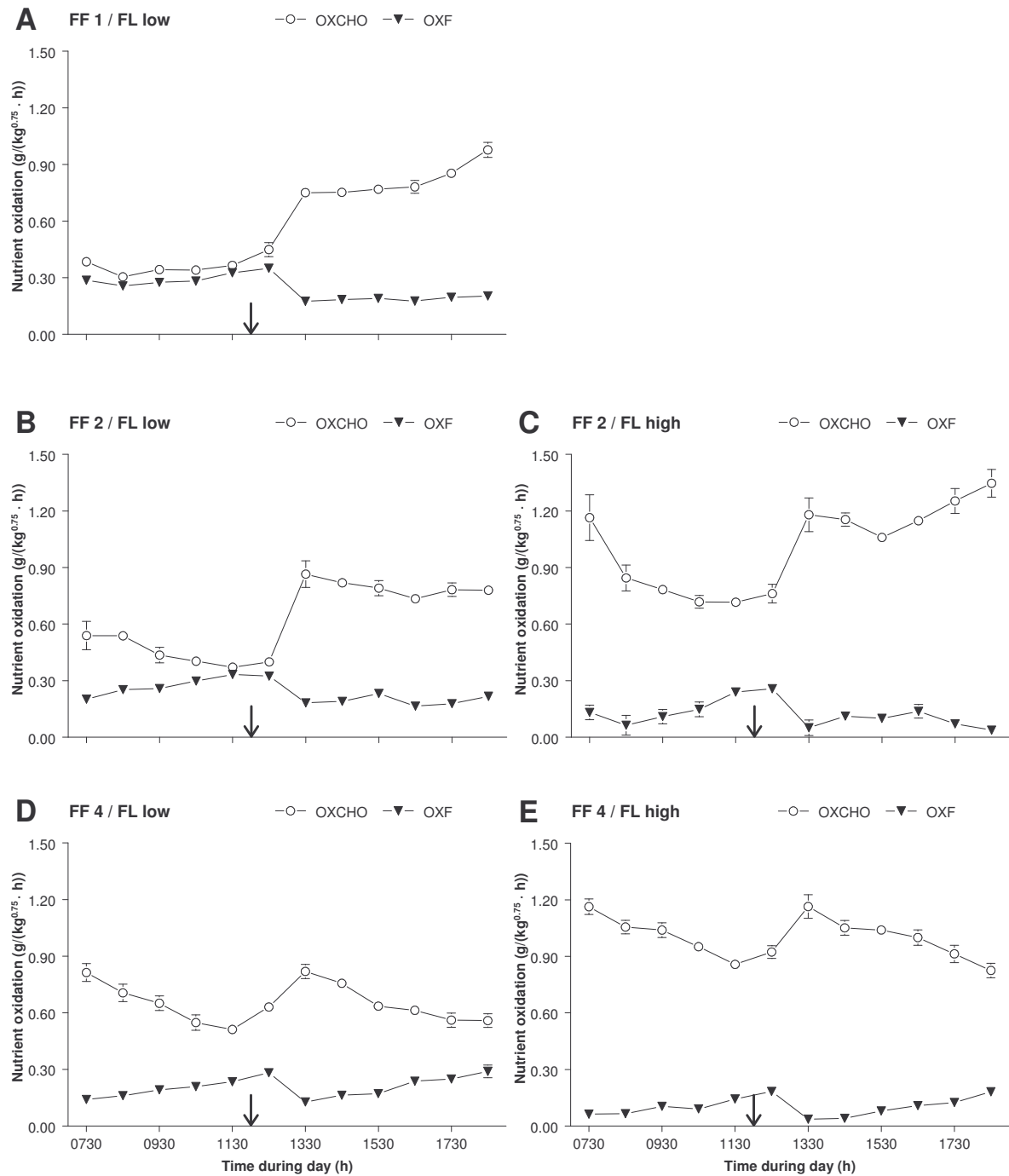
Figure 2. Influence of feeding frequency 1 (FF 1; panel A), 2 (FF2; panel B and C) and 4 (FF 4; panel D and E) at 690 kJ ME/(kg$^{0.75}$ · d) (FL low; panel A, B and D) and at 1150 kJ ME/(kg$^{0.75}$ · d) (FL high; panel C and E) on the circadian pattern of oxidation of amino acids (OXP), glucose (OXCHO) and fatty acids (OXF) in heavy preruminant calves. Substrate oxidation was calculated from gas exchange (O_2 and CO_2) in combination with urea production. Values are means ± SEM, n = 8 (FF 1), n = 5 (FF 2 at FL high), n = 6 (FF 2 at FL low), or n = 6 (FF 4). All calves were fed at 1200 h ($\downarrow$).

Glucose and fatty acid oxidation: indirect calorimetry

Hourly OXCHO values were greater ($P < 0.001$) at a higher FF until 1330 h, but decreased ($P < 0.001$) with greater FF thereafter (Figure 2). All hourly OXCHO values (except at 1330 h) were greater ($P < 0.001$) at the higher FL. Hourly OXF values were greater ($P < 0.01$) at the lower FF until 1330 h, whereas fatty acid oxidation increased ($P < 0.05$) with greater FF after 1630 h. All hourly OXF values (except 0730 and 1230 h) were lower ($P < 0.05$) at the higher FL.

Daily OXP as percentage of the protein intake decreased ($P = 0.038$) with increasing FF, but was not affected by FL (Figure 3). Daily OXCHO as percentage of the lactose intake was generally high: on average 94.2% of the carbohydrate intake was oxidized at the low FL and 91.5% at the high FL, but was not affected by either FF ($P = 0.563$) or FL ($P = 0.844$). Daily OXF as percentage of the fat intake tended ($P = 0.058$) to decrease with an increase in FF, and 77.7% of the fat intake was oxidized at the low FL compared with 30.6% at the high FL ($P < 0.001$).
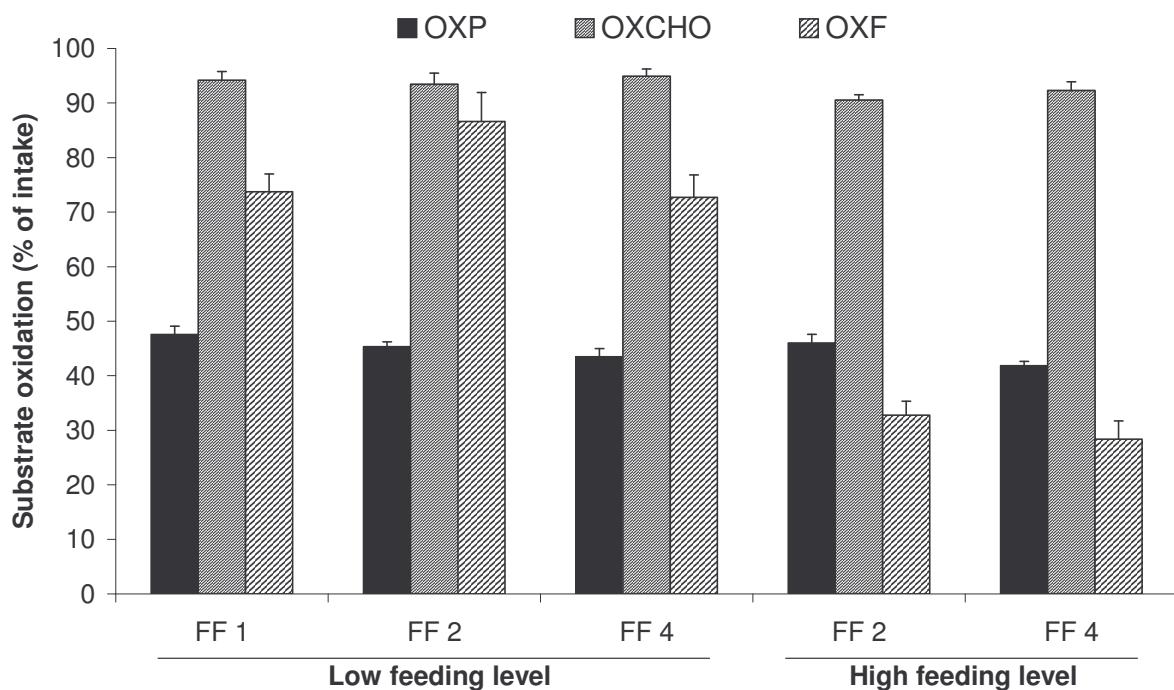
Figure 3. Influence of feeding frequency (FF) at a low and a high feeding level (690 and 1150 kJ ME/(kg$^{0.75}$ · d), respectively) on the oxidation of amino acids (OXP), glucose (OXCHO) and fatty acids (OXF) expressed as percentage of their intake in heavy preruminant calves. Values are means ± SEM, n = 8 (FF 1), n = 5 (FF 2, low FL), n = 6 (FF 2, high FL), or n = 6 (FF 4).

Table 2. Influence of feeding frequency (FF; 1, 2 or 4 meals/d) and feeding level (FL; high vs. low) on the 12 h urea production rate, the cumulative urea production over a 6 h period after feed intake (AUC_{12-18}) and the AUC_{12-18} in excess of preprandial values (ΔAUC_{12-18}).

Item	FF 1	FF 2		FF 4			P-value		
	FL low	FL low	FL high	FL low	FL high	SEM	FF	FL	FF × FL
Urea production									
12 h rate, mmol/(kg$^{0.75}$ · 12 h)	13.6	13.2	20.1	12.5	21.5	0.75	0.586	<0.001	0.959
AUC_{12-18}, mmol/(kg$^{0.75}$ · 6 h)	8.4	8.1	12.2	6.4	11.3	0.62	0.088	0.001	0.549
ΔAUC_{12-18}, mmol/(kg$^{0.75}$ · 6 h)	4.0	4.5	5.7	0.3	2.1	1.19	0.017	0.636	0.812
Glucose oxidation (OXCHO)[1]									
12 h rate, g/(kg$^{0.75}$ · 12 h)	7.1	7.5	11.1	7.8	12.0	0.23	0.051	<0.001	0.300
AUC_{12-18}, g/(kg$^{0.75}$ · 6 h)	4.4	4.4	6.6	4.0	6.1	0.09	<0.001	<0.001	0.653
ΔAUC_{12-18}, g/(kg$^{0.75}$ · 6 h)	2.2	2.1	2.3	0.8	0.7	0.16	<0.001	0.801	0.298
Fatty acid oxidation (OXF)[1]									
12 h average, g/(kg$^{0.75}$ · 12 h)	2.9	2.8	1.4	2.5	1.2	0.17	0.150	0.003	0.539
AUC_{12-18}, g/(kg$^{0.75}$ · 6 h)	1.3	1.3	0.7	1.2	0.6	0.09	0.575	0.006	0.556
ΔAUC_{12-18}, g/(kg$^{0.75}$ · 6 h)	-0.6	-0.6	-0.4	-0.1	-0.1	0.09	0.001	0.543	0.302

[1]Calculated from gas exchange rates and correction based on urea production rates.

Table 3. Influence of feeding frequency (1, 2 or 4 meals/d) and feeding level (high vs. low) on oxidation of orally provided L-[1-^{13}C]leucine, [U-^{13}C]glucose and [2-^{13}C]glucose in heavy preruminant calves. Recoveries and maxima are corrected (1st line) or uncorrected (2nd line) for bicarbonate sequestration.

	FF 1	FF 2		FF 4			P-value		
Item	FL low	FL low	FL high	FL low	FL high	SEM	FF	FL	FF × FL
L-[1-^{13}C]leucine oxidation									
Recovery as $^{13}CO_2$, % of dose	39.3	41.8	49.7	58.6	38.2	0.52	0.059	0.190	<0.001
	30.2	30.9	33.4	43.7	28.6	0.37	0.041	0.107	<0.001
Maximum $^{13}CO_2$ production, % of dose	4.2	6.5	7.3	9.3	7.2	0.20	0.068	0.971	0.063
	3.3	4.9	4.9	7.0	5.3	0.14	0.010	0.662	0.014
Time to peak, min	281	169	204	129	163	3.5	<0.001	0.442	0.838
[U-^{13}C]glucose oxidation									
Recovery as $^{13}CO_2$, % of dose	75.1	84.6	84.7	79.3	73.8	1.08	0.065	0.093	0.502
	57.6	57.8	56.3	56.6	54.9	0.65	0.893	0.300	0.991
Maximum $^{13}CO_2$ production, % of dose	6.8	9.6	9.4	11.6	11.5	0.13	<0.001	0.266	0.948
	5.2	6.6	6.2	8.2	8.5	0.05	<0.001	0.522	0.310
Time to peak, min	403	290	301	205	217	3.0	<0.001	0.190	0.858
[2-^{13}C]glucose oxidation									
Recovery as $^{13}CO_2$, % of dose	73.3	88.2	86.4	74.2	72.0	1.21	0.144	0.782	0.953
	56.3	56.0	59.6	48.9	55.5	0.73	0.336	0.463	0.684
Maximum $^{13}CO_2$ production, % of dose	6.9	10.3	9.1	10.9	11.7	0.21	0.001	0.858	0.177
	5.3	6.9	6.2	8.8	7.5	0.08	<0.001	0.662	0.024
Time to peak, min	393	297	300	243	235	4.4	<0.001	0.904	0.604

L-[1-^{13}C]leucine oxidation

Curve fits for L-[1-^{13}C]leucine were good and the MPE averaged 17.6% (r = 0.94).

The majority of the prediction error (> 95%) was attributed to random variation. For [^{13}C]bicarbonate, curve fits were also good (MPE: 16.3%; r = 0.97) and recovery of the bolus intravenous dose of sodium [^{13}C]bicarbonate as $^{13}CO_2$ averaged 72% and did not differ between treatments.

The proportion of the L-[1-^{13}C]leucine oxidized was greater with increasing FF at the low FL, but decreased with increasing FF at the high FL (FF × FL; $P < 0.001$) (Table 3). Although the maximum $^{13}CO_2$ excretion was increased ($P < 0.001$) with increasing FF, this was less clear when values were corrected for bicarbonate sequestration. The time until maximum $^{13}CO_2$ excretion was reached decreased ($P < 0.001$) with increasing FF: from 281 min at FF 1 to 129 min at FF 4 at a low FL and from 204 min at FF 2 to 163 min at FF 4 at a high FL.

Glucose oxidation: [U-^{13}C]glucose and [2-^{13}C]glucose

Curve fits were good and gave an MPE of 17.0% for [U-^{13}C]glucose (r = 0.95) and 17.6% for [2-^{13}C]glucose (r = 0.92). The majority of the prediction error (> 95%) was attributed to random variation. Corrected recoveries of the [U-^{13}C]glucose dose as $^{13}CO_2$ were high (on average 79.5%) and were unaffected by FF and FL (Table 3). The maximum $^{13}CO_2$ production was greater ($P < 0.001$) with increasing FF. The time to maximum decreased ($P < 0.001$) substantially with increasing FF. Corrected recoveries of the [2-^{13}C]glucose dose as $^{13}CO_2$ averaged 78.8%, were similar ($P > 0.10$) to those of [U-^{13}C]glucose and were unaffected by FF and FL (Table 3). The maximum $^{13}CO_2$ production was greater ($P < 0.001$) with increasing FF, while the time to maximum decreased ($P < 0.001$).

Discussion

During the past decades, skimmed milk protein in calf milk replacer has largely been replaced by more rapidly hydrolysed protein sources, such as vegetable proteins and whey. As a consequence, large amounts of amino acids are absorbed in a relatively short time span, and also lactose is rapidly digested and absorbed (i.e. as glucose and galactose) in preruminant calves (Verdonk et al., 1999). Along with a low FF (twice daily is common practice) and a high FL, this results in large amounts of nutrients absorbed at particular times within a day.

Calves need adaptive mechanisms to cope with these high loads of nutrients, which partition either towards nutrient deposition or oxidation. Heavy preruminant calves utilize dietary protein with a low efficiency (Van den Borne et al., 2006a), and they often develop insulin resistance and hyperglycemia (Blum & Hammon, 1999). This indicates that the capacity to process large amounts of nutrients at particular times of the day may be insufficient. In the current experiment, therefore, we have studied how the distribution of nutrient intake within a day impacts on nutrient oxidation in preruminant calves.

Urea production and L-[1-^{13}C]leucine oxidation

In this study, amino acid oxidation was assessed by two approaches, ureagenesis and oxidation of orally provided L-[1^{13}C]leucine. Measurement of urea production rates provides a quantitative estimate of amino acid oxidation within the day. However, measurements of changes within the day may be complicated by changes in body pool size during the dilution process. On the other hand, leucine oxidation only describes the metabolism of one amino acid and, as used here, is not a quantitative estimate of whole body leucine oxidation. Nonetheless, it is sensitive to changes in oxidation of one specific amino acid and can show qualitative (i.e. pattern) responses.

Urea production rates showed marked fluctuations within a day. In general, urea production increased after ingestion of the meal at 1200 h. Postprandial urea production (AUC_{12-18}) was greater with increased FL and tended to be greater with decreasing FF (meal size). After the 1200 h meal, the hourly urea production rate approximately doubled in calves fed 1 or 2 meals daily. In rats, larger meals tended to increase amino acid oxidation (and thus urea production) in the postprandial state (Weijs et al., 1995). An increase in urea production in response to a meal was also found in pigs (Deutz et al., 1998) and in man (Forslund et al., 1998). The larger increase in urea production in the current study than in the pig and human studies was probably due to the higher FL and the use of a rapidly hydrolysed protein source. The 12 h urea production rates did not differ between FF. This disputes our hypothesis that urea production rates would decrease with increasing FF. For calves at FF 2 and 4, at least one complete interval between two meals was covered in the measurements. Extrapolation of the 12 h urea production rate to daily urea production was therefore sensible for calves on FF 2 and 4 but not for those fed only once daily. The difference in type of measurement could explain the absence of an effect of FF on urea production whereas N-balance was greater with increasing FF. N-balance is based on differences between fluxes, of which urea production is

the most important. The 1-d measurement of urea production was associated with relatively large errors compared with the differences between fluxes from the 10-d measurement of N-balance. The N-balance differences may therefore lie within the error associated with the dynamic measurement of urea production. Nonetheless, the daily urea-N production showed a strong positive correlation (r = 0.98; $P < 0.001$) with the average daily urinary N excretion (Figure 4).
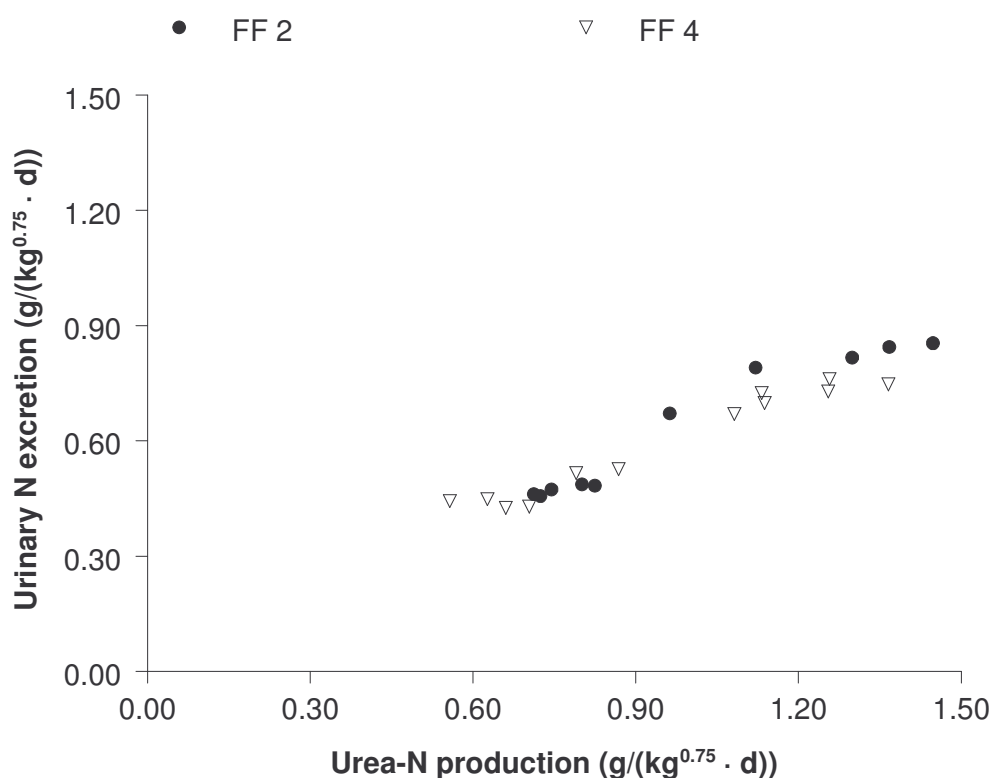
Figure 4. Correlation of the daily urinary N excretion with the (extrapolated) daily urea-N production in heavy preruminant calves (y = 0.54 · x + 0.09; r = 0.98; $P < 0.001$). Calves were fed identical nutrient intakes in two (FF 2) or four meals (FF 4) daily in equidistant time intervals at a low or a high feeding level (690 and 1150 kJ ME/(kg$^{0.75}$ · d), respectively).

As expected, urea production increased with increasing FL, which is commonly observed across species (Forslund et al., 1998; Sarraseca et al., 1998; Russell et al., 2000). The urea-N production exceeded the urinary N excretion by 85%. When assuming that 75% of the urinary N originated from urea and a urea entry into the gastro-intestinal lumen of 20% in preruminant calves (Gerrits, unpublished), it appears that urinary urea excretion is approximately twofold lower than hepatic urea production. Urinary N excretion is usually lower than the urea-N production due to hydrolysis of urea in the gut lumen. In ruminants, 40 to 80% of the produced urea is returned to the gastrointestinal tract (Lapierre & Lobley,

2001), but this recycling is usually lower in non-ruminants. In exclusively milk-fed calves, hydrolysis of urea was expected to be low due to the lack of fermentable substrates in the gastrointestinal tract. It is therefore surprising that only 50% of the urea production was excreted. On the other hand, milk-fed neonates excreted only 20% of the produced urea in urine (Wheeler et al., 1991).

The metabolic fate of dietary amino acids has often been studied by adding ^{13}C or ^{14}C labelled free amino acids to a meal (Schreurs et al., 1992; Krawielitzki et al., 1996; Tauson et al., 2000; Labayen et al., 2004). If just the excretion of $^{13}CO_2$ or $^{14}CO_2$ is measured in breath, the proportion of the dose oxidized can be calculated. A bicarbonate recovery factor can be used to correct for the incomplete recovery of the produced CO_2 in the exhaled air (e.g. Van Hall, 1999). The recovery of [^{13}C]bicarbonate averaged 72%, similar to recoveries in human (Armon *et al.*, 1990; Leijssen & Elia, 1996) and animal studies (Junghans et al., 2000; Moehn et al., 2004) after a bolus dose.

In response to increasing FF, recovery of the L-[1-^{13}C]leucine dose as $^{13}CO_2$ increased at the low FL, but decreased at the high FL (FF × FL; $P < 0.001$). The increased oxidation at the low FL does not correspond with protein utilization (Van den Borne et al., 2006b) or unchanged urea production and contradicts our hypothesis that less L-[1-^{13}C]leucine would be oxidized with increasing FF. Indeed, recovery as exhaled $^{13}CO_2$ from L-[1-^{13}C]leucine showed no correlation (r = 0.11; P = 0.545) with OXP (calculated from urinary N excretion) when expressed as a percentage of the daily protein intake (Figure 5). Nonetheless, the correlation was considerably better at the high FL (r = 0.77; P = 0.003) than at the low FL (r = -0.34; P = 0.149). Mechanisms responsible for the discrepancy between L-[1-^{13}C]leucine oxidation and OXP may include the following. First, leucine oxidation is a function of the proportion oxidized multiplied with the flux (not measured) through the free leucine pool. The pool is relatively small in relation to the flux (Waterlow, 2006). Fluctuations in the flux therefore strongly affect whole body leucine oxidation. Motil et al (1981), for example, described proportional leucine oxidation rates of 16.3, 18.2 and 29.3% of the flux, whereas absolute oxidation rates were 11.8, 21.6 and 46.3 μmol/(kg · h) respectively, indicating that the percentage oxidized not necessarily corresponds with the absolute oxidation rate. Second, ingestion of labelled leucine creates an isotopic gradient with the higher leucine enrichment in the gastrointestinal tract and this would overemphasize the contribution of enterocytes to oxidation. Leucine can be extensively oxidized by enterocytes (Stoll et al., 1998; Lobley et al., 2003) but the proportion oxidized decreases with increasing FL (Lapierre et al., 1999). This makes a larger proportion of the L-[1-^{13}C]leucine available for peripheral tissues, which

may explain the better correlation with OXP at the high FL. Third, a time lag between the absorption of free amino acids, such as the added L-[1-^{13}C]leucine, and those originating from protein digestion may have resulted in an amino acid imbalance and an increased recovery of L-[1-^{13}C]leucine as $^{13}CO_2$. Also the oxidation patterns differ between free amino acids and intrinsically labelled proteins (in rats: Nolles, 2006).

In summary, although the ^{13}C-leucine breath test may useful for clinical diagnosis (Schadewaldt et al., 1998) or studying the fate of dietary free amino acids (Nolles, 2006), it is not a suitable method to measure postprandial amino acid oxidation in preruminant calves.
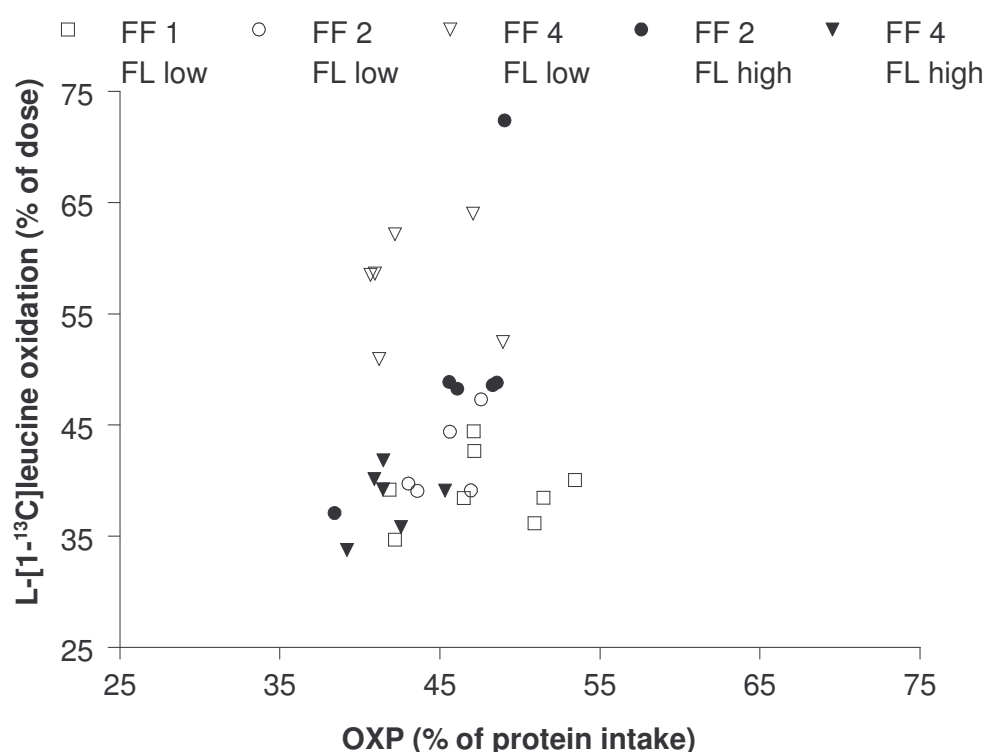
Figure 5. Correlation of amino acid oxidation (OXP) calculated from urinary N excretion and expressed as percentage of the protein intake with L-[1-^{13}C]leucine oxidation expressed as percentage of the oral dose in heavy preruminant calves (r = 0.11; *P* = 0.545). Calves were fed identical nutrient intakes in one (FF 1), two (FF 2) or four meals (FF 4) daily in equidistant time intervals at a low or a high feeding level (FL low, 690 kJ ME/(kg$^{0.75}$ · d); or FL high, 1150 kJ ME/(kg$^{0.75}$ · d), respectively).

Glucose and fatty acid oxidation & *de novo* fatty acid synthesis

Calculation of substrate oxidation from indirect calorimetry in fast-growing animals is often complicated by a high protein-free RQ, indicating *de novo* fatty acid synthesis from glucose (Chwalibog & Thorbek, 2000; Wolfe & Chinkes, 2005). In the current study, the RQ did not exceed 1. This allowed a good estimation of the rates of glucose and fatty acid oxidation.

Gluconeogenesis from non-carbohydrate precursors (e.g. alanine) results in a low RQ (Wolfe & Chinkes, 2005) and may affect estimations of substrate oxidation calculated from indirect calorimetry. In fact, this would lead to an overestimation of fatty acid oxidation and underestimation of glucose oxidation, therefore the estimates of the latter should be considered as minimal values.

A preruminant calf of 150 kg usually ingests more than 500 g of lactose in a single meal, and portal glucose and galactose appearance is virtually complete within a few hours after feeding (Verdonk et al., 1999). Temporary storage of glucose (as glycogen) is limited and therefore glucose is either extensively oxidized or used as a precursor for *de novo* fatty acid synthesis. From the high ^{13}C recovery of [U-^{13}C]glucose as $^{13}CO_2$ (on average 80%) and the high OXCHO as a percentage of the lactose intake (on average 94%), it appears that carbohydrates are predominantly oxidized, unaffected by FF or FL. This is not consistent with the hypothesis that an increased protein utilization with increasing FF would be the result of an increased availability of protein-free energy as glucose. The lower glucose oxidation derived from the tracer compared with the gas exchange method may be a consequence of the label sequestration through the glycogen interchange. It can be calculated that liver and muscle tissue of a 150 kg calf can store approximately 700-800 g of glycogen. If glycogen stores became more depleted with decreasing FF prior to the labelled glucose ingestion, this may have concealed an effect of FF on glucose oxidation. The gas exchange method does not discriminate between oxidation of exogenous and endogenous glucose, and probably represents net oxidation of glucose.

Fatty acid oxidation decreased markedly with increased FL in preruminant calves, indicating that fatty acids are spared from oxidation by increased glucose supply (and oxidation). From an energetic point of view this would make sense, because the energetic efficiency of triglyceride synthesis from fatty acids is considerably greater than that from glucose (90 vs. 74%; Black, 1995). However, regression of fatty acid oxidation versus glucose oxidation over the two FL showed that 1 kJ increase in heat production from glucose resulted in only 0.5 kJ decrease in heat production from fatty acids (r = -0.79; $P < 0.001$). Thus, oxidation of these two nutrients is not iso-energetically exchanged with increased FL, indicating that the high glucose oxidation does not exclusively result from sparing of fatty acids from oxidation.

Generally, animals on a low FF tend to have a greater lipogenic capacity and exhibit increased rates of *de novo* fatty acid synthesis in the immediate postprandial period, especially when carbohydrate-rich diets are offered (Dawson, 1999). This is indicated by increased enzyme activities associated with glucose and lipid metabolism and to improved sensitivity of adipose

tissue to insulin (reviewed by Dawson, 1999). However, it is expected that *de novo* fatty acid synthesis from glucose is negligible in preruminant calves, regardless of the FF, because glucose oxidation was virtually complete in the current study. *De novo* fatty acid synthesis from glucose was calculated by subtracting glucose oxidation and urinary glucose excretion (which varied between 0 to 91 g/d) from the digestible lactose intake plus gluconeogenesis from amino acids (as described by Chwalibog & Thorbek, 2000). The calculated *de novo* fatty acid synthesis was low (5.6 kJ/(kg$^{0.75}$ · d)), and unaffected by FF but also by FL. This rejects our hypothesis that substantial amounts of glucose are used for *de novo* fatty acid synthesis in preruminant calves and does not correspond with studies in pigs. Increasing the FL in pigs (using a 5% fat diet) resulted in an increase in *de novo* fatty acid synthesis; almost 700 g of the ingested starch was retained as fat (Chwalibog & Thorbek, 2000). Even when the energy supply was close to maintenance requirements, pigs used substantial amounts (~150 g) of carbohydrates for fatty acid synthesis, whereas simultaneously body fat was oxidized (Chwalibog & Thorbek, 2000).

In addition, recoveries of [U-^{13}C]glucose and [2-^{13}C]glucose did not differ. This indicates that all [^{13}C]acetate that is formed from [^{13}C]pyruvate (originating from [^{13}C]glucose) enters the citric acid cycle and is released as $^{13}CO_2$ instead of being used for *de novo* fatty acid synthesis. Therefore, both from gaseous exchange and tracer data it would appear that conversion of glucose to fat is not quantitatively important in heavy preruminant calves.

Conclusions

Total urea production and $^{13}CO_2$ recovery from orally provided L-[1-^{13}C]leucine did not (significantly) decrease with increasing FF. This is in contrast with an increased protein deposition (from N-balance measurements). Urea production correlated well with urinary N excretion, whereas $^{13}CO_2$ recovery from orally provided L-[1-^{13}C]leucine showed no correlation with urinary N excretion (as proportion of intake).

Orally provided glucose was almost completely oxidized and this was unaffected by both FF and FL, indicating that the increased protein utilization with increasing FF does not result from a increased protein-free energy availability as glucose.

Fatty acid synthesis *de novo* from glucose was negligible in preruminant calves. The increase in fat deposition with increased feed intake almost exclusively originates from a reduced oxidation of fatty acids. Potential mechanisms for the high glucose oxidation (and low *de novo* fatty acid synthesis) remain to be studied in preruminant calves.

References

Alferink SJJ, Van den Borne JJGC, Gerrits WJJ, Lammers-Wienhoven SCW & Heetkamp MJW (2003) On-line, continuous determination of $^{13}CO_2/^{12}CO_2$ ratios by non-dispersive infrared absorption in indirect calorimetry facilities. *In: Progress in Research on Energy and Protein Metabolism, Rostock-Warnemünde, Germany.* [WB Souffrant and CC Metges, editors]. Wageningen Academic Publishers, pp. 465-468.

Armon Y, Cooper DM, Springer C, Barstow TJ, Rahimizadeh H, Landaw E & Epstein S (1990) Oral [^{13}C]bicarbonate measurement of CO_2 stores and dynamics in children and adults. *J Appl Physiol* **69**, 1754-1760.

Berg JM, Tymoczko JL, Stryer L & Clarke ND (2002) *Biochemistry*, 5th ed. New York, NY, USA: Freeman.

Bibby J & Toutenburg H (1977) *Prediction and improved estimation in linear models*. Chichester, UK: John Wiley & Sons.

Black JL (1995) The evolution of animal growth models. *In: Modelling the growth in the pig, EAAP Publication no. 78*, [PJ Moughan, MWA Verstegen and MI Visser-Reyneveld, editors]. Wageningen, The Netherlands. Wageningen Press, pp. 3-9.

Blum JW & Hammon HM (1999) Endocrine and metabolic aspects in milk-fed calves. *Domest Anim Endocrinol* **17**, 219-230.

Chwalibog A, Jakobsen K, Henckel S & Thorbek G (1992) Estimation of quantitative oxidation and fat retention from carbohydrate, protein and fat in growing pigs. *J Anim Physiol Anim Nutr* **68**, 123-135.

Chwalibog A & Thorbek G (2000) Estimation of net nutrient oxidation and lipogenesis in growing pigs. *Arch Anim Nutr* **53**, 253-271.

CVB (2000) Veevoedertabel. *Centraal Veevoeder Bureau*.

Dawson JM (1999) Variation in nutrient supply and effects on whole body anabolism. *In: Proceedings of the VIIIth International Symposium on Protein Metabolism and Nutrition, Aberdeen, UK.* [GE Lobley, A White and JC MacRae, editors]. Wageningen Pers, pp. 101-126.

Deutz NEP, Bruins MJ & Soeters PB (1998) Infusion of soy and casein protein meals affects interorgan amino acid metabolism and urea kinetics differently in pigs. *J Nutr* **128**, 2435-2445.

Forslund AH, Hambraeus L, Olsson RM, El-Khoury AE, Yu YM & Young VR (1998) The 24-h whole body leucine and urea kinetics at normal and high protein intakes with exercise in healthy adults. *Am J Physiol* **275**, E310-E320.

Gerrits WJJ, Tolman GH, Schrama JW, Tamminga S, Bosch MW & Verstegen MWA (1996) Effect of protein and protein-free energy intake on protein and fat deposition rates in preruminant calves of 80 to 240 kg live weight. *J Anim Sci* **74**, 2129-2139.

Groop LC & Ferrannini E (1993) Insulin action and substrate competition. *Baillieres Clin Endocrinol Metab* **7**, 1007-1032.

Hammond AC, Waldo DR & Rumsey (1990) Prediction of body composition in Holstein steers using urea space. *J Dairy Sci* **73**, 3141-3145.

Harmeyer J, Várady J & Martens H (1973) Der Harnstoff-verteilungsraum bei kleinen Wiederkaeuern bei Fuetterung und bei Hunger. *Arch Tierernahr* **23**, 537-553.

Jenkins DJA (1997) Carbohydrate tolerance and food frequency. *Br J Nutr* **77**, S71-81.

Junghans P, Chudy A, Voigt J, Klein M, Beyer M, Derno M, Jentsch W, Kuhla S, Hagemeister H, Schneider F & Schwerin M (2000) Recovery of ^{13}C in breath CO_2 in German Holstein and Charolais bulls after a bolus dose of $NaH^{13}CO_2$. *In: The 15th symposium on energy metabolism in animals, Snekkersten, Denmark*. [A Chwalibog and K Jakobsen, editors]. Wageningen Pers, The Netherlands, pp. 27-30.

Krawielitzki K, Kreienbring F, Schadereit R, Gabel M & Schreurs VVAM (1996) Estimation of protein and amino acid metabolism using [15N] and [14C] labeled tracer amino acids. *J Anim Feed Sci* **5**, 187-199.

Labayen I, Díez N, Parra D, González A & Martínez JA (2004) Basal and postprandial substrate oxidation rates in obese women receiving two test meals with different protein content. *Clin Nutr* **23**, 571-578.

Lapierre H, Bernier JF, Dubreuil P, Reynolds CK, Farmer C, Oeullet DR & Lobley GE (1999) The effect of intake on protein metabolism across splanchnic tissues in growing beef steers. *Br J Nutr* **81**, 457-466.

Lapierre H & Lobley GE (2001) Nitrogen cycling in the ruminant: a review. *J Dairy Sci* **84**, E223-E236.

Leijssen DPC & Elia M (1996) Recovery of $^{13}CO_2$ and $^{14}CO_2$ in human bicarbonate studies: A critical review with original data. *Clin Sci* **91**, 665-677.

Lobley GE, Bremner DM & Zuur G (2000) Effects of diet quality on ure fates in sheep as assessed by refined non-invasive [$^{15}N^{15}N$]urea kinetics. *Br J Nutr* **84**, 459-468.

Lobley GE, Shen X, Le G, Bremner DM, Milne E, Calder AG, Anderson SE & Dennison N (2003) Oxidation of essential amino acids by the ovine gastrointestinal tract. *Br J Nutr* **89**, 617-629.

McClelland ISM & Jackson AA (1996) Urea kinetics in healthy young women: Minimal effect of stage of menstrual cycle, contraceptive pill and protein intake. *Br J Nutr* **76**, 199-209.

Meakins TS & Jackson AA (1996) Salvage of exogenous urea nitrogen enhances nitrogen balance in normal men consuming marginally inadequate protein diets. *Clin Sci* **90**, 215-225.

Moehn S, Bertolo RFP, Pencharz PB & Ball RO (2004) Pattern of carbon dioxide production and retention is similar in adult pigs when fed hourly, but not when fed a single meal. *BMC Physiol* **4**, 1-8.

Mosenthin R, Sauer WC, Henkel H, Ahrens F & De Lange CFM (1992) Tracer studies of urea kinetics in growing pigs: II. The effect of starch infusion at the distal ileum on urea recycling and bacterial nitrogen excretion. *J Anim Sci* **70**, 3467-3472.

Motil KJ, Matthews DE, Bier DM, Burke JF, Munro HN & Young VR (1981) Whole-body leucine and lysine metabolism: response to dietary protein intake in young men. *Am J Physiol* **240**, E712-E721.

Nolles JA (2006) Postprandial fate of amino acids - adaptation to molecular forms. PhD thesis, Wageningen University.

NRC (1998) Nutrient requirements of swine (10th edition). *National Academy Press*.

Roehrig K, Nestor KE & Palmquist DL (1988) ATP citrate lyase activity in liver and adipose tissue of veal or ruminating calves (*Bos Taurus*). *Comp Biochem Physiol* **90B**, 147-149.

Romsos DR & Leveille GA (1974) Effect of meal frequency and diet composition on glucose tolerance in the rat. *J Nutr* **104**, 1503-1512.

Rook AJ, France J & Dhanoa MS (1993) On the mathematical description of lactation curves. *J Agric Sci* **121**, 97-102.

Russell K, Lobley GE, Rawlings J, Millward DJ & Harper EJ (2000) Urea kinetics of a carnivore, Felis silvestris catus. *Br J Nutr* **84**, 597-604.

Sarraseca A, Milne E, Metcalf MJ & Lobley GE (1998) Urea recycling in sheep: Effects of intake. *Br J Nutr* **79**, 79-88.

Schadewaldt P, Bodner A, Brosicke H, Hammen HW & Wendel U (1998) Assessment of whole body L-leucine oxidation by noninvasive L-[1- 13C]leucine breath tests: a reappraisal in patients with maple syrup urine disease, obligate heterozygotes, and healthy subjects. *Pediatr Res* **43**, 592-600.

Schreurs VVAM, Boekholt HA, Koopmanschap RE & Weijs PJM (1992) The metabolic utilization of amino acids: potentials of $^{14}CO_2$ breath test measurements. *Br J Nutr* **67**, 207-214.

Stoll B, Henry J, Reeds PJ, Yu H, Jahoor F & Burrin DG (1998) Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets. *J Nutr* **128**, 606-614.

Tauson AH, Abdalla A, Kanska K, Sobczynska K & Chwalibog A (2000) Breath test measurements in combination with indirect calorimetry for estimation of 13C-leucine oxidation in mink *(Mustela Vison)*. *Thermochimica Acta* **349**, 53-59.

Van den Borne JJGC, Verdonk JMAJ, Schrama JW & Gerrits WJJ (2006a) Reviewing the low efficiency of protein utilization in heavy preruminant calves – a reductionist approach. *Reprod Nutr Dev* **46**, 121-137.

Van den Borne JJGC, Verstegen MWA, Alferink SJJ, Giebels RMM & Gerrits WJJ (2006b) Effects of feeding frequency and feeding level on nutrient utilization in heavy preruminant calves. *J Dairy Sci* **89**, 3578-3586.

Van Hall G (1999) Correction factors for ^{13}C-labelled substrate oxidation at whole-body and muscle level. *Proc Nutr Soc* **58**, 979-986.

Verdonk JMAJ, Gerrits WJJ, Beelen GM & Jansman AJM (1999) Effect of protein source on portal nutrient fluxes in pre-ruminant calves. *In: The VIIIth International Symposium on Protein Metabolism and Nutrition, Aberdeen, UK*. [GE Lobley, A White and JC MacRae, editors]. Wageningen Pers, The Netherlands, pp. 47 (Abstr.).

Verstegen MWA, Van der Hel W, Brandsma H, Henken AM & Bransen AM (1987) The Wageningen respiration unit for animal production research: a description of the equipment and its possibilities. *In: Energy Metabolism in Farm Animals: Effects of Housing, Stress and Disease*, [MWA Verstegen and AM Henken, editors]. Dordrecht, The Netherlands. Martinus Nijhoff Publishers, pp. 21-48.

Waterlow JC (2006) *Protein turnover*. Wallingford, UK: CAB International.

Weijs PJM, Schreurs VVAM & Grooten HNA (1995) Meal feeding and leucine utilization in pregnant rats. *Br J Nutr* **73**, 253-258.

Wheeler RA, Jackson AA & Griffiths DM (1991) Urea production and recycling in neonates. *J Pediatr Surg* **26**, 575-579.

Williams PEV, Fallon RJ, Brockway JM, Innes GM & Brewer AC (1986) The effect of frequency of feeding milk replacer to pre-ruminant calves on respiratory quotient and the efficiency of food utilization. *Anim Prod* **43**, 367-375.

Wolfe RR & Chinkes DL (2005) *Isotope tracers in metabolic research - Principles and practice of kinetic analysis*, 2nd ed. ed. Hobroken, NJ, USA: John Wiley & Sons.

Yki-Järvinen H, Bogardus C & Howard BV (1987) Hyperglycemia stimulates carbohydrate oxidation in humans. *Am J Physiol* **253**, 376-382.

Chapter 5

Synchronizing the availability of amino acids and glucose increases protein retention in pigs

JJGC van den Borne[1], JW Schrama[2], MJW Heetkamp[3],
MWA Verstegen[1] & WJJ Gerrits[1]

[1]*Animal Nutrition Group,* [2]*Aquaculture and Fisheries Group,* [3]*Adaptation Physiology Group,*
Department of Animal Sciences, Wageningen University, P.O. Box 338, 6700 AH
Wageningen, The Netherlands

Abstract

Effects of synchronizing the availability of amino acids and glucose within a day on protein and energy metabolism were studied in growing pigs. Ten pigs of 54 (SEM 1.0) kg were assigned to each of two dietary treatments (synchronous vs. asynchronous nutrient supply) in a change-over design. At the synchronous treatment (SYN), pigs received two balanced meals: one at 08.00 h and one at 16.00 h. At the asynchronous treatment (ASYN), pigs received virtually all protein at 08.00 h and all carbohydrates at 16.00 h. Daily intakes of all nutrients and dietary ingredients were similar for both treatments. Pigs were housed individually in respiration chambers. Apparent faecal nutrient digestibility was determined and nitrogen and energy balances were measured. Apparent faecal digestibility of energy, organic matter and non-starch polysaccharides was higher ($P<0.05$) for SYN than for ASYN. The efficiency of utilization of digestible protein for protein gain was higher ($P=0.001$) for SYN (56.7%) than for ASYN (47.1%). Heat production and energy retention as fat were not affected by nutrient synchrony. Activity related heat production tended to be lower for ASYN than for SYN ($P=0.10$). The substantial decrease ($P<0.05$) in respiratory quotient and ^{13}C enrichment of the expired CO_2 after the morning meal indicated an increased amino acid oxidation for ASYN compared with SYN. In conclusion, an asynchronous availability of glucose and amino acids within a day increases amino acid oxidation, resulting in a substantial reduction in protein utilization but with virtually no effect on fat retention.

Abbreviations: ASYN, protein and carbohydrates fed separately; BW, body weight; DE, digestible energy; H_{act}, activity related heat production; H_{tot}, heat production; ME, metabolizable energy; ME_m; metabolizable energy required for maintenance; NSP, non-starch polysaccharides; RQ, respiratory quotient; SYN, protein and carbohydrates fed at same time.

Introduction

A balanced supply of amino acids and energy-yielding nutrients is generally perceived to be required for optimal protein deposition in growing animals. Relationships between digestible energy supply and protein deposition were described for many species (e.g. Heger & Frydrych, 1985; Campbell & Taverner, 1988; Gerrits *et al.*, 1996; Eits *et al.*, 2002) and are included in feed evaluation systems and mechanistic growth simulation models. Requirements for protein and energy are expressed as daily means, and a lack of synchronous availability of amino acids and protein-free energy within a day is usually assumed not to hamper protein utilization.

The availability of amino acids is, however, not always in synchrony with that of non-protein energy (i.e. mainly glucose). Asynchronous absorption patterns can be induced by either a separated intake of protein and carbohydrates in time (Barja et al., 1972; Arnal et al., 2000) or by supplying ingredients with different kinetics of digestion and absorption (Deutz *et al.*, 1998; Metges *et al.*, 2000; Englyst *et al.*, 2003; Mosoni & Patureau-Mirand, 2003). Moreover, the absorption kinetics of various ingredients can be more pronounced in particular species. In preruminant calves, for example, skimmed milk protein, coagulates in the abomasum, resulting in a delayed amino acid absorption compared to glucose (Longenbach & Heinrichs, 1997). In pigs, resistant starches can delay portal glucose appearance when compared with pregelatinized starch, thereby changing the synchrony of portal glucose and amino acid appearance (Cummings & Englyst, 1995; Van der Meulen *et al.*, 1997).

The effects of nutrient asynchrony have to some extent been addressed in literature (e.g. Munro, 1964), generally reporting a decrease in the efficiency of protein utilization in adult animals and man (Larson & Chaikoff, 1937; Cuthbertson & Munro, 1939; Cuthbertson *et al.*, 1940). A partial separation of protein and carbohydrate intake within a day did not result in significant changes in protein utilization in growing pigs (Eggert *et al.*, 1953; Yeo & Chamberlain, 1966) and growing rats (Bancroft et al., 1951). In some species, however, there may be benefits of inducing asynchronous absorption of amino acids and glucose by means of changing the meal composition within the day. Recently, we reported that in preruminant calves, protein utilization was unaffected, but that heat production decreased and fat deposition increased substantially, with increasing nutrient asynchrony (Van den Borne et al., 2006).

Under normal conditions, it can be expected that a decreased nutrient synchrony (i.e. a partial separation of amino acid and glucose availability in time) rather than a complete asynchrony

will occur. To quantify the potential impact of nutrient asynchrony on protein utilization it is, however, necessary to study the effects of a (virtually) complete separation.

It is hypothesized that in pigs, reducing nutrient synchrony will reduce the efficiency of protein utilization for growth, mainly due to increased amino acid oxidation after a protein meal. The net effect on fat deposition is unknown, and will depend on the balance of a hypothesized increase in fat deposition after the carbohydrate meal and a hypothesized reduction in fat deposition after the protein meal. In this study, effects of synchronizing amino acid and glucose availability on protein and energy metabolism were therefore quantified.

Materials and methods

Animals and housing

Ten crossbred barrows ([Finnish Landrace × Great Yorkshire] × Tybor-G) of approximately 50 kg body weight (BW) were used in five replicates of two pigs each. Each replicate consisted of two experimental periods of 7 d each. Within each experimental period, pigs were assigned to one of two treatments in a change-over design: a synchronous supply of protein and starch (SYN) or an asynchronous supply of protein and starch (ASYN) (Table 1). Each experimental period was preceded by a 7-d adaptation period on the experimental diets. During the adaptation and experimental periods, pigs were individually housed on a tenderfoot floor (150 × 60 cm) in one of two identical, size-adjustable climatic respiration chambers. To collect faeces quantitatively, a plastic bag was attached to the rear end of the pigs as described by Van Kleef *et al.* (1994). The ambient temperature was kept at 20°C, relative humidity was maintained at 65%, and air velocity was <0.2 m/s. Pigs were exposed to 12 h of light (300 lx from 07.00 to 19.00 h) and 12 h of darkness (10 lx). The experiment was approved by the Ethical Committee of Wageningen University.

Diets and feeding

Daily nutrient supply and daily ingredient supply were equal for both treatments. A contrast was created in the distribution of the nutrient intake over the two daily meals (at 08.00 and at 16.00 h). Pigs assigned to SYN received a balanced meal at 08.00 h and at 16.00 h, involving a constant ratio of protein to starch in both meals. For ASYN, protein and starch intake were as much as possible separated between the two daily meals. This meant that pigs assigned to ASYN received 95% of the daily protein and 0% of the daily starch at 08.00 h and 5% of the daily protein and 100% of the daily starch at 16.00 h (Table 1).

The protein and carbohydrate diets were formulated separately (i.e. basal diets) (Table 2) and the synchronous diet was created by mixing these two in a ratio of 1:2.6. The gross energy intakes at 08.00 and 16.00 h were equal between treatments and contributed 33 and 67% to the daily gross energy intake, respectively. The basal diets were formulated to differ in ^{13}C enrichment (1.0952 atom % and 1.0814 atom % for the protein and the carbohydrate diet, respectively), which allowed an improved specification of the within-day contribution of different nutrients to total nutrient oxidation after measuring ^{13}C enrichment of CO_2 in the expired air.

Table 1. Experimental treatments; distribution of the total daily nutrient intake over two daily meals

	Treatment	
	Synchronous	Asynchronous
Daily intake	g/kg $BW^{0.75}$	
Crude protein	16.8	16.8
Starch	44.5	44.5
Crude fat	4.4	4.4
Gross energy, kJ/kg $BW^{0.75}$	1449	1449
Morning meal (08.00 h)	g/kg $BW^{0.75}$	
Crude protein	5.5	16.0
Starch	14.6	0
Crude fat	1.5	1.7
Gross energy, kJ/kg $BW^{0.75}$	444	444
Afternoon meal (16.00 h)	g/kg $BW^{0.75}$	
Crude protein	11.3	0.8
Starch	29.9	44.5
Crude fat	3.0	2.7
Gross energy, kJ/kg $BW^{0.75}$	905	905

Pigs were fed according to their metabolic body weight ($BW^{0.75}$) at 2.1 times the digestible energy requirements for maintenance. Digestible energy requirements for maintenance were assumed to be 480 kJ/kg $BW^{0.75}$ per d. Feed intake was adjusted daily for a projected average daily gain of 500 g. At the start of the adaptation period, pigs were switched to the experimental diets within two days. Feed was provided as mash and was mixed with water (3 L/kg feed) immediately prior to feeding (viz. no soaking time).

Table 2. Ingredient and nutrient composition of the basal diets[1]

Ingredient	Diet Protein	Diet Carbohydrate	Nutrient[3]	Diet Protein	Diet Carbohydrate
	g/kg			g/kg	
Gelatinized maize starch	0	750	Dry matter	906	894
Potato protein	382	0	Crude ash	62	45
Soy protein isolate	368	0	Crude protein (N × 6.25)	672	20
Oat hulls	90	90	Crude fat	43	20
Sugar beet pulp	60	60	Starch	21	663
Maize oil	20	20	Sugars[4]	3	14
Soybean oil	20	20	NSP[5]	105	131
$CaCO_3$	12.0	12.0	Gross energy, MJ/kg	22.1	17.2
$CaHPO_4.2H_2O$	10.0	10.0			
NaCl	0.5	0.5	[13]C enrichment, atom %	1.0814	1.0952
$KHCO_3$	12.0	12.0			
$NaHCO_3$	4.0	4.0			
MgO	0.8	0.8			
DL-methionine	0.7	0.7			
Diamol (SiO_2)	10.0	10.0			
Premix[2]	10.0	10.0			

[1] Basal diets were used as single diets for the asynchronous treatment (ASYN). For the synchronous treatment (SYN), the protein and carbohydrate diet were mixed in a ratio of 1 to 2.6.

[2] Provided per kg of the experimental diet: 5,000 IU retinol; 1,000 IU cholecalciferol; 7.5 mg α-tocopherol; 15 μg cyanocobalamin; 0.4 mg phylloquinone; 3.5 mg riboflavin; 20 mg niacinamid; 5 mg d-pantothenic acid; 200 mg choline chloride; 1 mg $CoSO_4.7H_2O$; 0.5 mg KI; 0.06 mg organic Se; 400 mg $FeSO_4.7H_2O$; 80 mg $CuSO_4.5H_2O$; 70 mg MnO_2; 200 mg $ZnSO_4.H_2O$.

[3] Analyzed content, unless indicated otherwise.

[4] Mono- and disaccharides as glucose units.

[5] Non-starch polysaccharide (NSP) content was calculated by subtracting the crude protein, fat, starch, sugars and ash content from the dry matter content.

Measurements

Body weight was measured weekly, directly before and after the experimental period. Gas exchange was measured in 9-min intervals by measuring the exchange of oxygen, carbon dioxide and methane (Verstegen et al., 1987) and physical activity was recorded by a radar-Doppler device (Wenk & Van Es, 1976). On day 2 and 6 in each experimental period, air was sampled in 30-min intervals during a 24-h period from the respiration chambers in evacuated tubes (Vacutainer, Becton Dickinson, Rutherford, NJ, USA) for analysis of the ^{13}C enrichment in CO_2 using a Finnigan Delta C continuous-flow isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany).

Faeces were collected quantitatively in plastic bags which were connected to the pigs and stored at -20°C pending analyses. Urine was collected below the tenderfoot floor via two funnels in two buckets which contained 125 ml of 4.5 M sulphuric acid each. Aerial NH_3 was quantitatively trapped in 4.5 M sulphuric acid and in water that condensed on the heat exchanger (Verstegen et al., 1987). For calculations, nitrogen in aerial NH_3 and in water that condensed on the heat exchanger were assumed of urinary origin. Feed was sampled during each experimental period. Feed refusals were collected prior to the next meal and stored at -20°C pending analyses.

For determination of the dry matter content, feed, feed refusals and fresh faeces were freeze-dried according to ISO 6496 (1998b). Following freeze-drying, faeces and feed were ground to pass a 1 mm screen and kept for analyses. Air-dry faeces and feed were dried in a forced air oven at 103ºC to a constant weight according to ISO 6496 (1998b). Kjeldahl nitrogen content was measured according to ISO 5983 (1997) in fresh feed, feed refusals, faeces, urine and in NH_4^+-containing sulphuric acid and water that condensed on the heat exchanger. Crude fat content was determined after acid hydrolysis in feed and in freeze-dried faeces according to ISO 6492 (1999). Crude ash content was determined in feed and in freeze-dried faeces. Samples were incinerated at 550ºC in a muffle furnace according to ISO 5984 (2002). The starch content in freeze-dried feed and faeces was analyzed enzymatically as described by Rijnen *et al.* (2001). Gross energy content was analyzed in freeze-dried feed, faeces, and urine using adiabatic bomb calorimetry (IKA-calorimeter C7000, Staufen, Germany) according to ISO 9831 (1998a).

Experimental diets were ground in a ball mill (Retsch MM 2000, Retsch GmbH & Co., Haan, Germany) and ^{13}C-enrichment was measured after combustion in an elemental analyser using the continuous flow isotope ratio mass spectrometer. All analyses were carried out in duplicate, except nitrogen content in urine which was determined in triplicate.

Calculations

For each balance period, the metabolizable energy intake (ME) was calculated per chamber as the difference between digestible energy intake and the sum of urinary energy losses and methane production. From the gaseous exchanges, heat production (H_{tot}) was calculated according to the formula of Brouwer (1965). For each pig within a balance period, the energy costs per unit of physical activity were estimated by regression of physical activity against H_{tot}. Activity related heat production (H_{act}) was calculated (Heetkamp et al., 1995). Heat production not related to physical activity was derived by subtracting H_{act} from H_{tot}. Energy retention was calculated by subtracting H_{tot} from ME intake. Retention of nitrogen was calculated from N in feed and excreta. Aerial NH_3 and NH_4^+ in water that condensed on the heat exchanger were included in the urinary N excretion. Crude protein content was calculated as N × 6.25. Energy retained as protein was calculated from retained protein assuming 23.6 kJ/g of protein. Energy retention as fat was calculated by subtracting energy retained as protein from energy retention. From the ME intake and energy retention as protein and as fat, ME_m was calculated, assuming an efficiency of utilization of ME for protein and fat deposition of 54% and 74%, respectively (ARC, 1981). The respiratory quotient (RQ) was calculated by expressing the CO_2 production relative to the O_2 consumption. Balance period means were calculated for all variables and hourly means were calculated for RQ, H_{tot} and H_{act}. In addition, the areas under the curve were calculated for H_{tot} and H_{act} relative to their baseline to quantify the contribution of variation in H_{act} to variation in H_{tot}.

Statistical analysis

Apparent faecal digestibility and nitrogen and energy balance traits were analyzed for the effect of treatment (SYN or ASYN) by ANOVA using the following model:

$$Y_{ij} = \mu + P_i + T_j + \varepsilon_{ij} \qquad [1]$$

where Y_{ij} = dependent variable; μ = average intercept; T_i = effect of treatment i (i = 1, 2); P_j = effect of pig (j = 1,…, 4 or 10); and ε_{ij} = error term. The statistical analysis for energy metabolism traits included only four pigs due to technical problems in the measurements of gas exchange. For the analysis of all other dependent variables, all ten pigs could be used. Effects of the sequence of both treatments were separately tested but were not significant and are therefore not included in the model. Treatment effects on H_{tot}, H_{act}, RQ and [13]C

enrichment of expired CO_2 were tested for each hour separately. The SAS software package version 9·1 (SAS Inst. Inc., Cary, NC, USA) was used in all statistical evaluations. Results are presented as least-square means with their SEM.

Results

General

At the start of the experimental period, pigs weighed on average 54 (SEM 1.0) kg. Average daily gain did not differ (*P*>0.10) between treatments and averaged 515 (SEM 26.8) g. One pig was excluded and replaced by another pig at the third day of the first adaptation period. The feed refusals, as a percentage of the feed supply, were generally low, but were twofold higher for the carbohydrate diet (5.6%) than for the protein diet (2.8%) in asynchronously fed pigs. Due to continuous leakage of air into the respiration chambers in the first three replicates, data on gas exchange for these replicates were excluded. Data from all replicates, however, were included to test the effects on nutrient digestibility and nitrogen balance traits.

Digestibility

Apparent faecal digestibility of dry matter, organic matter and energy was 87-90% and decreased when nutrient synchrony decreased (*P*=0.019) (Table 3). Fat, ash and protein digestibility was not affected by treatment and averaged 71.5 (SEM 1.70), 51.7 (SEM 1.38) and 90.8 (SEM 0.57) %, respectively. Starch was not detected in faeces. The apparent faecal digestibility of dietary non-starch polysaccharides (NSP) increased (*P*<0.001) from 45.9% for ASYN to 58.3% for SYN.

Protein metabolism

Nitrogen intake was 5% higher for ASYN than for SYN (*P*=0.003) (Table 4). The higher nitrogen intake for ASYN than for SYN was caused by a higher measured protein content in the protein diet than calculated (672 g/kg vs. 620 g/kg) in combination with more feed refusals for the carbohydrate diet than for the protein diet in asynchronously fed pigs. Asynchronously fed pigs lost 198 mg/kg $BW^{0.75}$ per d more nitrogen via urine than synchronously fed pigs (*P*<0.001), which ultimately resulted in a 127 mg/kg $BW^{0.75}$ per d lower nitrogen retention for ASYN than for SYN (*P*=0.017). The efficiency of digestible nitrogen utilization was 10 percentage units higher (*P*<0.001) for SYN (56.7%) than for ASYN (47.1%).

Table 3. Effects of synchronizing amino acid and glucose availability on animal performance and on apparent faecal nutrient digestibility in pigs[1]

Trait	Treatment		SEM	*P*-value
	Synchronous	Asynchronous		
No. of pigs	10	10	-	-
Initial body weight, kg	54.0	54.3	1.67	0.408
Average daily feed intake, g	1247	1272	32.8	0.595
Average daily gain, g	537	493	39.6	0.472
Apparent faecal digestibility	%			
Dry matter	88.1	86.6	0.52	0.019
Crude ash	51.1	52.3	1.38	0.379
Organic matter	90.1	88.7	0.48	0.011
Crude protein	91.0	90.6	0.57	0.508
Crude fat	73.7	69.2	1.70	0.112
Starch	100.0	100.0	-	-
NSP[2]	58.3	45.9	0.35	<0.001
Energy	88.5	86.9	0.61	0.016

[1] Values are least-square means, n=10 for each treatment.

[2] NSP=non-starch polysaccharides; NSP content in feed and faeces was calculated by subtracting the crude protein, crude fat, starch, sugars and ash content from the dry matter content.

Energy metabolism

Data on energy partitioning and circadian patterns of gas exchange include two out of five replicates. Gross energy intake did not differ between treatments (Table 4), but DE intake was about 5% higher for SYN than for ASYN (*P*=0.007). Urinary energy losses were higher for ASYN than for SYN (*P*=0.023), and consequently, as percentage of DE intake, metabolizability was lower for ASYN than for SYN (*P*=0.025). Heat production was not affected by nutrient synchrony (*P*>0.10). Energy retention and energy retained as protein tended to be higher (*P*<0.10) for SYN than for ASYN. Energy retained as fat did not differ (*P*>0.10) between treatments. Metabolizable energy requirements for maintenance were not affected by nutrient synchrony (*P*>0.10).

In accordance with average H_{tot}, the circadian patterns of H_{tot} and H_{act} were generally not affected by treatment (Figure 1A). Circadian variation in H_{tot} was largely caused by variation in H_{act}. The circadian rhythm in RQ showed a considerably larger amplitude for ASYN (0.28 units) than for SYN (0.17 units) (*P*<0.05; Figure 1B). The RQ did not increase after a protein meal, whereas a balanced meal and a carbohydrate meal resulted in an increase in RQ. The ^{13}C enrichment of CO_2 in the expired air was relatively constant (1.0915-1.0929 atom %)

during the day in synchronously fed pigs (Figure 1C) and always exceeded the natural enrichment of the control diet (1.0910 atom %). In asynchronously fed pigs, however, the pattern of ^{13}C enrichment of CO_2 within the day clearly reflected the ^{13}C enrichment of the meal supplied.

Table 4. Effects of synchronizing amino acid and glucose availability on protein and energy metabolism in growing pigs

Trait	Treatment		SEM	*P*-value
	Synchronous	Asynchronous		
Protein metabolism[1]	mg/kg BW$^{0.75}$ per d			
Nitrogen intake	1823	1918	16.4	0.003
Faecal nitrogen excretion	160	183	7.1	0.050
Digestible nitrogen intake	1663	1735	19.4	0.028
Urinary nitrogen excretion	718	917	22.7	<0.001
Nitrogen retention	945	818	30.9	0.017
Efficiency of nitrogen utilization	%			
as percentage of nitrogen intake	51.7	42.6	1.36	0.001
as percentage of digestible nitrogen intake	56.7	47.1	1.46	0.001
Energy metabolism[2]	kJ/kg BW$^{0.75}$ per d			
Gross energy intake	1099	1095	3.3	0.386
Digestible energy intake (DE)	979	929	10.4	0.007
Urinary energy	28	40	2.7	0.023
Methane production	3.7	2.0	0.55	0.048
Metabolizable energy intake (ME)	947	886	12.7	0.009
ME:DE, %	96.7	95.4	0.33	0.025
Heat production	653	642	13.8	0.618
Activity related heat production	110	96	4.2	0.100
Activity corrected heat production	543	546	14.7	0.886
Energy retention	294	244	28.9	0.058
Energy retention as protein	138	115	8.2	0.055
Energy retention as fat	156	129	27.3	0.263
ME for maintenance	484	501	30.6	0.719
Respiratory quotient (RQ)	0.96	0.97	0.008	0.338
Δ RQ	0.17	0.28	0.002	0.027

[1] Values are least-square means, n=10 for each treatment.

[2] Values are least-square means, n=4 for each treatment.

Discussion

Digestibility

The apparent faecal digestibility of dry matter, organic matter and energy ($P<0.05$) increased with increasing nutrient synchrony. This mainly originated from the increased apparent digestibility of NSP ($P<0.001$) and from the numerically increased apparent digestibility of protein and fat. The increased apparent faecal digestibility of NSP with increasing nutrient synchrony is related to an increased fermentation and corresponds with the higher methane production for SYN than for ASYN ($P<0.05$; Table 4). This is in accordance with a higher methane production in synchronously than in asynchronously fed sows (Müller & Kirchgessner, 1996). It is speculated that the large circadian fluctuations in the substrate supply for ASYN may have changed the ratio between available carbon and nitrogen for the microflora. This may have required continuous adaptation of the microflora to their environment and decreased the ability of (large) intestinal microflora to degrade NSP. The distribution of NSP intake over the morning and afternoon meal differed only slightly between SYN (33 vs. 67%, respectively) and ASYN (24 and 76%, respectively). Protein digestibility was not affected by treatment ($P>0.10$) and exceeded 90%, which is in accordance with tabulated values for soy protein isolate and potato protein (CVB, 2000).

Protein metabolism

Despite the slightly higher N intake for ASYN than for SYN, nitrogen retention and the efficiency of utilization of digestible protein for protein gain were substantially lower for ASYN (818 mg/kg $BW^{0.75}$ per d and 47.1%, respectively) than for SYN (945 mg/kg $BW^{0.75}$ per d and 56·7%, respectively). This does not correspond with studies in growing pigs in which within-day separation of corn (Eggert et al., 1953) or barley (Yeo & Chamberlain, 1966) intake from the dietary protein intake did not affect nitrogen deposition. Reasons for the difference may include the genetic progress during the past 40 to 50 years and the exclusion of between-animal variation in the current study. Moreover, the degree of nutrient asynchrony was much larger in the present study than in the other studies in growing pigs (Eggert *et al.*, 1953; Yeo & Chamberlain, 1966).
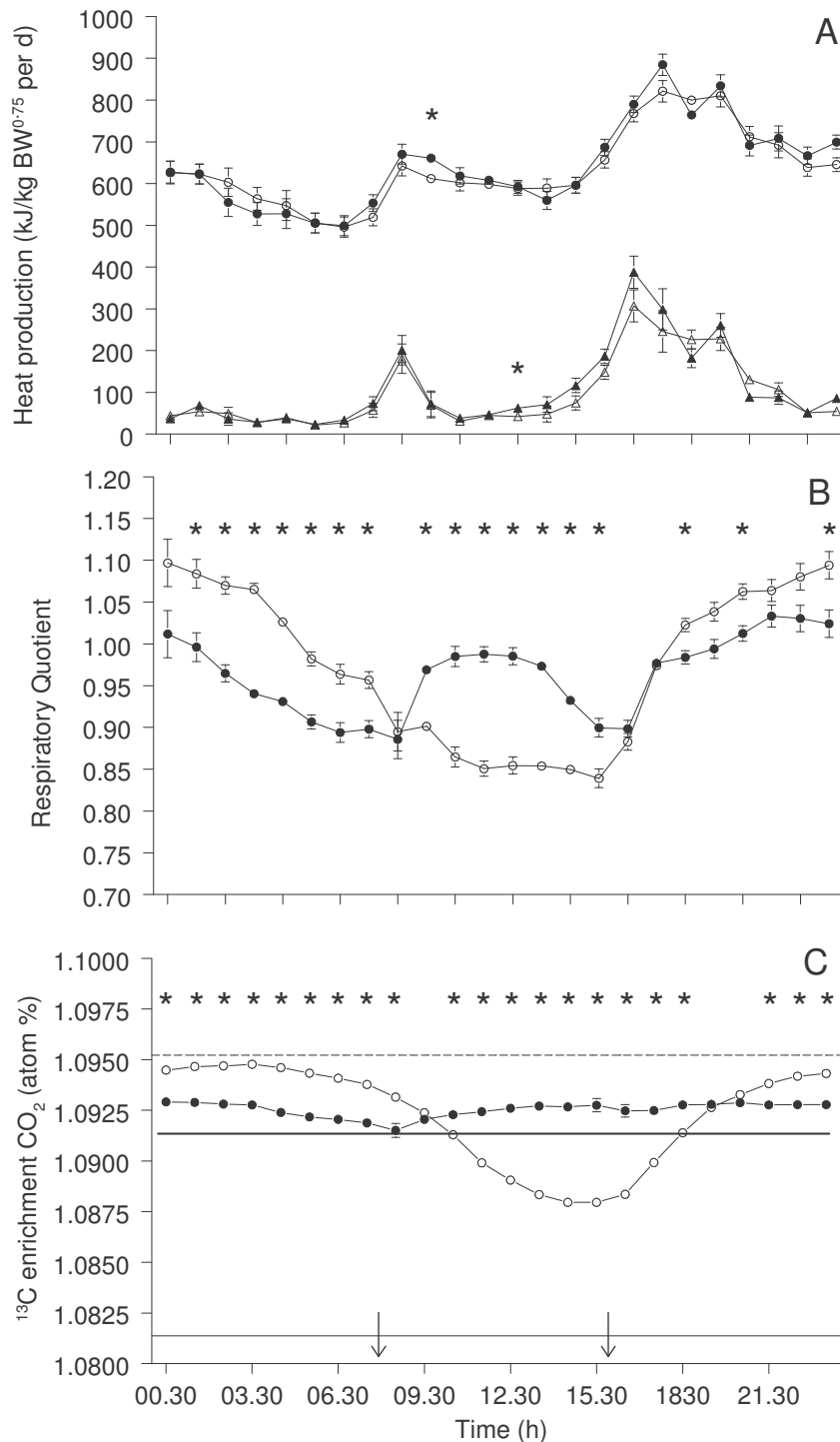
Figure 1. Effects of synchronous (●, ▲) or asynchronous (○, △) availability of amino acids and glucose in growing pigs on the circadian rhythms of (A) heat production (——) and activity related heat production (- - - -); (B) the respiratory quotient; and (C) the ^{13}C enrichment of the expired CO_2. Stars indicate significance ($P<0.05$). Results are expressed as least-square means ± SEM, n=4 for each treatment. Arrows represent feeding times. Asynchronously fed pigs received a protein meal in the morning and a carbohydrate meal in the afternoon. Horizontal lines in Figure 1C represent ^{13}C enrichment of the carbohydrate meal (——; 1.0952 atom %), the protein meal (- - - -; 1.0814 atom %) and the balanced meal (— - — -; 1.0910 atom %).

Our results are, however, in accordance with a decreased protein retention in children (Barja et al., 1972) and growing rats (Bancroft et al., 1951) when protein and carbohydrate intake were partially separated. Similar effects were found in protein-depleted rats, that had a higher nitrogen retention and growth rate during repletion when protein and carbohydrates were synchronously fed compared with asynchronous feeding (Geiger et al., 1950). The efficiency of protein utilization in rats also decreased (from 87 to 69%) when continuous intravenous infusion of amino acids (during 12 h/d) was separated from the infusion of fat and carbohydrates (during the remaining 12 h/d) compared with a continuous infusion (24 h/d) of all nutrients simultaneously (Martins et al., 1985). Cuthbertson *et al.* (1940) did not find an effect of nutrient synchrony on nitrogen balance in growing rats, but the growth rates observed were very low (1 g/d) and it was argued that there may have been a dietary deficiency in their study (Geiger, 1948). In growing, ruminant lambs, an asynchronous abomasal infusion of amino acids and energy (i.e. triglycerides of acetate and butyrate) increased urea production compared with a synchronous infusion (Randles, 2001). An increased separation of protein and lactose intake in time did, however, not decrease protein utilization in preruminant calves (Van den Borne et al., 2006).

In adult subjects, protein utilization generally decreases when nutrient synchrony decreases. In adult dogs, nitrogen balance decreased when carbohydrates were given more than 4 hours before or after a high protein meal (Larson & Chaikoff, 1937). Similar results were described when nutrient asynchrony was imposed in adult man (Cuthbertson & Munro, 1939) and adult rats (Cuthbertson et al., 1940; Munro, 1949; Van Dam-Bakker et al., 1958). In non-growing sows, however, separation of protein and carbohydrate intake for 33, or even for 48 h, did not depress nitrogen balance (Müller & Kirchgessner, 1996; Kirchgessner & Müller, 1998). In 26 y old women, a partial separation of protein and carbohydrate intake did not clearly affect protein retention, although it increased numerically with increased nutrient synchrony (Arnal et al., 2000). Surprisingly, in 68 y old women at a high protein intake, protein utilization increased with decreasing nutrient synchrony (Arnal et al., 1999). This may be caused by an age-related impairment of the response of both protein synthesis and protein breakdown to feeding as described in adult (11 mo old) and old (23 mo old) rats (Arnal et al., 2002). As a consequence, asynchronous nutrient intake was suggested to stimulate protein retention in old but not in adult rats (Arnal et al., 2002). The effect of nutrient synchrony may therefore be influenced by both the degree of nutrient synchrony and the stage of maturity of the animals. Effects of the degree of nutrient synchrony and the sequence of the high protein and high lactose meal remain to be studied in growing pigs. A virtually complete separation of the

protein and carbohydrate intake within a day decreased the efficiency of digestible protein utilization in pigs increased from 57 to 47%.

Regulation of protein utilization by nutrient synchrony

The increased urinary nitrogen losses for ASYN compared with SYN have likely resulted from an increased amino acid oxidation after the protein meal. The decreasing RQ after a protein meal (Figure 1B) suggests an increased oxidation of fat (RQ=0.70) and amino acids (RQ=0.81) due to a lack of glucose (RQ=1) availability. Similar changes in the RQ after carbohydrate and protein meals in growing pigs were described by Charlet-Lery and Morel (1977). The increased amino acid oxidation after the protein meal was more clearly demonstrated by the circadian rhythm of the ^{13}C enrichment of expired CO_2, which was nearly invariable for SYN but showed clear fluctuations for ASYN (Figure 1C). Although firm evidence can only be obtained from direct measurement of the amino oxidation flux, the decrease in ^{13}C enrichment for ASYN during daytime strongly suggests that the contribution of amino acid oxidation to total substrate oxidation was substantially increased during this period. The ^{13}C enrichment of body protein, glycogen and adipose tissues were not measured and the contribution of their oxidation to total substrate oxidation could not be quantified. It can, however, quite safely be assumed that the enrichment of the protein diet is reflected in body protein. The ^{13}C enrichment of body fat can be assumed to be only slightly lower than the enrichment of the carbohydrate diet, because fat deposition mainly originates from dietary glucose (high enrichment) and dietary fat (intermediary enrichment). It is therefore concluded from the circadian rhythms of RQ and CO_2 enrichment that amino acids are oxidized in absence of glucose. Several potential mechanisms for the increased amino acid oxidation with decreasing nutrient synchrony can be suggested.

First, amino acids may be used to provide ATP for maintenance and for protein deposition in the absence of glucose from dietary origin. The dietary glucose availability at 08.00 h has probably been insignificant, since it has been shown that after a 65% maize starch meal, the 10-h net portal glucose flux accounts for 97% of the ileal digestible glucose in pigs (Van der Meulen et al., 1997). Fat intake in the morning meal (~46 kJ DE/kg BW$^{0.75}$) only provided energy to cover maintenance requirements (assuming 20 kJ DE/kg BW$^{0.75}$ per h) for about 2.5 h, suggesting that amino acids, glycogen and fatty acids from body fat should then be oxidized to produce ATP.

Second, amino acids may have been required for gluconeogenesis. Especially when dietary glucose supply is limited, i.e. prior to the carbohydrate meal for ASYN, glycogenolysis and

gluconeogenesis will have to provide glucose for essential physiological processes (e.g. fuel for brain and erythrocytes). The excessively available amino acids during this period of the day would be obvious precursors for gluconeogenesis. This may have contributed to the decreased RQ in the morning, because gluconeogenesis from amino acids results in a low RQ (RQ=0.13; Wolfe & Chinkes, 2005), because HCO_3^- is used to produce urea.

Third, glucose may interact with amino acid metabolism via endocrine responses. In growing pigs, carbohydrate-induced insulin secretion is known to stimulate nitrogen retention (Fuller et al., 1977), although this effect is not consistently found in non-growing subjects (discussed by Millward, 2004). To affect the metabolic fate of dietary amino acids, however, insulin secretion should be in synchrony with the postabsorptive supply of amino acids. A low insulin response after the protein meal may have decreased the utilization of amino acids for protein retention and increased gluconeogenesis (see e.g. Barthel & Schmoll, 2003).

Fourth, the presence of dietary carbohydrates may affect amino acid metabolism in the intestinal tissues with concurrent implications for the kinetics of the systemic amino acid availability. A slow release of amino acids from the gut to extra-intestinal tissues (Mariotti et al., 2000; Soeters et al., 2001) prevents large variation in plasma amino acid concentrations and decreases urea production compared with a fast release of amino acids (Deutz et al., 1995; Bos et al., 2003). The intestinal tissues may affect the kinetics of availability of amino acids for other tissues by acting as a temporary buffer for amino acids after a meal and releasing them during a postabsorptive phase (Mariotti et al., 2000; Soeters et al., 2001). The retention of amino acids in the portal drained viscera is increased when carbohydrates are added to a protein meal, which results in a slower amino acid release into the portal vein (Deutz et al., 1995; Fouillet et al., 2001).

In summary, ATP and glucose requirements likely have induced oxidation of amino acids in asynchronously fed pigs. In addition, a separate supply of protein and carbohydrates may have decreased amino acid utilization by altering the endocrine regulation and the kinetics of amino acid availability to extra-intestinal tissues.

Energy metabolism

Gross energy intake did not differ between treatments, but DE intake was 50 kJ/kg $BW^{0.75}$ per d lower for ASYN than for SYN ($P<0.01$). The 61 kJ/kg $BW^{0.75}$ per d lower ME intake combined with a 11 kJ/kg $BW^{0.75}$ per d lower heat production for ASYN than for SYN resulted in a tendency for a lower energy retention (-50 kJ/kg $BW^{0.75}$ per d) for ASYN than for SYN. The efficiency of digestible protein utilization corresponded between the two

replicates included in the data on energy partitioning and all five replicates; 56.6 vs. 56.7% for SYN and 46.9 vs. 47.1% for ASYN respectively. Despite the 61 kJ/kg $BW^{0.75}$ per d lower ME intake for ASYN than for SYN, H_{tot} was only 11 kJ/kg $BW^{0.75}$ per d lower for ASYN than for SYN. If H_{act} was excluded, the corrected heat production was numerically even higher for ASYN (547 kJ/kg $BW^{0.75}$ per d) than for SYN (542 kJ/kg $BW^{0.75}$ per d). This indicates that a substantial part of the increased quantity of deaminated amino acids for ASYN when compared with SYN was oxidized and not deposited as fat.

Average daily fat deposition was similar for SYN and ASYN (*P*>0.10). From Figure 1B, however, it is clear that there is considerable variation in RQ between treatments within the day. Conversion of glucose into fat occurred when the RQ exceeded unity and this happened after the 16.00 h meal for 11 h for ASYN, while the total time of *de novo* fatty acid synthesis was considerably shorter for SYN (5 h/d). A low RQ, on the other hand, suggests increased rates of fatty acid (and amino acid) oxidation in the morning and early afternoon for ASYN when compared with SYN (Figure 1B). As a result, energy retained as fat was numerically even 17% lower for ASYN than for SYN.

Circadian rhythms of H_{tot} were similar for SYN and ASYN. Activity related heat production varied between 23 and 280 kJ/kg $BW^{0.75}$ per d and explains most of the variation in H_{tot}. The contribution of variation in H_{act} to variation in H_{tot} varied, however, strongly over the day. After the morning meal, only 30% of the increase in H_{tot} could be explained by an increase in H_{act}, while an increased H_{act} contributed for about 70% to the increase in H_{tot} in the late afternoon. Although not always significant, H_{act} was numerically higher for SYN than for ASYN during the period 12.00-17.00 h. This may be due to the difference in the satiating effects of macronutrients (protein>carbohydrates>fat; Stubbs & Elia, 2001).

In conclusion, a virtually complete separation of protein and carbohydrate intake within a day decreased the apparent faecal digestibility of dry matter, organic matter, energy and NSP. The efficiency of digestible protein utilization for protein retention decreased from 57% to 47% with decreasing nutrient synchrony in growing pigs. The energy yield from the increased amino acid degradation was largely lost as heat, resulting in no net effect on energy retention as fat. Within the day, however, more prolonged periods of *de novo* fatty acid synthesis occurred for ASYN than for SYN, combined with increased rates of fatty acid oxidation for ASYN than for SYN during the remainder of the day. Heat production related to physical activity tended to be lower, mainly after the protein meal, for ASYN than for SYN.

Acknowledgements

References

ARC (1981) The nutrient requirements of pigs. *Agricultural Research Council, Commonwealth Agricultural Bureaux, Slough, UK*.

Arnal MA, Mosoni L, Boirie Y, Houlier ML, Morin L, Verdier E, Ritz P, Antoine JM, Prugnaud J, Beaufrère B & Mirand PP (1999) Protein pulse feeding improves protein retention in elderly women. *Am J Clin Nutr* **69**, 1202-1208.

Arnal MA, Mosoni L, Boirie Y, Houlier ML, Morin L, Verdier E, Ritz P, Antoine JM, Prugnaud J, Beaufrère B & Patureau-Mirand P (2000) Protein feeding pattern does not affect protein retention in young women. *J Nutr* **130**, 1700-1704.

Arnal MA, Mosoni L, Dardevet D, Ribeyre MC, Bayle G, Prugnaud J & Patureau-Mirand P (2002) Pulse protein feeding pattern restores stimulation of muscle protein synthesis during the feeding period in old rats. *J Nutr* **132**, 1002-1008.

Bancroft RW, Geiger E & Hagerty EB (1951) Nitrogen-sparing effect of carbohydrate related to time factor with hypophysectomized and diabetic rats. *Endocrinology* **49**, 149-153.

Barja I, Araya H, Munoz P, Vega L, Arteaga A & Tagle MA (1972) Effect of spacing protein intake on nitrogen balance in normal children. *Am J Clin Nutr* **25**, 506-511.

Barthel A & Schmoll D (2003) Novel concepts in insulin regulation of hepatic gluconeogenesis. *Am J Physiol* **285**, E685-692.

Bos C, Metges CC, Gaudichon C, Petzke KJ, Pueyo ME, Morens C, Everwand J, Benamouzig R & Tomé D (2003) Postprandial kinetics of dietary amino acids are the main determinant of their metabolism after soy or milk protein ingestion in humans. *J Nutr* **133**, 1308-1315.

Brouwer E (1965) Report of sub-committee on constants and factors. In *Energy Metabolism*, pp. 441-443 [KL Blaxter, editor]. London, UK: Academic Press.

Campbell RG & Taverner MR (1988) Genotype and sex effects on the relationship between energy intake and protein deposition in growing pigs. *J Anim Sci* **66**, 676-686.

Charlet-Lery G & Morel MT (1977) Influence of diet on respiratory quotients and fat deposition in growing pigs. *Ann Biol Anim Bioch Biophys* **17**, 897-904.

Cummings JH & Englyst HN (1995) Gastrointestinal effects of food carbohydrate. *Am J Clin Nutr* **61**, 938S-945S.

Cuthbertson DP, McCutcheon A & Munro HN (1940) The relationship of carbohydrate metabolism to protein metabolism. 2. Note on the effect of separation in time of the protein and carbohydrate moieties of the diet of the adult and growing rat. *Biochem J* **34**, 1002-1007.

Cuthbertson DP & Munro HN (1939) The relationship of carbohydrate metabolism to protein metabolism. I. The roles of dietary carbohydrate and of surfeit carbohydrate in protein metabolism. *Biochem J* **33**, 128-142.

CVB (2000) Veevoedertabel. *Centraal Veevoeder Bureau, Lelystad, The Netherlands*.

Deutz NEP, Bruins M & Soeters PB (1998) Infusion of soy and casein protein meals affects interorgan amino acid metabolism and urea kinetics differently in pigs. *J Nutr* **128**, 2435-2445.

Deutz NEP, Ten Have GAM, Soeters PB & Moughan PJ (1995) Increased intestinal amino-acid retention from the addition of carbohydrates to a meal. *Clin Sci* **14**, 354-364.

Eggert RG, Brinegar MJ & Anderson CR (1953) Delayed protein supplementation of corn diets for growing swine. *J Nutr* **50**, 469-477.

Eits RM, Kwakkel RP, Verstegen MWA, Stoutjesdijk P & De Greef KH (2002) Protein and lipid deposition rates in male broiler chickens: Separate responses to amino acids and protein-free energy. *Poultry Sci* **81**, 472-480.

Englyst KN, Vinoy S, Englyst HN & Lang V (2003) Glycaemic index of cereal products explained by their content of rapidly and slowly available glucose. *Br J Nutr* **89**, 329.

Fouillet H, Gaudichon C, Mariotti F, Bos C, Huneau J-F & Tomé D (2001) Energy nutrients modulate the splanchnic sequestration of dietary nitrogen in humans: a compartmental analysis. *Am J Physiol* **281**, E248-E260.

Fuller MF, Weekes TEC, Cadenhead A & Bruce JB (1977) The protein-sparing effect of carbohydrate 2. The role of insulin. *Br J Nutr* **38**, 489-496.

Geiger E (1948) The importance of the time element in feeding of growing rats. Experiments with delayed supplementation of protein. *Science* **108**, 42-43.

Geiger E, Bancroft RW & Hagerty EB (1950) The nitrogen-sparing effect of dietary carbohydrate in its relation to the time factor: experiments with repletion of protein-depleted adult rats. *J Nutr* **42**, 577-585.

Gerrits WJJ, Tolman GH, Schrama JW, Tamminga S, Bosch MW & Verstegen MWA (1996) Effect of protein and protein-free energy intake on protein and fat deposition rates in preruminant calves of 80 to 240 kg live weight. *J Anim Sci* **74**, 2129-2139.

Heetkamp MJW, Schrama JW, de Jong L, Swinkels JW, Schouten WG & Bosch MW (1995) Energy metabolism in young pigs as affected by mixing. *J Anim Sci* **73**, 3562-3569.

Heger J & Frydrych Z (1985) Efficiency of utilization of essential amino acids in growing rats at different levels of intake. *Br J Nutr* **54**, 499-508.

ISO (1997) Animal feeding stuffs. Determination of nitrogen content and calculation of crude protein content. Kjeldahl method. ISO 5983. International Organization for Standardization.

ISO (1998a) Animal feeding stuffs, animal products, and faeces or urine. Determination of gross calorific value. ISO 9831. International Organization for Standardization.

ISO (1998b) Animal feeding stuffs. Determination of moisture and other volatile matter content. ISO 6496. International Organization for Standardization.

ISO (1999) Animal feeding stuffs. Determination of fat content. ISO 6492. International Organization for Standardization.

ISO (2002) Animal feeding stuffs. Determination of crude ash. ISO 5984. International Organization for Standardization.

Kirchgessner M & Müller HL (1998) Dietary separation of protein and carbohydrate intake. *Energy Metabolism in Farm Animals*.

Larson PS & Chaikoff IL (1937) The influence of carbohydrate on nitrogen metabolism in the normal nutritional state. *J Nutr* **13**, 287-304.

Longenbach JI & Heinrichs AJ (1997) A review of the importance and physiological role of curd formation in the abomasum of young calves. *Anim Feed Sci Technol* **73**, 85-97.

Mariotti F, Huneau J-F, Mahé S & Tomé D (2000) Protein metabolism and the gut. *Curr Opin Clin Nutr Metab Care* **3**, 45-50.

Martins FM, Sandberg G, Ekman L & Lindmark L (1985) Metabolic response of simultaneous versus sequential intravenous administration of amino acids and energy substrates to rats. *Am J Clin Nutr* **42**, 61-68.

Metges CC, El-Khoury AE, Selvaraj AB, Tsay RH, Atkinson A, Regan MM, Bequette BJ & Young VR (2000) Kinetics of L-1-13C-Leucine when ingested with free amino acids, unlabeled or intrinsically labelled casein. *Am J Physiol* **278**, E1000-E1009.

Millward DJ (2004) Macronutrient intakes as determinants of dietary protein and amino acid adequacy. *J Nutr* **134**, 1588S-1596S.

Mosoni L & Patureau-Mirand P (2003) Type and timing of protein feeding to optimize anabolism. *Curr Opin Clin Nutr Metab Care* **6**, 301-306.

Müller HL & Kirchgessner M (1996) Effect of chronological separation of protein and carbohydrates by up to 48 h on the energy metabolism [in German]. *J Anim Physiol Anim Nutr* **76**, 1-8.

Munro HN (1949) The relationship of carbohydrate metabolism to protein metabolism. *J Nutr* **39**, 375-391.

Munro HN (1964) General aspects of the regulation of protein metabolism by diet and by hormones. In *Mammalian protein metabolism*, pp. 381-481 [HN Munro and JB Allison, editors]. London: Academic Press.

Randles WG (2001) The effects of different temporal patterns of post-ruminal energy and protein supply on nitrogen metabolism in growing lambs, Doctoral thesis, University of Aberdeen, Aberdeen, UK.

Rijnen MMJA, Verstegen MWA, Heetkamp MJW & Schrama JW (2001) Effects of dietary fermentable carbohydrates on energy metabolism in group-housed sows. *J Anim Sci* **79**, 148-154.

Soeters PB, Dejong CH & Deutz NEP (2001) The protein sparing function of the gut and the quality of food protein. *Clin Nutr* **20**, 97-99.

Stubbs RJ & Elia M (2001) Macronutrients and appetite control with implications for the nutritional management of the malnourished. *Clin Nutr* **20**, S129-S139.

Van Dam-Bakker AWI, De Groot AP & Luyken R (1958) The influence of alternate high-protein and low-protein feeding on growth and reproduction and on regeneration of haemoglobin in rats. *Br J Nutr* **12**, 259-266.

Van den Borne JJGC, Verstegen MWA, Alferink SJJ, Van Ass FHM & Gerrits WJJ (2006) Synchronizing the availability of amino acids and glucose decreases fat retention in heavy preruminant calves. *J Nutr* **136**, 2181-2187.

Van der Meulen J, Bakker JGM, Smits B & De Visser H (1997) Effect of source of starch on net portal flux of glucose, lactate, volatile fatty acids and amino acids in the pig. *Br J Nutr* **78**, 533-544.

Van Kleef DF, Deuring K & Van Leeuwen P (1994) A new method for faeces collection in the pig. *Lab Anim* **28**, 78-79.

Verstegen MWA, Van der Hel W, Brandsma H, Henken AM & Bransen AM (1987) The Wageningen respiration unit for animal production research: a description of the equipment and its possibilities. In *Energy Metabolism in Farm Animals: Effects of Housing, Stress and Disease*, pp. 21-48 [MWA Verstegen and AM Henken, editors]. Dordrecht, The Netherlands: Martinus Nijhoff Publishers.

Wenk C & Van Es AJH (1976) Eine Methode zur Bestimmung des Energieauswandes für die körperlich Aktivität von wachsenden Küken. *Schweiz Landwirtsch Monatsh* **54**, 232.

Wolfe RR & Chinkes DL (2005) *Isotope tracers in metabolic research: principles and practice of kinetic analysis*, 2nd ed. Hoboken, New Jersey, USA: John Wiley & Sons, Inc.

Yeo ML & Chamberlain AG (1966) Delayed protein supplementation of barley diets for weanling pigs. *Proc Nutr Soc* **25**, 41 (Abstr).

Chapter 6

Synchronizing the availability of amino acids and glucose decreases fat retention in heavy preruminant calves

JJGC van den Borne, MWA Verstegen, SJJ Alferink, FHM van Ass & WJJ Gerrits

Animal Nutrition Group, Department of Animal Sciences, Wageningen University, P.O. Box 338, 6700 AH Wageningen, The Netherlands

Abstract

Effects of synchronizing the availability of amino acids and glucose within a day on protein and energy metabolism were studied in heavy preruminant calves. Thirty-six preruminant calves (148 ± 1.6 kg body weight) were assigned to one of six degrees of nutrient synchrony (SYN, 1-6) and to one of two meal sequences (i.e. the high protein meal in the morning or in the evening). Calves at SYN 1 received two balanced meals: one at 0600 and one at 1800. Nutrient synchrony decreased stepwise from SYN 1 to 6 in which calves received 85% of the daily protein supply in one meal. The digestible energy intakes at 0600 and 1800 were equal between treatments. Daily intakes of all nutrients and dietary ingredients were similar for all treatments. Calves were housed individually in respiration chambers. Apparent fecal nutrient digestibility and nitrogen and energy balances were measured. Apparent nutrient digestibility decreased when more than 71% of the dietary protein was fed in one meal. Nutrient synchrony did not affect the efficiency of digestible protein utilization in calves at a similar digestible nutrient intake. Heat production decreased from 691 to 629 $kJ/(kg^{0.75}{\cdot}d)$ ($P < 0.05$) and energy retained as fat increased from 116 to 184 $kJ/(kg^{0.75}{\cdot}d)$ ($P < 0.01$) with decreasing nutrient synchrony. Meal sequence did not affect any of the traits. In conclusion, synchronizing the availability of amino acids and glucose within a day did not increase the efficiency of protein utilization, but substantially decreased fat retention in heavy preruminant calves.

Abbreviations: H_{act}, activity related heat production; H_{tot}, total heat production; ME, metabolizable energy; ME_m, metabolizable energy required for maintenance; RQ, respiratory quotient; SEQ, meal sequence; SYN, degree of nutrient synchrony.

Introduction

Dietary protein is efficiently utilized for body protein deposition in growing animals and man. During the first four weeks of life, about 80% of the milk protein provided above maintenance is deposited as body protein in rats, pigs, sheep and man (Reeds et al., 2000). After weaning, slightly lower values are reached for the marginal efficiency of digestible protein utilization in growing rats and pigs (Gahl et al., 1996; Mnilk et al., 1996). In heavy preruminant calves, however, the marginal efficiency of protein utilization for growth is very low (~30%) as compared with other species (Van den Borne *et al.*, 2006a). Several potential mechanisms to explain this low efficiency have been explored and discussed: (i) a protein to energy imbalance (Gerrits et al., 1996), (ii) an imbalanced amino acid profile (Gerrits et al., 1998), and (iii) the use of amino acids for ammonia detoxification (Gerrits et al., 1999), but none of these were responsible for the inefficient protein utilization in preruminant calves (Van den Borne *et al.*, 2006a). It was suggested that the problem may be of multi-factorial origin, and that a lack of nutrient synchrony may be involved (Van den Borne *et al.*, 2006a).

A decreased nutrient synchrony, i.e. a nearly complete separation of protein and carbohydrate intake in time, was shown to decrease the efficiency of protein utilization from 57 to 47% of the digestible protein intake in growing pigs (JJGC van den Borne, JW Schrama, MJW Heetkamp, MWA Verstegen, and WJJ Gerrits, unpublished results). In preruminant calves, nutrient synchrony may be decreased when milk replacers are based on skimmed milk protein. Casein, representing 80% of the skimmed milk protein, coagulates in the abomasum and is released gradually during the day, while absorption of glucose and galactose peaks within 1 hour post-feeding (Verdonk et al., 1999). This separation of amino acid and monosaccharide availability in time can result in within-day deficiencies or excesses of amino acids relative to glucose and may prevent an efficient protein deposition in milk-fed calves. Therefore, it is hypothesized that a lack of post-absorptive synchrony between nutrients restricts the efficiency of protein utilization in preruminant calves.

When compared with amino acids, the body has only limited capacity to store glucose. With diurnal variation in metabolic processes related to physical activity being evident (Schrama *et al.*, 1994; Rijnen *et al.*, 2003), the body may prefer glucose oxidation over amino acid oxidation to generate energy during the day period. The meal sequence of a high protein meal and a high carbohydrate meal may therefore affect protein retention. Moreover, it is speculated that the response of metabolism to a separation of glucose and amino acid availability within a day depends on the degree of nutrient synchrony.

In this paper it is hypothesized that, at equal daily nutrient intakes, a decreased synchrony of the protein and lactose supply over two meals decreases the efficiency of protein utilization in heavy preruminant calves. The aim of the experiment was to test the linearity of these effects and to study the impact of the hypothesized reduction in protein deposition on fat deposition. In addition, the effect of the sequence of the high protein and high lactose meals on protein utilization and energy partitioning was studied.

Materials and methods

Animals and housing

Thirty-six male, Holstein Friesian calves were used in 18 trials of two calves each. In a 6 × 2 factorial design calves were assigned to one of six degrees of nutrient synchrony (SYN, 1-6)[3] and to one of two meal sequences (SEQ, A or B). Allocation of SYN and SEQ to calves was balanced in time. Daily nutrient intakes were similar for all treatments. The degree of synchrony determined the distribution of protein and lactose over the two daily meals. Animals at SYN 1 were fed the daily amount of protein and lactose equally divided over both meals, while animals at SYN 6 received 85% of the daily protein supply in one meal and the remaining 15% in the other meal (Table 1). Other treatments (SYN 2 to 5) were intermediate. Protein and lactose were exchanged based on digestible energy, which resulted in identical digestible energy intakes in morning and evening meals across treatments. Protein and lactose supply were step-wise less synchronous from treatment 1 to 6. For calves at meal sequence A (SEQ A), the high protein meal was fed in the morning and the high lactose meal in the evening. For SEQ B, this sequence was reversed.

Calves arrived at the experimental facility at the age of two weeks and were raised for ten weeks on a commercial milk replacer, after which they were adapted to the experimental treatments and housing conditions for four weeks. Harnesses for the fecal collection bags were attached five days before the start of the experiment. The experimental period consisted of a 7-d balance period. Calves were individually housed in one of two identical climatic respiration chambers set to 2.5 × 1.5 × 2.0 m (L × W × H). Within each chamber, calves were housed in metabolic cages (1.85 × 0.75 m). Calves in the two separate chambers could see each other. Temperature was maintained at 18°C, relative humidity at 65% and air velocity was < 0.2 m/s. Calves were exposed to 13 h of light (50 lx; from 0530 to 1830) and 11 h of partial darkness (6 lx). The experiment was approved by the Ethical Committee of Wageningen University.

Table 1. Experimental treatments; distribution of the nutrient intake over two daily meals

Treatment (SYN)	1	2	3	4	5	6
CON[1]	50	57	64	71	78	85
Morning meal[2] (0600)			$g/kg^{0.75}$			
Protein	4.6	5.3	5.9	6.5	7.1	7.7
Lactose	10.0	8.9	7.8	6.7	5.6	4.5
Fat	4.6	4.6	4.6	4.6	4.6	4.6
Evening meal (1800)			$g/kg^{0.75}$			
Protein	4.6	4.0	3.4	2.8	2.2	1.6
Lactose	10.0	11.1	12.2	13.4	14.5	15.6
Fat	4.6	4.5	4.5	4.5	4.5	4.5

[1] CON = Contribution of the high protein meal to the daily protein supply (50% = two identical, balanced meals; 100% = all daily protein in one meal).

[2] Treatments are presented for meal sequence A. For meal sequence B, the nutrient intake in the morning and evening meal was reversed.

Diets and feeding

Calves were fed according to their metabolic body weight ($kg^{0.75}$), adjusted daily for a projected daily gain of 1000 g. Metabolizable energy requirements for maintenance (ME_m) were assumed to be 460 $kJ/(kg^{0.75}\cdot d)$ (Van Es *et al.*, 1967; Gerrits *et al.*, 1996). The feeding level was $2.0 \times ME_m$. Two basal diets were produced for the most asynchronous meals (SYN 6) and diets for the other five treatments (SYN 1-5) were created by mixing the two basal diets in a ratio appropriate to realize the experimental treatments as indicated in Table 1. All calves received 9.3 g crude protein, 9.1 g crude fat and 20.1 g lactose per $kg^{0.75}$ daily. The ingredient and analyzed nutrient composition of the basal diets are shown in Table 2. Milk replacer was reconstituted with water (140 g/L) and supplied at a temperature of about 40°C in a bucket. Roughage was not supplied. Calves were fed individually at 0600 and 1800. Calves were allowed 15 min to consume the meal.

Measurements

A balance trial was performed to measure apparent fecal nutrient digestibility and energy and protein retention. Feces were collected quantitatively in plastic bags which were harnessed to the calves and were stored at -20°C. For each balance period, morning and evening feces were pooled separately over days and pH was measured in morning and evening feces. After mixing the morning and evening feces, pH was measured again and feces were sampled for further analyses. Urine was collected in a pit containing 500 mL of 4.5 mol/L sulphuric acid.

Aerial NH_3 was quantitatively trapped in 4.5 mol/L sulphuric acid and in water that condensed on the heat exchanger. Feed refusals were collected, registered and frozen at -20ºC pending further analyses. Feed was sampled for each balance period and stored at 4ºC pending further analyses.

Table 2. Ingredient and analyzed nutrient composition of the two basal diets[1]

Item	High protein diet	High lactose diet
Ingredient	*g/kg*	
Whey protein concentrate	464.3	68.6
Soy oil	209.7	175.7
Coconut oil	52.4	43.9
Lactose	214.6	662.3
Premix[2]	54.6	45.8
Iron-supplement	4.4	3.7
Nutrient	*g/kg*	
Dry matter	985.5	992.5
Crude protein	364.5	62.1
Crude fat	217.8	178.0
Lactose	211.4	617.1
Ash	62.0	36.3

[1] Basal diets were the high protein and high lactose diet for treatment SYN 6 as indicated in Table 1. Diets were mixed as 45.6% of the high protein diet and 54.4% of the high lactose diet for SYN 1. With decreasing degree of nutrient synchrony, the contribution of the high protein diet to the high protein meal increased with 2.1 $g/kg^{0.75}$, while the contribution of the high lactose diet simultaneously decreased with 2.5 $g/kg^{0.75}$.

[2] Provided per kg of the experimental diet: 26.1 µmol retinol; 0.13 µmol cholecalciferol; 0.18 µmol α-tocoferol; 47.8 mg iron; 8.5 mg copper; 1.47 g magnesium; 13.3 mg manganese; 81.7 mg zinc; 1.9 mg cobalt; 0.1 mg selenium.

For determination of the dry matter content, feed refusals and fresh feces were freeze-dried, feed samples were vacuum-dried at 80ºC, and air-dry feces were dried in a forced air oven at 103ºC. All samples were dried to a constant weight according to ISO 6496 (1998b). Following freeze-drying, feces were ground to pass a 1 mm screen and kept for analyses. Nitrogen content was measured in fresh feed, feed refusals, fresh feces, urine, sulphuric acid containing aerial NH_3 and water of condensation containing aerial NH_3 according to ISO 5983 (1997). For calculations, nitrogen in aerial NH_3 was assumed of urinary origin. Crude fat content was determined in feed and in freeze-dried feces after acid hydrolysis according to

ISO 6492 (1999). Crude ash content was determined in feed and in freeze-dried feces. Samples were carefully incinerated in a muffle furnace by slowly increasing the temperature from 20ºC to 550ºC to prevent foaming, and subsequent incineration occurred according to ISO 5984 (2002). Lactose content was analyzed enzymatically in feed and in freeze-dried feces (Enzytec, Diffchamb Biocontrol, Nieuwerkerk aan den IJssel, The Netherlands). Gross energy content was analyzed in feed and freeze-dried feces and urine using adiabatic bomb calorimetry (IKA-calorimeter C7000, Staufen, Germany) according to ISO 9831 (1998a). All analyses were carried out in duplicate, except nitrogen content in urine which was determined in triplicate.

Gas exchange was measured in 6-min intervals by measuring the exchange of oxygen, carbon dioxide, and methane as described by Verstegen et al. (1987). Posture of calves was measured every minute by infrared beam interruption and expressed as lying (i.e. lying during the complete 6-min interval) or non-lying (i.e. standing during at least 1 min of the 6-min interval). Physical activity was recorded with a radar-Doppler device according to the method described by Wenk & Van Es (1976).

Calculations

For each balance period, intake of ME per calf was calculated as the difference between digestible energy intake and the sum of urinary energy losses and methane production. From the gaseous exchanges, heat production (H_{tot}) was calculated according to the formula of Brouwer (1965). Energy retention was calculated by subtracting H_{tot} from ME intake. Retention of nitrogen was calculated from N in feed and N in excreta. Energy retained as protein was derived from retained N assuming 23.6 kJ/g of protein. Energy retention as fat was calculated by subtracting energy retained as protein from energy retention. The respiratory quotient (RQ) was calculated as the CO_2 production divided by the O_2 consumption. For each calf within a balance period, the energy costs per unit of physical activity were estimated by regression of physical activity against heat production and subsequent calculation of heat production for physical activity (H_{act}) as described by Van den Borne et al. (2006b). Balance period means were calculated for all variables and hourly means were calculated for H_{tot}, H_{act} and RQ.

Statistical analysis

The effects of the degree of nutrient synchrony and the interaction between the degree of nutrient synchrony and meal sequence on apparent fecal digestibilities, energy and nitrogen

balance parameters, and circadian rhythms of heat production traits and RQ were analyzed in a mixed model, using the degree of nutrient synchrony as a regressor, according to the following model:

$$Y_{ij} = \mu + \beta_1 \times X_j + \beta_{2i} \times S_i X_j + \varepsilon_{ij} \qquad [1]$$

where Y_{ij} = dependent variable over the whole period (or the hourly mean), μ = average intercept, β_1 = effect of degree of nutrient synchrony, expressed as percentage of the daily protein intake in the morning meal, β_{2i} = interaction between degree of nutrient synchrony and meal sequence i, X_j = degree of nutrient synchrony (expressed as percentage of the daily protein intake in the high protein meal) for calf j, ε_{ij} = error term, i = 1, 2, and j = 1…18. Treatment effects on H_{tot}, H_{act} and RQ were tested for each hour separately. Differences were considered significant at $P < 0.05$. Results are presented as means with their SEM.

Results

General

The initial body weight and daily gain did not differ between treatments (Table 3) and averaged 148 ± 1.6 kg and 1209 ± 46.3 g respectively. Two calves were excluded from the analysis, because of feed refusals or illness. Feed intake was similar for all treatments. Digestive problems (i.e. diarrhea) occurred for calves at SYN 5 and 6. Compared with feces of calves at SYN 1 to 4, feces of calves at SYN 5 and 6 were characterized by a lower dry matter content (11.9 vs. 18.8%; $P < 0.01$) and a reduced pH (5.9 vs. 7.8; $P < 0.01$) (Figure 1). Feces collected after the morning meal did not differ in pH from feces collected after the evening meal (data not shown). Consequently, calves at SYN 5 and 6 had a lower ($P < 0.001$) digestible nutrient intake than calves at SYN 1 to 4. Linear regression was, therefore, performed for SYN 1 to 6 and for SYN 1 to 4 separately. The meal sequence did not affect any of the weekly averaged balance traits and interactions between SYN and SEQ were not present. Therefore, results are presented as pooled data without differentiation for SEQ A and B.
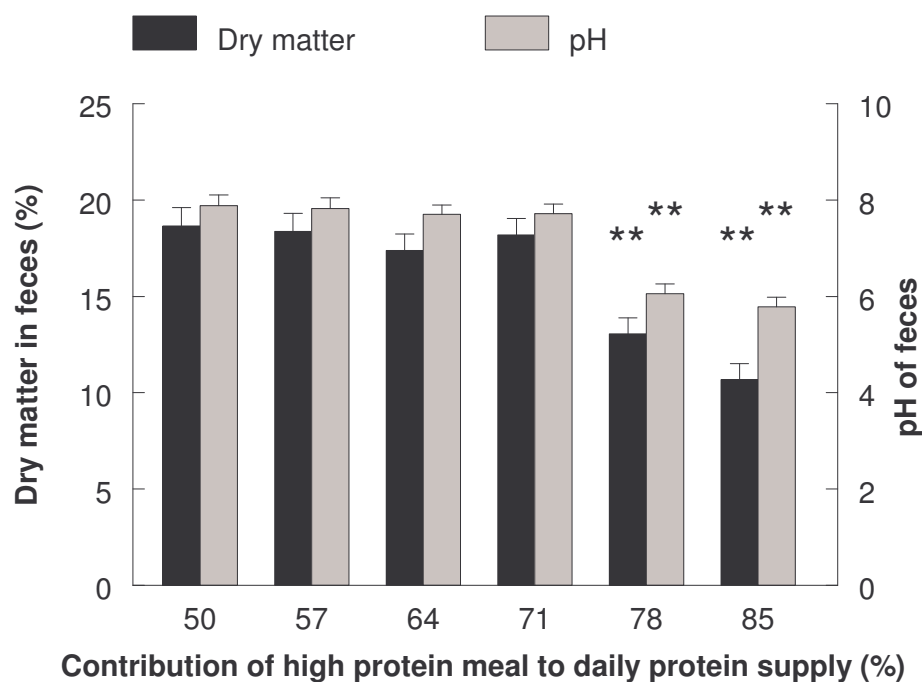
Figure 1. Effect of nutrient synchrony on fecal dry matter content and fecal pH in heavy preruminant calves. Stars indicate differences (**: $P < 0.01$) of respectively 78% of the daily protein in the high protein meal (i.e. SYN 5) and 85% of the daily protein in the high protein meal (i.e. SYN 6) versus 50-71% of the daily protein in the high protein meal (i.e. SYN 1-4). Values are means ± SEM, $n = 5$ (SYN 1-2) or 6 (SYN 3-6).

Digestibility

Apparent fecal nutrient digestibility, except for ash, decreased with decreasing nutrient synchrony ($P < 0.01$). This decrease was due to low nutrient digestibilities for SYN 5 and 6. Nutrient digestibility was not affected when only SYN 1 to 4 were taken into account. In accordance, the daily digestible intake of individual nutrients (data not shown) was similar for SYN 1 to 4, but substantially lower for SYN 5 and 6 ($P < 0.01$).

Nitrogen metabolism

Nitrogen intake did not differ between treatments and fecal nitrogen excretion was not affected (SYN 1-4) by decreasing nutrient synchrony (Table 4). Therefore, digestible nitrogen intake was similar between treatments ($P < 0.01$; SYN 1-4). Urinary nitrogen excretion and nitrogen retention were not affected by the degree of nutrient synchrony. The degree of nutrient synchrony did also not affect nitrogen retention when expressed as percentage of either nitrogen intake or digestible nitrogen intake.

Table 3. Effects of nutrient synchrony on initial body weight, feed intake, daily gain and apparent fecal nutrient digestibility in heavy preruminant calves[1]

| Treatment (SYN) | 1 | 2 | 3 | 4 | 5 | 6 | SYN 1-6 | | | SYN 1-4 | | |
CON[2]	50	57	64	71	78	85	SEM	b[3]	*P*-value[4]	SEM	b	*P*-value
No. of observations	5	5	6	6	6	6	-	-	-	-	-	-
Initial body weight, *kg*	147	147	145	150	143	155	1.8	0.2	0.326	2.0	0.1	0.711
Feed intake, *g/d*	2007	2031	1995	2063	1965	2109	17.8	1.6	0.299	20.5	2.0	0.483
Feed intake, *g/(kg$^{0.75}$·d)*	46.5	47.1	46.7	47.0	46.8	47.0	0.11	0.007	0.445	0.12	0.015	0.324
Daily gain, *g*	1208	1307	1297	1387	941	1133	46.3	-6.0	0.135	39.2	7.2	0.131
Digestibility				%				· 10^{-3}			· 10^{-3}	
Dry matter	95.8	95.6	95.7	95.4	92.1	93.0	0.31	-1.0	<0.001	0.22	0.1	0.706
Protein	94.2	93.2	93.3	93.7	86.2	88.3	0.78	-2.1	<0.001	0.57	-0.2	0.796
Ash	70.0	71.0	73.8	71.5	64.0	71.7	0.98	-0.6	0.464	1.24	1.1	0.510
Fat	95.1	94.2	95.1	93.6	89.7	91.8	0.52	-1.3	0.002	0.44	-0.5	0.356
Lactose	100.0	100.0	100.0	100.0	93.0	78.0	2.73	-5.5	<0.001	-	-	-
Energy	96.3	95.8	96.0	95.6	91.5	92.7	0.38	-1.3	<0.001	0.25	-0.3	0.405

[1] Values are means ± SEM.

[2] CON = Contribution of the high protein meal to the daily protein supply (50% = two identical, balanced meals; 100% = all daily protein in one meal).

[3] Regression coefficient (y = a + b · x), representing the change in response parameter per % increase of the contribution of the high protein meal to the daily protein supply.

[4] Probability for test if the regression coefficient (b) equals 0. Effects of meal sequence and interactions between meal sequence and degree of nutrient synchrony were non-significant and were therefore excluded from the model.

Table 4. Effects of nutrient synchrony on protein metabolism in heavy preruminant calves[1]

Treatment (SYN)	1	2	3	4	5	6	SYN 1-6			SYN 1-4		
CON[2]	50	57	64	71	78	85	SEM	b[3]	P-value[4]	SEM	b	P-value
				$mg/(kg^{0.75} \cdot d)$				$\cdot 10^{-3}$			$\cdot 10^{-3}$	
Nitrogen intake (NI)	1484	1508	1505	1502	1480	1502	4.9	0.1	0.888	3.8	0.6	0.254
Fecal nitrogen	98	107	111	107	201	144	8.3	2.1	0.002	7.2	0.5	0.642
Digestible nitrogen intake (DNI)	1386	1401	1394	1395	1279	1359	9.6	-2.0	0.012	8.1	0.1	0.901
Urinary nitrogen	749	752	693	701	735	713	10.1	-0.8	0.345	14.1	-2.9	0.117
Nitrogen retention	637	648	701	694	544	646	14.3	-1.2	0.335	15.9	3.0	0.149
Efficiency of nitrogen utilization				%								
as percentage of NI	43.0	43.0	46.6	46.2	36.8	43.0	0.95	-0.08	0.312	1.08	0.18	0.200
as percentage of DNI	45.9	46.3	50.3	49.7	42.5	47.5	0.85	-0.02	0.765	1.04	0.21	0.120

[1] Values are means ± SEM, n = 5 (SYN 1-2) or 6 (SYN 3-6).

[2] CON = Contribution of the high protein meal to the daily protein supply (50% = two identical, balanced meals; 100% = all daily protein in one meal).

[3] Regression coefficient (y = a + b · x), representing the change in response parameter per % increase of the contribution of the high protein meal to the daily protein supply.

[4] Probability for test if the regression coefficient (b) equals 0. Effects of meal sequence and interactions between meal sequence and degree of nutrient synchrony were non-significant and were therefore excluded from the model.

Table 5. Effects of nutrient synchrony on energy metabolism in heavy preruminant calves[1]

Treatment (SYN)	1	2	3	4	5	6	SYN 1-6			SYN 1-4		
CON[2]	50	57	64	71	78	85	SEM	b[3]	P-value[4]	SEM	b	P-value
			$kJ/(kg^{0.75} \cdot d)$									
Gross energy intake	985	996	995	994	989	995	2.3	0.15	0.473	2.5	0.29	0.391
Digestible energy intake (DE)	949	955	957	950	907	922	4.6	-1.18	0.002	3.6	0.00	0.998
Urinary energy	45	42	36	32	54	56	3.4	0.39	0.195	3.2	-0.60	0.136
Methane production	2.8	2.6	3.2	2.7	2.2	1.9	0.18	-0.03	0.090	0.25	0.00	0.998
Metabolizable energy intake (ME)	900	910	918	915	849	864	5.9	-1.54	0.001	4.2	0.61	0.265
ME:DE, %	94.9	95.3	95.9	96.2	93.7	93.7	0.37	-0.05	0.157	0.34	0.06	0.137
Total heat production	691	677	666	629	652	631	6.9	-1.66	0.003	8.9	-2.80	0.012
Activity related heat production	72	79	67	61	66	69	2.7	-0.23	0.328	3.4	-0.64	0.151
Activity corrected heat production	619	598	599	567	586	562	5.9	-1.43	0.003	7.8	-2.16	0.024
Total energy retention	209	233	251	286	198	233	8.2	0.12	0.870	10.3	3.41	0.006
Energy retention as protein	94	95	103	102	80	95	2.1	-0.18	0.333	2.3	0.45	0.146
Energy retention as fat	116	138	148	184	117	138	6.7	0.30	0.606	8.7	2.96	0.004
							$\cdot 10^{-3}$	$\cdot 10^{-3}$		$\cdot 10^{-3}$	$\cdot 10^{-3}$	
Respiratory quotient	0.85	0.86	0.87	0.87	0.86	0.84	2.0	-0.02	0.892	2.5	0.71	0.028

[1] Values are means ± SEM, $n = 5$ (SYN 1-2) or 6 (SYN 3-6).

[2] CON = Contribution of the high protein meal to the daily protein supply (50% = two identical, balanced meals; 100% = all daily protein in one meal).

[3] Regression coefficient ($y = a + b \cdot x$), representing the change in response parameter per % increase of the contribution of the high protein meal to the daily protein supply.

[4] Probability for test if the regression coefficient (b) equals 0. Effects of meal sequence and interactions between meal sequence and degree of nutrient synchrony were non-significant and were therefore excluded from the model.

Energy metabolism

When considering treatments SYN 1 to SYN 4, intakes of gross, digestible and metabolizable energy were not affected by the degree of nutrient synchrony (Table 5). Heat production, however, decreased by 62 kJ/(kg$^{0.75}$ · d) from SYN 1 to SYN 4 ($P < 0.01$). Activity related heat production was not affected by nutrient synchrony, but activity corrected heat production decreased by 52 kJ/(kg$^{0.75}$ · d) from SYN 1 to SYN 4 ($P < 0.01$). Energy retention increased gradually from 209 kJ/(kg$^{0.75}$ · d) for SYN 1 to 286 kJ/(kg$^{0.75}$ · d) for SYN 4 ($P < 0.01$). Energy retained as protein was not affected ($P = 0.146$), but energy retained as fat increased ($P < 0.01$) with decreasing nutrient synchrony. The RQ increased slightly ($P < 0.05$) with decreasing nutrient synchrony (SYN 1 to 4).

The circadian rhythm of heat production indicated that the decreased heat production with decreasing nutrient synchrony mainly occurred during the first 3-5 hours after feeding (Figure 2A; $P < 0.05$), after the high protein as well as after the high lactose meal. The activity related heat production was higher for SYN 1 than for SYN 4 at 6 hours after the high lactose meal ($P < 0.05$), but was unaffected by nutrient synchrony during the remainder of the day. The circadian rhythm of the RQ differed substantially between SYN 1 and SYN 4 (Figure 2B). The RQ was higher ($P < 0.05$) for SYN 4 than for SYN 1 during nine hours per day. The RQ was only higher ($P < 0.05$) for SYN 1 than for SYN 4 at 5 hours after the high protein meal. The increase in RQ was considerably stronger after the high lactose than after the high protein meal for SYN 4.

Discussion

General

The high moisture content and decreased pH in feces of calves at the two most asynchronous treatments (SYN 5 and 6) suggested that osmotic diarrhea had occurred in those calves. For an appropriate assessment of effects of nutrient synchrony on nitrogen utilization and energy partitioning, treatments should be compared at similar digestible nutrient intakes. Therefore, this discussion focuses mainly on the effects observed from treatments SYN 1 to SYN 4, with a maximal experimental contrast of 71 and 32% of the daily protein and lactose intakes, respectively in one and the remainder in the other meal (SYN 4).
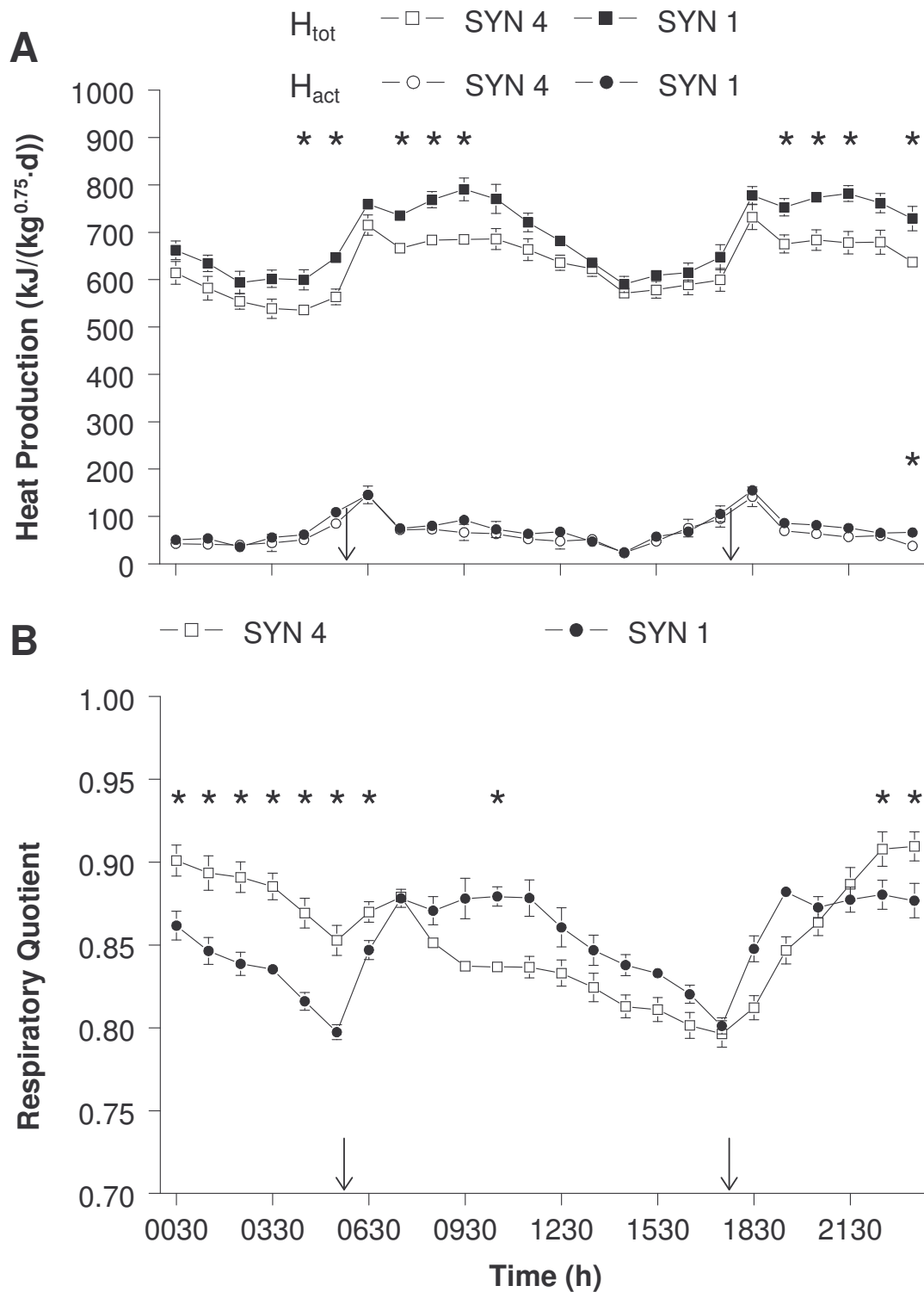
Figure 2. Effects of synchronous (SYN 1; $n = 5$) or asynchronous (SYN 4; $n = 6$) availability of amino acids and glucose in heavy preruminant calves on the circadian rhythms of (A) heat production (H_{tot}) and activity related heat production (H_{act}), and (B) the respiratory quotient. As meal sequence, nor interactions with meal sequence, were significant, data are pooled over meal sequences and presented as the high protein meal in the morning and the high lactose meal in the evening. Stars indicate differences ($P < 0.05$) between treatments. Arrows represent feeding times. Results are expressed as means ± SEM.

Digestibility

Apparent nutrient digestibility was generally high and is in accordance with values in other studies (Gerrits *et al.*, 1996; Diaz *et al.*, 2001). Decreasing nutrient synchrony (SYN 1-4) did not affect nutrient digestibility. When protein and carbohydrate intake were separated further (SYN 5 and 6), nutrient digestibility decreased. Apparent fecal lactose digestibility was incomplete for SYN 5 (93%) and SYN 6 (78%), suggesting the occurrence of osmotic diarrhea. Weijers and Van de Kamer (1965) stated that, in man, a low fecal pH in combination with a low dry matter content usually indicates carbohydrate fermentation, whereas loose feces in combination with an high pH, is related to putrefactive diarrhea (Weijers & Van de Kamer, 1965). In preruminant calves, the relationship between the origin of diarrhea and the associated fecal characteristics seems to be less clear (Roy, 1969; Radostits & Bell, 1970). Nonetheless, an excessive daily lactose intake (> 10 g/(kg BW · d)) in young milk-fed calves resulted in diarrhea and a low fecal pH (Hof, 1980). Daily lactose intake in the present study averaged only 6 g/(kg BW · d) and diarrhea occurred if more than 4 g/kg BW was provided in one of the two daily meals. Therefore, an extreme separation of protein and carbohydrate intake within a day affects digestion and gut health in heavy preruminant calves.

Protein metabolism

In contrast to our hypothesis, nitrogen retention in heavy preruminant calves was not reduced by a decreased nutrient synchrony. The efficiency of digestible nitrogen utilization even increased numerically with decreasing nutrient synchrony: from 45.9% for SYN 1 to 49.7% for SYN 4 ($P = 0.138$). This implies that an asynchronous nutrient availability does not explain the low efficiency of protein utilization in heavy milk-fed calves. Nutrient availability was more asynchronous in the present study than in practical diets containing skimmed milk protein. In the present study, the molar ratio between amino acids and lactose varied from 0.6 in the high lactose meal to 2.7 in the high protein meal for SYN 4. This ratio was probably slightly lower in portal blood, because absorbed amino acids are usually oxidized to a larger extent by intestinal tissues than absorbed glucose (Wu, 1998). The asynchrony was, however, substantially higher than when skimmed milk protein was fed to heavy preruminant calves which resulted in a molar ratio of amino acids relative to glucose of 1.8 (Verdonk et al., 1999).

The lack of response of nitrogen retention to an increased separation of protein and carbohydrate intake in time is in contrast with effects in growing pigs. In pigs, a virtually

complete separation of protein and carbohydrate intake over two daily meals substantially decreased the efficiency of nitrogen utilization (JJGC van den Borne, JW Schrama, MJW Heetkamp, MWA Verstegen, and WJJ Gerrits, unpublished results). Similar effects were found in growing rats (Geiger, 1948) and growing lambs (using volatile fatty acids instead of carbohydrate) (Randles, 2001). A partial separation of protein and carbohydrate intake decreased protein utilization in growing boys (Barja et al., 1972), but not in growing pigs (Eggert *et al.*, 1953; Yeo & Chamberlain, 1966). It can therefore be concluded that protein metabolism in preruminant calves responded differently to a decreased nutrient synchrony compared with most other studies in the literature. Several reasons for this discrepancy can be suggested.

First, the response of protein retention to an asynchronous nutrient availability can be non-linear and thus become evident only when protein and carbohydrate intake are more separated. The degree of nutrient synchrony decreased stepwise, but did not ultimately result in a complete separation of the protein and carbohydrate intake in calves. The presence of lactose in the high protein diet may, to some extent, have prevented amino acids from being oxidized to meet the energy requirements for maintenance. Lactose in the high protein diet may also have spared amino acid utilization for gluconeogenesis.

Second, insulin resistance can explain the absence of an increase in protein retention with increasing nutrient synchrony in heavy preruminant calves. Insulin sensitivity in preruminant calves decreases markedly towards the end of the fattening period (Doppenberg & Palmquist, 1991; Hugi *et al.*, 1997) and possibly results in glucosuria. Substantial amounts of glucose were detected in urine of calves in the current study (data not shown), indicating insulin resistance. In insulin-sensitive animals, however, insulin (strongly induced by glucose availability) stimulates protein synthesis, provided that amino acids are present. The effects of insulin and amino acids in stimulating protein synthesis are synergistic and protein synthesis in muscle is unchanged if only glucose is supplied (Volpi et al., 2000). Thus, it would be expected that a synchronous supply of amino acids and glucose would increase the insulin-mediated amino acid utilization. Due to insulin resistance, this increased utilization with increasing nutrient synchrony may be absent in preruminant calves. Alternatively, it can be speculated that protein retention was increased with decreasing nutrient synchrony. A higher increase of plasma free amino acid levels (i.e. after the high protein meal) may be required to stimulate muscle protein synthesis in insulin-resistant animals. In elderly women (Arnal et al., 1999) and rats (Arnal et al., 2002), which often have a decreased insulin sensitivity (Fukagawa *et al.*, 1988; Arnal *et al.*, 2000), protein utilization increased when the majority of

the daily protein intake was consumed in one meal (i.e. separated from carbohydrate intake) compared with distribution over four meals.

Third, fatty acid oxidation has probably contributed significantly to the energy requirements for maintenance. The fat content in milk replacer diets is generally high (~20%) and fatty acid oxidation can have limited oxidation of amino acids to provide ATP for maintenance processes after the high protein meal. The source of non-protein energy (fat or carbohydrates) does probably not affect protein retention in preruminant calves (Tikofsky et al., 2001).

In summary, nutrient synchrony in heavy preruminant calves did not increase protein retention as hypothesized and as observed in other species. The incomplete separation of protein and carbohydrate, insulin resistance and a high dietary fat content may explain the lack of effect in this study.

Energy metabolism

Metabolizable energy intake increased numerically by 15 kJ/(kg$^{0.75}$ · d) and H$_{tot}$ decreased by 62 kJ/(kg$^{0.75}$ · d) ($P < 0.05$) when nutrient synchrony decreased from SYN 1 to SYN 4. As a result, energy retention increased by 77 kJ/(kg$^{0.75}$ · d) with decreasing degree of nutrient synchrony (SYN 1-4). Protein and fat retention contributed for respectively 11 and 89% to the total increase in energy retention.

The increased fat deposition rate in asynchronously fed calves is expected to originate from an increased incorporation of dietary fatty acids in adipose tissue rather than from an increased *de novo* fatty acid synthesis. Synthesis of fatty acids from glucose is probably less important in preruminant calves than in pigs, because the RQ did not exceed 0.91 within the day for calves at SYN 4, whereas the RQ exceeded 1 (viz. indication of net fatty acid synthesis) for several hours per day in growing pigs after a high starch diet (Charlet-Lery & Morel, 1977). Furthermore, there is evidence that preruminant calves may not even be capable of *de novo* fatty acid synthesis from glucose. Potential ruminants have, for example, low activities of citrate lyase and NADP-malate dehydrogenase (Hanson & Ballard, 1967). A reduced *de novo* fatty acid synthesis is consistent with the observation that daily fat intake exceeded daily fat deposition by two-fold, allowing the possibility that fat deposition completely originated from dietary fat. Within the present study, RQ increased ($P < 0.05$) from SYN 1 to 4 which also indicates a higher contribution of glucose relative to fatty acid oxidation, leaving more fat to be deposited. The low variability in RQ within a day (Figure 2B) possibly originates from the high dietary fat content, but also the previously mentioned

peculiarities of glucose metabolism (i.e. insulin resistance and low *de novo* fatty acid synthesis) may contribute to a lack of flexibility to select substrates for oxidation.

Noticeably, the increased fat retention seems not to be related to one particular meal in asynchronously fed calves, as the reduced H_{tot} with increasing nutrient asynchrony occurred after both meals (Figure 2A). The increase in RQ after ingestion of both daily meals was steeper for SYN 1 than for SYN 4 (Figure 2B). This was expected for the high protein meal, but not for the high lactose meal. Differences in the kinetics of digestive processes (gastric emptying, digestion and absorption) or in post-absorptive kinetics of metabolic processes (release of nutrients into the portal vein, intracellular nutrient uptake and oxidation) may be involved. More detailed studies about quantitative interactions between glucose and fat metabolism in preruminant calves may explain the mechanism of an increased fat deposition.

In conclusion, an extreme decrease in nutrient synchrony in preruminant calves was shown to cause diarrhea and to reduce nutrient digestibility. Supplying 71 and 32% of the daily protein and lactose intakes respectively in one, and the remainder in the other meal could be realized without negatively affecting nutrient digestibility. Separating protein and carbohydrate intake within a day to this extent did not reduce the efficiency of digestible nitrogen utilization for protein retention in heavy preruminant calves. Remarkably, heat production decreased and fat retention increased substantially with a decreasing degree of nutrient synchrony. With the large experimental contrast realized in this study, it is unlikely that an asynchronous nutrient availability contributes to the low efficiency of protein utilization often observed in heavy preruminant calves. The findings from this study may be used in devising new feeding strategies for preruminant calves when increased rates of fat deposition are desired.

Acknowledgements

References

Arnal MA, Mosoni L, Boirie Y, Gachon P, Genest M, Bayle G, Grizard J, Arnal M, Antoine JM, Beaufrère B & Patureau Mirand P (2000) Protein turnover modifications induced by the protein feeding pattern still persist after the end of the diets. *Am J Physiol* **278**, E902-E909.

Arnal MA, Mosoni L, Boirie Y, Houlier ML, Morin L, Verdier E, Ritz P, Antoine JM, Prugnaud J, Beaufrère B & Mirand PP (1999) Protein pulse feeding improves protein retention in elderly women. *Am J Clin Nutr* **69**, 1202-1208.

Arnal MA, Mosoni L, Dardevet D, Ribeyre MC, Bayle G, Prugnaud J & Patureau-Mirand P (2002) Pulse protein feeding pattern restores stimulation of muscle protein synthesis during the feeding period in old rats. *J Nutr* **132**, 1002-1008.

Barja I, Araya H, Munoz P, Vega L, Arteaga A & Tagle MA (1972) Effect of spacing protein intake on nitrogen balance in normal children. *Am J Clin Nutr* **25**, 506-511.

Brouwer E (1965) Report of sub-committee on constants and factors. *In: Energy Metabolism*, [KL Blaxter, editor]. London, UK. Academic Press, pp. 441-443.

Charlet-Lery G & Morel MT (1977) Influence of diet on respiratory quotients and fat deposition in growing pigs. *Ann Biol Anim Bioch Biophys* **17**, 897-904.

Diaz MC, Van Amburgh ME, Smith JM, Kelsey JM & Hutten EL (2001) Composition of growth of Holstein calves fed milk replacer from birth to 105-kilogram body weight. *J Dairy Sci* **84**, 830-842.

Doppenberg J & Palmquist DL (1991) Effect of dietary fat level on feed intake, growth, plasma metabolites and hormones of calves fed dry or liquid diets. *Livest Prod Sci* **29**, 151-158.

Eggert RG, Brinegar MJ & Anderson CR (1953) Delayed protein supplementation of corn diets for growing swine. *J Nutr* **50**, 469-477.

Fukagawa NK, Minaker KL, Rowe JW, Matthews DE, Bier DM & Young VR (1988) Glucose and amino acid metabolism in aging man: differential effects of insulin. *Metabolism* **37**, 371-377.

Gahl MJ, Finke MD, Crenshaw TD & Benevenga NJ (1996) Efficiency of lysine or threonine retention in growing rats fed diets limiting in either lysine or threonine. *J Nutr* **126**, 3090-3099.

Geiger E (1948) The importance of the time element in feeding of growing rats. Experiments with delayed supplementation of protein. *Science* **108**, 42-43.

Gerrits WJJ, Dijkstra J, Verdonk JMAJ, Beelen GM & Boer H (1999) Effects of ammonia and starch infusion in the colon of preruminant calves. *In: The VIIIth International Symposium on Protein Metabolism and Nutrition*, [GE Lobley, A White and JC MacRae, editors]. Aberdeen, UK. pp. 55 (Abstr).

Gerrits WJJ, Schrama JW & Tamminga S (1998) The marginal efficiency of utilization of all ileal digestible indispensible amino acids for protein gain is lower than 30% in preruminant calves between 80 and 240 kg live weight. *J Nutr* **128**, 1774-1785.

Gerrits WJJ, Tolman GH, Schrama JW, Tamminga S, Bosch MW & Verstegen MWA (1996) Effect of protein and protein-free energy intake on protein and fat deposition rates in preruminant calves of 80 to 240 kg live weight. *J Anim Sci* **74**, 2129-2139.

Hanson RW & Ballard FJ (1967) The relative significance of acetate and glucose as precursors for lipid synthesis in liver and adipose tissue from ruminants. *Biochem J* **105**, 529-536.

Hof G (1980) An investigation into the extent to which various dietary components, particularly lactose, are related to the incidence of diarrhoea in milk-fed calves, PhD thesis, Wageningen Agricultural University, The Netherlands.

Hugi D, Bruckmaier RM & Blum JW (1997) Insulin resistance, hyperglycemia, glucosuria, and galactosuria in intensively milk-fed calves: dependency on age and effects of high lactose intake. *J Anim Sci* **75**, 469-482.

ISO (1997) Animal feeding stuffs. Determination of nitrogen content and calculation of crude protein content. Kjeldahl method. ISO 5983. International Organization for Standardization.

ISO (1998a) Animal feeding stuffs, animal products, and faeces or urine. Determination of gross calorific value. ISO 9831. International Organization for Standardization.

ISO (1998b) Animal feeding stuffs. Determination of moisture and other volatile matter content. ISO 6496. International Organization for Standardization.

ISO (1999) Animal feeding stuffs. Determination of fat content. ISO 6492. International Organization for Standardization.

ISO (2002) Animal feeding stuffs. Determination of crude ash. ISO 5984. International Organization for Standardization.

Mnilk B, Harris CI & Fuller MF (1996) Lysine utilization by growing pigs: simultaneous measurement of protein accretion and lysine oxidation. *Br J Nutr* **75**, 57-67.

Radostits OM & Bell JM (1970) Nutrition of the pre-ruminant dairy calf with special reference to the digestion and absorption of nutrients: a review. *Can J Anim Sci* **50**, 405-452.

Randles WG (2001) The effects of different temporal patterns of post-ruminal energy and protein supply on nitrogen metabolism in growing lambs, PhD thesis, University of Aberdeen, UK.

Reeds PJ, Burrin DG, Davis TA, Fiorotto ML, Stoll B & Van Goudoever JB (2000) Protein nutrition of the neonate. *Proc Nutr Soc* **59**, 87-97.

Rijnen MMJA, Verstegen MWA, Heetkamp MJW & Schrama JW (2003) Effects of two different dietary fermentable carbohydrates on activity and heat production in group-housed growing pigs. *J Anim Sci* **81**, 1210-1219.

Roy JHB (1969) Diarrhoea of nutritional origin. *Proc Nutr Soc* **28**, 160-170.

Schrama JW, Noordhuizen JPTM, Arieli A, Brandsma HA, Van der Linden JM & Verstegen MWA (1994) Circadian fluctuation in heat production of young calves at different ambient temperatures in relation to posture. *J Anim Sci* **72**, 598-605.

Tikofsky JN, Van Amburgh ME & Ross DA (2001) Effect of varying carbohydrate and fat content of milk replacer on body composition of Holstein bull calves. *J Anim Sci* **79**, 2260-2267.

Van den Borne JJGC, Verdonk JMAJ, Schrama JW & Gerrits WJJ (2006a) Reviewing the low efficiency of protein utilization in heavy preruminant calves – a reductionist approach. *Reprod Nutr Dev* **46**, 121-137.

Van den Borne JJGC, Verstegen MWA, Alferink SJJ, Giebels RMM & Gerrits WJJ (2006b) Effects of feeding frequency and feeding level on nutrient utilization in heavy preruminant calves. *J Dairy Sci* **89**, 3578-3586.

Van Es AJH, Nijkamp HJ, Van Weerden EJ & Van Hellemond KK (1967) Energy, carbon and nitrogen balance experiments with veal calves. *In: Energy metabolism of farm animals, Newcastle-upon-Tyne, UK*. [KL Blaxter, J Kielanowski and G Thorbek, editors]. Oriel Press, pp. 197-201.

Verdonk JMAJ, Gerrits WJJ, Beelen GM & Jansman AJM (1999) Effect of protein source on portal nutrient fluxes in pre-ruminant calves. *In: The VIIIth International Symposium on Protein Metabolism and Nutrition, Aberdeen, UK*. [GE Lobley, A White and JC MacRae, editors]. Wageningen Pers, The Netherlands, pp. 47 (Abstr.).

Verstegen MWA, Van der Hel W, Brandsma H, Henken AM & Bransen AM (1987) The Wageningen respiration unit for animal production research: a description of the equipment and its possibilities. *In: Energy Metabolism in Farm Animals: Effects of Housing, Stress and Disease*, [MWA Verstegen and AM Henken, editors]. Dordrecht, The Netherlands. Martinus Nijhoff Publishers, pp. 21-48.

Volpi E, Mittendorfer B, Rasmussen BB & Wolffe RR (2000) The response of muscle protein anabolism to combined hyperaminoacidemia and glucose-induces hyperinsulinemia is impaired in the eldery. *J Clin Endocrinol Metab* **85**, 4481-4490.

Weijers HA & Van de Kamer JH (1965) Alteration of intestinal bacterial flora as a cause of diarrhoea. *Nutr Abstr Rev* **35**, 591-604.

Wenk C & Van Es AJH (1976) Eine Methode zur Bestimmung des Energieauswandes für die körperlich Aktivität von wachsenden Küken. *Schweiz Landwirtsch Monatsh* **54**, 232.

Wu G (1998) Intestinal mucosal amino acid catabolism. *J Nutr* **128**, 1249-1252.

Yeo ML & Chamberlain AG (1966) Delayed protein supplementation of barley diets for weanling pigs. *Proc Nutr Soc* **25**, 41 (Abstr.).

Chapter 7

Whole body and muscle energy metabolism in preruminant calves: effects of nutrient synchrony and physical activity

JJGC van den Borne[1], JF Hocquette[2], MWA Verstegen[1]
& WJJ Gerrits[1]

[1]*Animal Nutrition Group, Department of Animal Sciences, Wageningen University, P.O. Box 338, 6700 AH Wageningen, The Netherlands*

[2]*Unité de Recherches sur les Herbivores, INRA, Clermont-Ferrand/Theix, 63122 Saint-Genès-Champanelle, France*

Abstract

The effects of asynchronous availability of amino acids and glucose on muscle composition and enzyme activities in skeletal muscle were studied in preruminant calves. It was hypothesized that decreased oxidative enzyme activities in muscle would explain a decreased whole body heat production with decreasing nutrient synchrony. Preruminant calves were assigned to one of six degrees of nutrient synchrony, step-wise separating the intake of protein and lactose over the two daily meals. Calves at the most synchronous treatment received two identical meals daily. At the most asynchronous treatment, 85% of the daily protein and 20% of the daily lactose supply was fed in one meal and the remainder in the other meal. Daily intakes of all dietary ingredients were identical for all treatments. Oxidative enzyme activities and fat content increased with decreasing nutrient synchrony in *M. Rectus Abdominis* (RA), but not in *M. Semitendinosus*. Cytochrome-c-oxidase activity was positively correlated with fat content in RA ($r=0.49$; $P<0.01$). Oxidative enzyme activities in both muscles were not correlated with average daily heat production, but citrate synthase activity in RA was positively correlated ($P<0.01$) with the circadian amplitude ($r=0.53$) and maximum ($r=0.61$) of heat production related to physical activity. In conclusion, intramuscular fat and oxidative enzyme activities increased with decreasing nutrient synchrony in RA. Muscle oxidative enzyme activities were not correlated with average daily heat production, but citrate synthase activity in RA was positively correlated with within-day variation in activity related heat production.

Abbreviations: ADG, average daily gain; BW, body weight; COX, cytochrome-*c*-oxidase; CS, citrate synthase; H_{act}, physical activity related heat production; H_{cor}, heat production corrected for physical activity; H_{tot}, total heat production; LDH, lactate dehydrogenase; ME_m, metabolizable energy requirements for maintenance; RA, *M. Rectus Abdominis*; RQ, respiratory quotient; SEQ, meal sequence; ST, *M. Semitendinosus*; SYN, degree of nutrient synchrony.

Introduction

Daily nutrient intake has been correlated with muscle enzyme activities in animals (Cassar-Malek et al., 2004) and man (Helge & Kiens, 1997), and with intramuscular fat and glycogen contents in animals (Pethick & Rowe, 1996; Gondret *et al.*, 2000). Regulation of muscle oxidative enzyme activities and muscle composition also depends on the supply of individual macronutrients (Hocquette *et al.*, 1998; Geelen *et al.*, 2001; Gondret & Lebret, 2002). Apart from variation in daily nutrient supply, within-day variation in the supply of different nutrients (i.e. synchrony) may induce these effects. Asynchronous absorption patterns can be induced by either a separated intake of protein and carbohydrates in time (e.g. dissociated diets) or by supplying ingredients with different kinetics of digestion and absorption. In preruminants, for example, asynchronous absorption of glucose and amino acids may result from differences in passage behaviour of clotting vs. non-clotting dietary ingredients (Guilloteau et al., 1986; Verdonk et al., 1999). Applying a theoretical approach, we separated the intake of glucose and amino acids within the day (i.e. across meals) in heavy preruminant calves (Van den Borne *et al.*, 2006b). Surprisingly, and in contrast to similar investigations in pigs (JJGC van den Borne, JW Schrama, MJW Heetkamp, MWA Verstegen & WJJ Gerrits, unpublished), increased separation of the amino acid and glucose availability within a day decreased whole body heat production (-9%), and increased whole body fat deposition (+59%), in heavy preruminant calves (Van den Borne *et al.*, 2006b). Muscle energy metabolism may contribute to these changes as it accounts for about 20% of the daily heat production in growing farm animals (Ortigues et al., 1995). Effects of nutrient synchrony on muscle energy metabolism and composition may have implications for muscle function, meat quality and development of metabolic disorders in calves.

The regulation of muscle energy metabolism is well documented (e.g. Hocquette et al., 1998). In non-growing man, oxidative enzyme activities in muscle are positively correlated with whole body heat production (Zurlo *et al.*, 1994; Doucet *et al.*, 2003). It is not known if a similar relationship exists in growing animals and if increased oxidative enzyme activities in muscle are associated with increased whole body heat production. In addition, it is unknown to what extent the demand for energy in muscle, i.e. for physical activity, drives the activity of the enzymes and hence regulates muscle energy metabolism in farm animals.

The aim of this study was therefore to gain insight in the regulation of whole body heat production by assessing the effects of nutrient synchrony and physical activity on muscle enzyme activities and muscle composition in heavy preruminant calves. Results on whole

body protein and energy utilization have been presented elsewhere (Van den Borne *et al.*, 2006b). It is hypothesized that oxidative enzyme activities decrease and intramuscular fat content increases with decreasing nutrient synchrony in preruminant calves. Oxidative enzyme activities in skeletal muscle were expected to correlate with whole body heat production.

Materials and methods

Animals, housing and experimental treatments

Thirty-six male, preruminant calves of the Holstein Friesian breed were used. The experimental treatments consisted of six degrees of nutrient synchrony (SYN 1 to 6) at each of two meal sequences (SEQ A and B) in a 6×2 factorial arrangement. The experiment was originally designed to study effects of SYN and SEQ on nutrient utilization (Van den Borne *et al.*, 2006b). Two identical respiration chambers were available and calves were studied in 18 trials of two calves each. Individual calves of approximately 130 kg body weight (BW) were assigned to an experimental treatment. The degree of nutrient synchrony was varied by a step-wise exchange of protein and lactose between the two daily meals (on a calculated digestible energy basis) which resulted in an equal distribution of digestible energy intake within a day (Table 1). Calves at SYN 1 received two identical meals daily. Calves at the most asynchronous treatment, SYN 6, received 85% of the daily protein supply in one meal and the remaining 15% in the other meal. A high protein meal was fed in the morning and a high lactose meal was fed in the evening for calves at SEQ A. For calves at SEQ B, this sequence was reversed. Allocation of calves to SYN and SEQ was balanced in time across trials.

Calves were fed the experimental diets for five weeks. During the first four weeks of study, calves were individually housed in cages (1.85×0.75 m) in a climate-controlled stable. Then they were moved to the respiration chambers where whole body heat production was measured during one week. The experiment complied with the Dutch law regarding the use of experimental animals.

Diets and feeding

Calves were fed according to their metabolic BW ($BW^{0.75}$), adjusted daily for a projected daily gain of 1000 g. Metabolizable energy requirements for maintenance (ME_m) were assumed to be 460 kJ/d per kg $BW^{0.75}$ (Van Es *et al.*, 1967; Gerrits *et al.*, 1996), and the feeding level was $2.0 \times ME_m$. Two basal milk replacers were produced for the most asynchronous treatment (SYN 6). All other experimental treatments were realized by mixing

the basal milk replacers in the appropriate ratio to create the experimental contrast presented in Table 1. The detailed nutrient and ingredient composition of the basal milk replacers are given by Van den Borne *et al.* (2006b). Briefly, diets included whey protein concentrate, lactose and coconut and soy oil as main ingredients. Diets provided on a daily basis 20.5% crude protein, 20.8% crude fat and 44.9% lactose. Daily intakes of all nutrients and dietary ingredients were similar between treatments. Milk replacer was reconstituted with water (140 g/L) and supplied at a temperature of about 40°C in a bucket. Roughage was not provided. Calves were fed individually at 06.00 h and 18.00 h, and were allowed 15 min to consume the meal. Feed intake was measured twice daily and BW was measured weekly throughout the experiment. Average daily gain and feed conversion ratio were calculated over the 5-week experimental period.

Table 1. Experimental treatments; division of the nutrient intake over two daily meals

Treatment (SYN)	1	2	3	4	5	6
CON	50	57	64	71	78	85
Morning meal* (06.00 h)			*g/kg BW$^{0.75}$*			
Protein	4.6	5.3	5.9	6.5	7.1	7.7
Lactose	10.0	8.9	7.8	6.7	5.6	4.5
Fat	4.6	4.6	4.6	4.6	4.6	4.6
Evening meal (18.00 h)			*g/kg BW$^{0.75}$*			
Protein	4.6	4.0	3.4	2.8	2.2	1.6
Lactose	10.0	11.1	12.2	13.4	14.5	15.6
Fat	4.6	4.5	4.5	4.5	4.5	4.5

CON, contribution of the high protein meal to the daily protein supply (50%=two identical, balanced meals; 100%=all daily protein in one meal).

* Treatments are presented for meal sequence A. For meal sequence B, the nutrient intake in the morning and evening meal was reversed.

Measurement of whole body heat production and physical activity

During the fifth week of the study, gas exchange (O_2, CO_2 and CH_4) was continuously measured during seven days in 6-min intervals by indirect calorimetry for estimation of whole body heat production. From the gaseous exchange, heat production (H_{tot}) was calculated according to the formula of Brouwer (1965) and the respiratory quotient (RQ) was calculated as the CO_2 production divided by the O_2 consumption. Posture of calves was measured every minute by infrared beam interruption. Physical activity was recorded with a radar-Doppler device according to the method described by Wenk and Van Es (1976). From these

measurements, heat production related to physical activity (H_{act}) and heat production corrected for physical activity (H_{cor}) were separately estimated (described by Van den Borne *et al.*, 2006a). The kinetics of whole body heat production were described by calculation of the hourly means of H_{tot}, H_{cor} and H_{act} for each calf. The circadian maximum and minimum were defined for each calf as the highest and lowest hourly mean within a day, respectively. The amplitudes of H_{tot}, H_{cor} and H_{act} were subsequently calculated as the difference between the circadian maximum and minimum for each calf. For calculating circadian means of heat production by the formula of Brouwer (1965), the nitrogen excretion was assumed to be constant throughout the day.

Measurement of muscle composition and enzyme activities

After five weeks on the experimental treatment, calves were transported to the slaughterhouse (20 min) and killed at 13.00 h (i.e. 7 h after the morning meal) by stunning and exsanguination. Within 15 min post-slaughtering, samples of the *M. Rectus Abdominis* (RA; oxido-glycolytic muscle) and *M. Semitendinosus* (ST; glycolytic muscle) were removed. The samples of skeletal muscle were immediately trimmed of visible fat and connective tissue. Samples were then cut into pieces, immediately frozen in liquid nitrogen and stored at -80ºC pending analyses. Samples for crude fat analysis (about 30 g for each muscle) were not frozen in liquid nitrogen, but ice-chilled and subsequently stored at -20ºC pending analyses.

Protein (Bradford, 1976) and DNA (Labarca & Paigen, 1980) contents were measured in muscle homogenates. Activity of the oxidative enzyme citrate synthase (CS; EC 4.1.3.7), which is involved in the substrate flux through the tricarboxylic acid cycle, was determined in sonicated homogenates by measuring the rate of initial reaction at 412 nm by means of the DTNB [5.5'-dithiobis(2-nitrobenzoate)] method as described by Shepherd and Garland (1969). Activity of the oxidative enzyme cytochrome-*c*-oxidase (COX; EC 1.9.3.1), which is involved in the substrate flux through the respiratory chain, was determined in sonicated homogenates by measuring the oxidation of reduced cytochrome-*c* as described by Van Hinsberg *et al.* (1978). Activities of CS and COX were expressed in μmol of coenzyme A liberated per min per g protein and in μmol of cytochrome-*c* oxidized per min per g protein at 25ºC respectively. Activity of the glycolytic enzyme lactate dehydrogenase (LDH; EC 1.1.1.2.7), which catalyzes the formation of lactate from pyruvate, was determined as described by Ansay (1974). Lactate dehydrogenase activity was expressed in μmol of NADH oxidized per min per g protein. Glycogen content was measured by the method of Carroll *et al.* (1955). Briefly, glycogen was extracted from the tissue by homogenization with 5%

trichloroacetic acid solution, precipitated from the extract by 95% ethanol and determined with the anthrone reagent in a colorimeter at 620 nm. Fat content was measured in muscle tissue after freeze-drying according to ISO 6492 (1999). Ratios for CS:LDH, COX:LDH (relative enzyme activities), protein:DNA (indicator for muscle fibre size) and COX:CS (indicator for the biochemical properties of the mitochondria) were calculated.

Statistical analysis

The effects of nutrient synchrony and the interaction between nutrient synchrony and meal sequence on muscle traits and whole body energy metabolism traits were analyzed by linear regression analysis, using the GLM procedure of the Statistical Analysis Systems statistical software package version 9.1 (SAS Institute, Cary, NC, USA). The degree of nutrient synchrony was included as a regressor (Eq. [1]).

$$Y_{ij} = \mu + \beta_1 \times X_j + \beta_{2i} \times S_i X_j + \varepsilon_{ij} \qquad [1]$$

where Y_{ij} = dependent variable over the whole period, μ = average intercept, β_1 = effect of degree of nutrient synchrony, expressed as percentage of the daily protein intake in the morning meal, β_{2i} = interaction between degree of nutrient synchrony and meal sequence i, S_i = fixed effect of meal sequence i, X_j = degree of nutrient synchrony (expressed as percentage of the daily protein intake in the high protein meal) for calf j, ε_{ij} = error term, i = 1, 2, and j = 1…18.

Pearson correlation coefficients were calculated for relationships between muscle energy metabolism traits within each muscle, for each trait between the two muscles and between muscle energy metabolism traits and whole body energy metabolism traits. Traits for the two muscles were compared by pair-wise comparisons.

Results

Animal performance

Two calves were excluded from analysis, because of feed refusals or illness. The initial BW of the calves was 128 (SEM 1.7) kg and ADG during the 5-wk experimental period was 885 (SEM 27.1) g. Average daily gain was lower (*P*<0.05) for calves at SYN 5 and 6 (729 and 815 g respectively) than for calves at SYN 1-4. Calves at SYN 1-4 had a similar daily gain

(on average 945 g). Feed intake did not differ between treatments and consequently the feed to gain ratio was higher ($P<0.01$) for calves at SYN 5 and 6 (2.56 and 2.48 respectively) than for calves at the other treatments (on average 2.02). However, calves at the most asynchronous treatments had a lower nutrient digestibility. The consequently lower digestible nutrient intake in calves at SYN 5 and 6 complicated interpretation of the results from SYN 1 to 6. Linear regression was therefore also performed for SYN 1 to 4 (n=22) separately, because calves at those treatments had identical digestible nutrient intakes

Muscle enzyme activities

In RA, activities of COX and CS respectively increased ($P=0.048$) and tended to increase ($P=0.075$), respectively, with decreasing nutrient synchrony (SYN 1-4; Table 2). Ratios of enzyme activities were not affected by nutrient synchrony. In ST, enzyme activities were not affected by nutrient synchrony (Table 3). The sequence of the high protein and high carbohydrate meals did not affect enzyme activities.

Muscle composition

Intramuscular fat content increased in RA ($P=0.037$) with decreasing nutrient synchrony at an identical digestible nutrient intake (Table 2), but nutrient synchrony did not affect intramuscular fat content in ST (Table 3). The fat content was substantially higher ($P<0.001$) in RA (18.9 mg/g tissue) than in ST (6.5 mg/g tissue). The sequence of the high protein and high carbohydrate meals did not affect intramuscular fat content.

Glycogen content in both muscles was not affected by nutrient synchrony (Tables 2 and 3), but a positive correlation between glycogen content in RA and ST was found (r=0.72; $P<0.001$). Glycogen content averaged 6.24 mg/g in RA and 7.01 mg/g in ST. An interaction between SEQ and SYN was found for intramuscular glycogen content in both muscles ($P<0.05$), indicating that glycogen content was higher after feeding the high protein diet than after the high lactose diet. The difference in glycogen content between meal sequences was more pronounced at SYN 2 (3.08 mg/g in RA and 6.08 mg/g in ST) and SYN 3 (4.63 mg/g in RA and 4.96 mg/g in ST) than at the other degrees of nutrient synchrony.

Whole body heat production

Average heat production and H_{cor} decreased with decreasing nutrient synchrony ($P<0.05$), while the RQ increased ($P=0.028$) (Table 4). A detailed description of whole body energy partitioning and protein deposition is presented and discussed elsewhere (Van den Borne *et*

al., 2006b). The maxima of the circadian patterns of H_{tot} and H_{cor} decreased with decreasing nutrient synchrony ($P<0.05$), but the maximum of the circadian pattern of H_{act} was not affected by nutrient synchrony. The amplitudes of the circadian patterns of H_{tot}, H_{cor} and H_{act} were not affected by the degree of nutrient synchrony. The sequence of the high protein and high carbohydrate meals did not affect any of the traits of whole body energy metabolism.

Correlations within muscle

In both muscles, CS and COX activities were positively correlated (r=0.35-0.36; *P*<0.05). Intramuscular fat content showed a positive correlation (r=0.49; *P*<0.01) with COX (and COX:LDH) in RA (data not shown).

Correlations between muscle energy metabolism and whole body energy metabolism

The decreased H_{tot} and H_{cor} with decreasing nutrient synchrony did not correspond with the increased (or unaffected) oxidative enzyme activities in muscle. In accordance, simple correlation analysis showed that average H_{tot}, H_{cor} and H_{act} were not correlated with enzyme activities in RA (Table 5) and ST (data not shown). Intramuscular fat content in RA was negatively correlated with average H_{tot} and H_{cor}.

The maximum of H_{act} and the amplitude of H_{tot} and H_{act} increased with increasing CS activity in RA in preruminant calves, resulting in positive correlations (Table 5). In ST, oxidative enzyme activities were not related to the maxima and amplitudes of heat production traits (data not shown).

Discussion

Animal performance

Feeding more than 71% of the daily protein in one meal and more than 68% of the daily lactose in the other one (i.e. SYN 5-6) induced diarrhoea, which was indicated by a lower faecal dry matter content, a lower faecal pH and a lower nutrient digestibility (Van den Borne *et al.*, 2006b). The lower daily gain and higher feed conversion ratio for calves at SYN 5 and 6 were therefore likely due to a decreased digestible nutrient intake. Comparison of calves at SYN 1-4 allows studying the effects of nutrient synchrony at identical intakes of digestible nutrients. Therefore, this discussion focuses on treatments SYN 1-4.

Table 2. Effects of nutrient synchrony on muscle enzyme activities and muscle composition in *M. Rectus Abdominis* of heavy preruminant calves (mean values for treatments with their standard errors; n=5 per treatment for SYN 1 and 2, and n=6 per treatment for SYN 3-6)

Treatment (SYN)	1	2	3	4	5	6	CON 50-85			CON 50-71		
CON	50	57	64	71	78	85	SEM	b*	*P*-value†	SEM	b	*P*-value
Enzyme activity, μmol/g protein per min												
Lactate dehydrogenase (LDH)	4317	4269	4502	4447	4358	4076	286.2	$-4.5 \cdot 10^{-3}$	0.673	344.4	$8.7 \cdot 10^{-3}$	0.710
Cytochrome-*c*-oxidase (COX)	119	97	119	154	121	99	13.7	$0.2 \cdot 10^{-4}$	0.960	14.1	$2.0 \cdot 10^{-3}$	0.048
Citrate synthase (CS)	54	52	57	67	58	51	5.3	$0.4 \cdot 10^{-4}$	0.850	5.0	$0.7 \cdot 10^{-3}$	0.075
Relative enzyme activity												
COX:CS	2.22	1.87	2.16	2.70	2.33	2.00	0.321	$2.3 \cdot 10^{-3}$	0.849	0.299	0.01	0.499
COX:LDH, $\cdot 10^{-3}$	27.8	24.6	27.7	35.3	28.4	24.2	3.92	$2.1 \cdot 10^{-6}$	0.989	4.32	$0.4 \cdot 10^{-3}$	0.191
CS:LDH, $\cdot 10^{-3}$	12.4	12.8	12.9	15.9	13.8	12.3	1.53	$0.2 \cdot 10^{-4}$	0.728	1.48	$0.2 \cdot 10^{-3}$	0.180
Muscle composition												
Glycogen, mg/g fresh tissue‡	5.9	7.6	6.2	6.0	5.5	6.1	1.14	-0.02	0.630	1.25	$-9.0 \cdot 10^{-3}$	0.907
Fat, mg/g fresh tissue	16.3	13.5	21.9	23.9	16.3	13.7	2.46	-0.03	0.802	2.43	0.44	0.037
Protein, mg/g fresh tissue	165	175	170	158	162	172	7.0	-0.07	0.764	7.7	-0.45	0.391
DNA, μg/g fresh tissue	1881	1972	2017	1996	1977	1989	55.1	1.95	0.309	47.9	5.75	0.088
Protein:DNA, mg/mg	88.1	88.8	84.8	79.4	82.3	87.0	4.39	$-0.1 \cdot 10^{-3}$	0.435	4.92	$-0.5 \cdot 10^{-3}$	0.140

CON, contribution of the high protein meal to the daily protein supply (50%=two identical, balanced meals; 100%=all daily protein in one meal)

* Regression coefficient (y = a + b · x), representing the change in response parameter per % increase of the contribution of the high protein meal to the daily protein supply.

† Probability for test if the regression coefficient (b) equals 0. Effects of meal sequence and interactions between meal sequence and degree of nutrient synchrony were non-significant and were therefore excluded from the model.

‡ An interaction between CON and the meal sequence on muscle glycogen content was found (*P*=0.023).

Table 3. Effects of nutrient synchrony on muscle enzyme activities and muscle composition in *M. Semitendinosus* of heavy preruminant calves

(mean values for treatments with their standard errors; n=5 per treatment for SYN 1 and 2, and n=6 per treatment for SYN 3-6)

Treatment (SYN)	1	2	3	4	5	6	CON 50-85			CON 50-71		
CON	50	57	64	71	78	85	SEM	b*	P-value†	SEM	b	P-value
Enzyme activity, μmol/g protein per min												
Lactate dehydrogenase (LDH)	4167	4618	4500	4738	4381	4562	242.2	$5.2 \cdot 10^{-3}$	0.543	272.9	$0.2 \cdot 10^{-4}$	0.240
Cytochrome-*c*-oxidase (COX)	113	94	78	103	90	96	12.7	$-0.2 \cdot 10^{-3}$	0.544	12.8	$-0.5 \cdot 10^{-3}$	0.525
Citrate synthase (CS)	46	53	43	61	54	58	4.9	$0.3 \cdot 10^{-3}$	0.095	5.4	$0.5 \cdot 10^{-3}$	0.166
Relative enzyme activity												
COX:CS	2.51	1.80	2.04	1.72	1.68	1.62	0.264	-0.02	0.051	0.326	-0.03	0.199
COX:LDH, $\cdot 10^{-3}$	29.4	21.2	17.8	22.0	20.9	21.6	3.91	$-0.1 \cdot 10^{-3}$	0.347	4.14	$-0.3 \cdot 10^{-3}$	0.267
CS:LDH, $\cdot 10^{-3}$	11.5	11.8	9.7	13.1	12.4	12.8	1.37	$0.1 \cdot 10^{-3}$	0.311	1.53	$0.6 \cdot 10^{-4}$	0.533
Muscle composition												
Glycogen, mg/g fresh tissue‡	6.7	6.6	8.2	6.4	6.8	6.8	1.06	$-1.4 \cdot 10^{-3}$	0.969	1.14	$-0.3 \cdot 10^{-3}$	0.881
Fat, mg/g fresh tissue	5.6	5.4	7.4	7.6	6.1	6.0	0.98	0.01	0.717	1.19	0.12	0.166
Protein, mg/g fresh tissue	177	171	171	166	172	175	5.5	-0.04	0.841	6.9	-0.42	0.283
DNA, μg/g fresh tissue	1964	1880	1908	1950	1944	1844	80.8	-1.57	0.594	84.9	-0.74	0.889
Protein:DNA, mg/mg	92.7	93.1	89.6	85.9	90.2	94.8	5.69	$0.1 \cdot 10^{-4}$	0.961	6.62	0.01	0.475

CON, contribution of the high protein meal to the daily protein supply (50%=two identical, balanced meals; 100%=all daily protein in one meal)

* Regression coefficient (y = a + b · x), representing the change in response parameter per % increase of the contribution of the high protein meal to the daily protein supply.

† Probability for test if the regression coefficient (b) equals 0. Effects of meal sequence and interactions between meal sequence and degree of nutrient synchrony were non-significant and were therefore excluded from the model.

‡ An interaction between CON and the meal sequence on muscle glycogen content was found (P=0.007).

Table 4. Effects of nutrient synchrony on whole body energy metabolism in heavy preruminant calves

(mean values for treatments with their standard errors; n=5 per treatment for SYN 1 and 2, and n=6 per treatment for SYN 3-6)

Treatment (SYN)	1	2	3	4	5	6	CON 50-85			CON 50-71		
CON	50	57	64	71	78	85	SEM	b*	P-value†	SEM	b	P-value
Average			kJ/d per kg $BW^{0.75}$									
Heat production	691	677	666	629	652	631	6.9	-1.66	0.003	8.9	-2.80	0.012
Activity related heat production	72	79	67	61	66	69	2.7	-0.23	0.328	3.4	-0.64	0.151
Activity corrected heat production	619	598	599	567	586	562	5.9	-1.43	0.003	7.8	-2.16	0.024
Respiratory quotient	0.85	0.86	0.87	0.87	0.86	0.85	0.002	$-0.02 \cdot 10^{-3}$	0.892	0.003	$0.71 \cdot 10^{-3}$	0.028
Maximum			kJ/d per kg $BW^{0.75}$									
Heat production	815	801	786	749	776	743	8.6	-1.90	0.007	10.9	-3.03	0.027
Activity related heat production	158	174	153	156	141	157	5.6	-0.38	0.410	6.8	-0.40	0.670
Activity corrected heat production	721	688	680	642	680	619	9.1	-2.31	0.002	11.0	-3.46	0.011
Respiratory quotient	0.89	0.90	0.91	0.92	0.91	0.92	0.003	$0.54 \cdot 10^{-3}$	0.036	0.003	$1.09 \cdot 10^{-3}$	0.006
Amplitude (Δ)			kJ/d per kg $BW^{0.75}$									
Heat production	242	228	227	226	236	204	5.8	-0.67	0.177	7.5	-0.66	0.511
Activity related heat production	138	151	135	142	120	130	5.3	-0.51	0.244	6.4	0.05	0.956
Activity corrected heat production	187	167	165	164	176	131	4.7	-1.04	0.007	4.7	-1.01	0.101
Respiratory quotient	0.10	0.10	0.09	0.11	0.11	0.12	0.008	$0.75 \cdot 10^{-3}$	0.005	0.008	$0.42 \cdot 10^{-3}$	0.304

CON, contribution of the high protein meal to the daily protein supply (50%=two identical, balanced meals; 100%=all daily protein in one meal)

* Regression coefficient (y = a + b · x), representing the change in response parameter per % increase of the contribution of the high protein meal to the daily protein supply.

† Probability for test if the regression coefficient (b) equals 0. Effects of meal sequence and interactions between meal sequence and degree of nutrient synchrony were non-significant and were therefore excluded from the model.

Table 5. Pearson correlation coefficients between oxidative enzyme activities and muscle composition in *M. Rectus Abdominis* and whole body energy metabolism traits in heavy preruminant calves (n=22).

Trait	CS	COX	CS:LDH	COX:LDH	Fat	Glycogen
Average						
Heat production	-0.06	-0.33	-0.22	-0.42	-0.49*	-0.06
Activity related heat production	0.04	-0.41	0.06	-0.29	-0.43*	0.09
Activity corrected heat production	-0.09	-0.20	-0.28	-0.36	-0.37	-0.11
Maximum						
Heat production	0.28	-0.29	0.19	-0.27	-0.35	0.08
Activity related heat production	0.53**	-0.12	0.58**	-0.10	-0.22	0.26
Activity corrected heat production	-0.12	-0.26	-0.27	-0.39	-0.44*	-0.14
Amplitude						
Heat production	0.63**	0.08	0.66***	0.10	-0.08	0.16
Activity related heat production	0.61**	-0.14	0.58**	-0.11	-0.20	0.19
Activity corrected heat production	-0.04	-0.14	-0.05	-0.14	-0.21	-0.26

CS: citrate synthase; COX: cytochrome-*c*-oxidase; LDH: lactate dehydrogenase.

* $P<0.05$; ** $P<0.01$; *** $P<0.001$

Effects of nutrient synchrony on muscle enzyme activities

Although the quantity (Brandstetter et al., 1998; Cassar-Malek et al., 2004) and the composition (Helge & Kiens, 1997; Geelen *et al.*, 2001; Cuvelier *et al.*, 2006) of the daily feed supply are known to affect oxidative and glycolytic enzyme activities in muscles, this study is one of the first to describe the effect of within-day distribution of nutrient availability on muscle metabolism. Ortigues-Marty *et al* (2003) found increased oxidative enzyme activities in muscle (ST) of preruminant calves when skimmed milk protein was replaced by soluble wheat and whey proteins. Feeding soluble wheat and whey proteins resulted in larger circadian fluctuations of the amino acid availability compared with skimmed milk protein. Therefore, it was suggested that a transiently high amino acid availability may require an increased capacity of oxidative enzymes in muscle to provide energy for protein synthesis when amino acids are available (Ortigues-Marty et al., 2003). However, potential reasons for the affected oxidative capacity in the study of Ortigues-Marty *et al* (2003) also include a lower intake of the skimmed milk diet than of the wheat and whey protein diet and the use of different dietary ingredients with different kinetics of fatty acid absorption (Petit *et al.*, 1988; Cruywagen *et al.*, 1990; Ortigues-Marty *et al.*, 2003).

A decreased nutrient synchrony (i.e. increased circadian fluctuations in nutrient supply) also increased oxidative enzyme activities in RA in the current study (Table 2), but not in ST.

Separating the availability of amino acids and glucose may have required temporarily high oxidative enzyme activities to provide energy for protein synthesis (see Ortigues-Marty *et al.* 2003) or to oxidize excessively available amino acids or glucose. As net protein utilization was unaffected and fat deposition was increased (Van den Borne *et al.*, 2006b), temporarily increased rates of glucose and/or amino acid oxidation should be accompanied by decreased oxidation rates of oxidation during the remainder of the day.

It is noteworthy that nutrient synchrony only affected muscle enzyme activities in an oxidative muscle (RA), but not in a glycolytic muscle (ST). This corresponds with previous studies which demonstrated an higher response of oxidative (mitochondria-rich) than glycolytic muscles to nutrient supply (Cassar-Malek et al., 2004; Jurie et al., 2006).

Effects of nutrient synchrony on muscle composition

Intramuscular fat content was almost threefold higher in RA than in ST, which is in line with the generally described higher fat content in oxidative than in glycolytic muscles (Gondret et al., 1998; Hocquette et al., 2003). The increased intramuscular fat content in RA with decreasing nutrient synchrony corresponds to an increased whole body fat deposition in these calves (Van den Borne *et al.*, 2006b) indicating that the extra fat is at least partly deposited in muscles. Intramuscular fat generally develops after abdominal, intermuscular and subcutaneous fat deposition (Vernon, 1981), which suggests that nutritional regulation of partitioning of fat into muscle is unlikely in relatively young animals (approximately 18 wks of age). On the other hand, fat deposition in muscle may have been selectively stimulated by the increased glucose supply after a high lactose meal. Smith & Crouse (1984) showed that glucose provides only 1-10% of the acetyl units to *in vitro* lipogenesis in subcutaneous adipose tissue of steers, but 50-75% of the acetyl units in the intramuscular adipocytes. This may be true for preruminants as well, thus providing an explanation for the effect of nutrient synchrony on intramuscular fat content of RA. However, care should be taken in extrapolating these results to the *in vivo* situation of preruminants, as they absorb much larger amounts of long-chain fatty acids and monosaccharides (glucose and galactose) than mature ruminants.

The intramuscular glycogen content was higher in both muscles after the high protein diet than after the high lactose diet, but interpretation of this interaction between SYN and SEQ on glycogen content is complicated by its non-linearity. The lower glycogen content when protein and carbohydrate intakes are further separated may be explained by a lower insulin response in more asynchronously fed calves (T Vicari, JJGC van den Borne, WJJ Gerrits, Y

Zbinden and JW Blum, unpublished results). This corresponds with human studies in which the insulin response as well as muscle glycogen accumulation was higher after a mixed protein and carbohydrate meal than after a carbohydrate meal (Zawadzki *et al.*, 1992; Van Loon *et al.*, 2000), but does not explain the low muscle glycogen content at the most synchronous treatment (SYN 1).

Correlations within muscle

Cytochrome-*c*-oxidase activity was positively correlated with muscle composition in RA (i.e. intramuscular fat) in the current study. This relationship between an oxidative enzyme and muscle fat content in RA seems paradoxical, but similar results were obtained in growing rabbits (Gondret et al., 2004) and steers (Hocquette et al., 2003). A high fat deposition and a high fatty acid oxidation can go together as the result of a high fatty acid flux. Although the fat intake with the two daily meals did not differ between treatments, the degree of nutrient synchrony may have (temporarily) increased the fatty acid flux by affecting the kinetics of fat digestion and/or the partitioning of fatty acids to individual tissues.

Correlations between muscle enzyme activities and whole body energy metabolism

In humans positive correlations between muscle enzyme activities and 24-h heat production or sleeping metabolic rate were found (Zurlo *et al.*, 1994; Doucet *et al.*, 2003). In the present study, however, average whole body heat production was not correlated with enzyme activities in RA and ST. Our initial hypothesis that the decreased whole body heat production with decreasing nutrient synchrony is associated with decreased muscle oxidative enzyme activities therefore has to be rejected. Oxidative enzyme activity in RA was positively correlated with the circadian maximum and amplitude of H_{act} in growing calves. A similar correlation was found with H_{tot}, which can be explained by the high contribution of variation in H_{act} to variation in H_{tot}.

The high feeding level in preruminant calves may explain why oxidative enzyme activity in muscle showed a better relationship with within-day variation in H_{act} than with average H_{tot}. Apart from muscle, several other tissues contribute to whole-body heat production. Feeding large amounts of nutrients results in a high diet-induced heat production predominantly caused by portal drained viscera and liver (Ortigues et al., 1995). This may also explain the different results in growing calves and non-growing man. The diet-induced heat production usually contributes for 30 to 35% to H_{tot} in growing farm animals (Collin et al., 2001; Le Bellego et al., 2001; Van Milgen et al., 2001), but only for 5 to 15% to H_{tot} in adult man

(Westerterp, 2004). Consequently, the overall contribution of H_{act} and muscle energy metabolism to average H_{tot} is likely to be higher in man than in calves. The individual housing conditions in the current study restricted the physical activity of calves, whereas calves under practical circumstances are housed in groups. Although group-housing in pigs only marginally increased H_{act} compared with individual housing (Rijnen, 2003), the housing conditions may have contributed to the lack of correlation between oxidative enzyme activity and H_{act}.

Oxidative enzyme activities in muscle were not correlated with the average H_{act} but only with the circadian fluctuations in H_{act} (maximum and amplitude) in preruminant calves. This may be explained by the measurement of enzyme activity, which truly reflects the maximum enzyme activity rather than the enzyme activity *in vivo*. Variation in fatty acid oxidation, for example, is not necessarily associated with variation in oxidative enzyme activities in muscle (Piot et al., 1998). Enzyme activities depend not only on the capacity of enzymes to convert substrates into new (co or end) products, but also on the availability of substrates. The availability of substrates for oxidative enzymes within a day is affected by nutrient supply, but also by particular energy requiring processes like physical activity. It can therefore be expected that the maximum oxidative enzyme activity is regulated by the kinetics of substrate availability rather than by the daily average substrate availability. Although the major aim of this study was to determine effects of variation in nutrient supply on muscle energy metabolism, it appeared that variation in H_{act} was positively correlated with CS activity in RA.

The positive correlation between physical activity and CS activity in muscle corresponds with results obtained in man (Rimbert et al., 2004), rats (Spangenberg et al., 2005), pigs (Petersen et al., 1997) and steers (Jurie et al., 2006). Similarly, muscle CS activity was 23% higher in group-housed calves than in individually housed calves (Ortigues-Marty et al., 2003) which likely relates to increased physical activity. Previous studies have, however, not related the muscle enzyme activity to *in vivo* energy expenditure. Therefore, the current study is one of the first to relate CS activity in muscle to physical activity related heat production (and hence to contractile activity in muscle tissue).

Conclusions

From the results of the present study it was concluded that a decreased nutrient synchrony increased oxidative enzyme activity and intramuscular fat content in the oxidative muscle RA

of preruminant calves. This indicates that the within-day distribution of macronutrient availability can affect muscle composition which may have consequences for muscle function and meat quality. In addition, CS activity in RA showed a positive correlation with the daily maximum and amplitude of activity related heat production, whereas COX activity was positively correlated to intramuscular fat content. In contrast with findings in humans, oxidative enzyme activities were not correlated with heat production rates when averaged over the day. This discrepancy may be due to the low contribution of H_{act} to H_{tot} in growing calves and to the measurement of enzyme maximum capacity (instead of the average *in vivo* activity). Therefore, oxidative enzyme activities in muscle are useless as an indicator of whole-body heat production in rapidly growing animals.

Acknowledgements

References

Ansay M (1974) Individualité musculaire chez le bovin: étude de l'équipement enzymatique de quelques muscles. *Ann Biol Anim Biochim Biophys* **14**, 471-486.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-254.

Brandstetter AM, Picard B & Geay Y (1998) Muscle fibre characteristics in four muscles of growing male cattle II. Effect of castration and feeding level. *Livest Prod Sci* **53**, 25-36.

Brouwer E (1965) Report of sub-committee on constants and factors. *In: Energy Metabolism*, [KL Blaxter, editor]. London, UK. Academic Press, pp. 441-443.

Carroll NV, Longley RW & Roe JH (1955) The determination of glycogen in liver and muscle by use of anthrone reagent. *J Biol Chem* **220**, 583–593.

Cassar-Malek I, Hocquette JF, Jurie C, Listrat A, Jailler R, Bauchart D, Briand Y & Picard B (2004) Muscle-specific metabolic, histochemical and biochemical responses to a nutritionally induced discontinuous growth path. *Anim Sci* **79**, 49-59.

Collin A, Van Milgen J, Dubois S & Noblet J (2001) Effect of high temperature and feeding level on energy utilization in piglets. *J Anim Sci* **79**, 1849-1857.

Cruywagen CW, Brisson GJ, Tremblay GF & Meissner HH (1990) Effect of curd suppression in a milk replacer on physiological parameters in calves. II. Selected blood profiles. *S Afr J Anim Sci* **20**, 239-243.

Cuvelier C, Cabaraux JF, Dufrasne I, Clinquart A, Hocquette JF, Istasse L & Hornick JL (2006) Performance, slaughter characteristics and meat quality of young bulls from Belgian Blue, Limousin and Aberdeen Angus breeds fattened with a sugar-beet pulp or a cereal-based diet. *Anim Sci* **82**, 125-132.

Doucet E, Tremblay A, Simoneau JA & Joanisse DR (2003) Skeletal muscle enzymes as predictors of 24-h energy metabolism in reduced-obese persons. *Am J Clin Nutr* **78**, 430-435.

Geelen SNJ, Blázquez C, Geelen MJH, Sloet van Oldruitenborgh-Oosterbaan MM & Beynen AC (2001) High fat intake lowers hepatic fatty acid synthesis and raises fatty acid oxidation in aerobic muscle in Shetland ponies. *Br J Nutr* **86**, 31-36.

Gerrits WJJ, Tolman GH, Schrama JW, Tamminga S, Bosch MW & Verstegen MWA (1996) Effect of protein and protein-free energy intake on protein and fat deposition rates in preruminant calves of 80 to 240 kg live weight. *J Anim Sci* **74**, 2129-2139.

Gondret F, Hocquette JF & Herpin P (2004) Age-related relationships between muscle fat content and metabolic traits in growing rabbits. *Reprod Nutr Dev* **44**, 1-16.

Gondret F, Lebas F & Bonneau M (2000) Restricted feed intake during fattening reduces intramuscular lipid deposition without modifying muscle fiber characteristics in rabbits. *J Nutr* **130**, 228-233.

Gondret F & Lebret B (2002) Feeding intensity and dietary protein level affect adipocyte cellularity and lipogenic capacity of muscle homogenates in growing pigs, without modification of the expression of sterol regulatory element binding protein. *J Anim Sci* **80**, 3184-3193.

Gondret F, Mourot J & Bonneau M (1998) Comparison of intramuscular adipose tissue cellularity in muscles differing in their lipid content and fiber type composition during rabbit growth. *Livest Prod Sci* **54**, 1-10.

Guilloteau P, Toullec R & Grongnet JF (1986) Digestion of milk, fish and soya-bean protein in the preruminant calf: Flow of digesta, apparent digestibility at the end of the ileum and amino acid composition of ileal digesta. *Br J Nutr* **55**, 571-592.

Helge JW & Kiens B (1997) Muscle enzyme activity in humans: role of substrate availability and training. *Am J Physiol* **272**, R1620-R1624.

Hocquette JF, Jurie C, Ueda Y, Boulesteix P, Bauchart D & Pethick DW (2003) The relationship between muscle metabolic pathways and marbling of beef. *In: Progress in Research on Energy and Protein Metabolism, EAAP publication 109, Rostock-Warnemünde, Germany.* [WB Souffrant and CC Metges, editors]. Wageningen Academic Publishers, pp. 513-516.

Hocquette JF, Ortigues-Marty I, Pethick D, Herpin P & Fernandez X (1998) Nutritional and hormonal regulation of energy metabolism in skeletal muscles of meat-producing animals. *Livest Prod Sci* **56**, 115-143.

ISO (1999) Animal feeding stuffs. Determination of fat content. ISO 6492. International Organization for Standardization.

Jurie C, Ortigues-Marty I, Picard B, Micol D & Hocquette JF (2006) The separate effects of the nature of diet and grazing mobility on metabolic potential of muscles from Charolais steers. *Livest Sci (In the Press)*.

Labarca C & Paigen K (1980) A simple rapid and sensitive DNA assay procedure. *Anal Biochem* **102**, 344-352.

Le Bellego L, Van Milgen J, Dubois S & Noblet J (2001) Energy utilization of low-protein diets in growing pigs. *J Anim Sci* **79**, 1259-1271.

Ortigues-Marty I, Hocquette JF, Bertrand G, Martineau C, Vermorel M & Toullec R (2003) The incorporation of solubilized wheat proteins in milk replacers for veal calves: effects on growth performance and muscle oxidative capacity. *Reprod Nutr Dev* **43**, 57-76.

Ortigues I, Martin C, Durand D & Vermorel M (1995) Circadian changes in energy expenditure in the preruminant calf: whole animal and tissue level. *J Anim Sci* **73**, 552-564.

Petersen JS, Henckel P, Maribo H, Oksbjerg N & Sørensen MT (1997) Muscle metabolic traits, post mortem-pH-decline and meat quality in pigs subjected to regular physical training and spontaneous activity. *Meat Sci* **46**, 259-275.

Pethick DW & Rowe JB (1996) The effect of nutrition and exercise on carcass parameters and the level of glycogen in skeletal muscle of Merino sheep. *Aust J Agric Res* **47**, 525-535.

Petit HV, Ivan M & Brisson GJ (1988) Digestibility and blood parameters in the preruminant calf fed a clotting or a nonclotting milk replacer. *J Anim Sci* **66**, 986-991.

Piot C, Veerkamp JH, Bauchart D & Hocquette JF (1998) Contribution of mitochondria and peroxisomes to palmitate oxidation in rat and bovine tissues. *Comp Biochem Physiol B* **121**, 185-194.

Rijnen MMJA (2003) Energetic utilization of dietary fiber in pigs. PhD Thesis, Wageningen University, The Netherlands.

Rimbert V, Boirie Y, Bedu M, Hocquette JF, Ritz P & Morio B (2004) Muscle fat oxidative capacity is not impaired by age but by physical inactivity: association with insulin sensitivity. *FASEB J* **18**, 737-739.

Shepherd D & Garland PB (1969) Citrate synthase from rat liver. *Methods Enzymol* **13**, 11-16.

Smith SB & Crouse JD (1984) Relative contributions of acetate, lactate and glucose to lipogenesis in bovine intramuscular and subcutaneous adipose tissue. *J Nutr* **114**, 792-800.

Spangenberg EMF, Augustsson H, Dahlborn K, Essén-Gustavsson B & Cvek K (2005) Housing-related activity in rats: Effects on body weight, urinary corticosterone levels, muscle properties and performance. *Lab Anim* **39**, 45-57.

Van den Borne JJGC, Verstegen MWA, Alferink SJJ, Giebels RMM & Gerrits WJJ (2006a) Effects of feeding frequency and feeding level on nutrient utilization in heavy preruminant calves. *J Dairy Sci* **89**, 3578-3586.

Van den Borne JJGC, Verstegen MWA, Alferink SJJ, Van Ass FHM & Gerrits WJJ (2006b) Synchronizing the availability of amino acids and glucose decreases fat retention in heavy preruminant calves. *J Nutr* **136**, 2181-2187.

Van Es AJH, Nijkamp HJ, Van Weerden EJ & Van Hellemond KK (1967) Energy, carbon and nitrogen balance experiments with veal calves. *In: Energy Metabolism of Farm Animals*, [KL Blaxter, J Kielanowski and G Thorbek, editors]. Newcastle-upon-Tyne, UK. Oriel Press, pp. 197-201.

Van Hinsberg VWM, Veerkamp JH & Bookelman H (1978) Palmitate oxidation by rat skeletal muscle mitochondria. Comparison of polarographic and radiochemical experiments. *Arch Biochem Biophys* **190**, 762-771.

Van Loon LJC, Saris WHM, Kruijshoop M & Wagenmakers AJM (2000) Maximizing postexercise muscle glycogen synthesis: carbohydrate supplementation and the application of amino acid or protein hydrolysate mixtures. *Am J Clin Nutr* **72**, 106-111.

Van Milgen J, Noblet J & Dubois S (2001) Energetic efficiency of starch, protein and lipid utilization. *J Nutr* **131**, 1309-1318.

Verdonk JMAJ, Gerrits WJJ, Beelen GM & Jansman AJM (1999) Effect of protein source on portal nutrient fluxes in pre-ruminant calves. *In: The VIIIth International Symposium on Protein*

Metabolism and Nutrition, [GE Lobley, A White and JC MacRae, editors]. Aberdeen, UK. Wageningen Pers, The Netherlands, pp. 47 (abstract).

Vernon RG (1981) Lipid metabolism in the adipose tissue of ruminants. *In: Lipid metabolism in ruminant animals*, [WW Christie, editor]. Oxford, UK. Pergamon Press, pp. 279-362.

Wenk C & Van Es AJH (1976) Eine Methode zur Bestimmung des Energieauswandes für die körperliche Aktivität von wachsenden Küken. *Schweiz Landwirtsch Monatsh* **54**, 232.

Westerterp KR (2004) Diet induced thermogenesis. *Nutr Metab* **1**, 1-5.

Zawadzki KM, Yaspelkis BB, 3rd & Ivy JL (1992) Carbohydrate-protein complex increases the rate of muscle glycogen storage after exercise. *J Appl Physiol* **72**, 1854-1859.

Zurlo F, Nemeth PM, Choksi RM, Sesodia S & Ravussin E (1994) Whole-body energy metabolism and skeletal muscle biochemical characteristics. *Metabolism* **43**, 481-486.

Chapter 8

General Discussion

Introduction

The emphasis of this thesis was on quantifying effects of two types of nutrient synchrony on nutrient deposition in preruminant calves. The review, in which a reductionist approach was presented for studying the low efficiency of protein utilization for growth in preruminant calves (Chapter 2), identified a lack of nutrient synchrony as one of the potential reasons for the lower marginal efficiency of protein utilization in calves than in other farm animals. Therefore, the effects of two types of nutrient synchrony on protein and energy metabolism were studied. A feeding frequency study was performed to quantify effects of a type 1 nutrient synchrony (i.e. synchrony of nutrient supply and nutrient requirements in time) on nitrogen and energy balance (Chapter 3) and on within-day nutrient oxidation (Chapter 4) in preruminant calves. Type 2 nutrient synchrony (i.e. synchrony of the supply glucose and amino acids) affected nutrient deposition in growing pigs (Chapter 5) differently from that in heavy preruminant calves (Chapter 6). In Chapter 7, effects of type 2 nutrient synchrony on muscle composition were reported and muscle energy metabolism was related to whole body energy metabolism in preruminant calves.

The main research questions were on protein and energy metabolism (Chapter 1). Nutrient synchrony in relation to whole body protein and energy metabolism has been discussed in the various chapters. After only briefly discussing effects of nutrient synchrony on protein deposition, the main focus in this section is on presenting and discussing effects on glucose metabolism. We previously speculated (Chapter 2) that glucose metabolism and its regulation could affect nutrient utilization in calves. Hence, supporting evidence may be gathered for identifying potential mechanisms involved in the regulation of whole body protein and energy metabolism in heavy preruminant calves. If possible, results were compared with those in pigs and man. Finally, our current and future work to obtain more insight in the physiology and nutrient partitioning in heavy milk-fed calves is outlined and the main conclusions of the thesis are summarized.

Effects of nutrient synchrony on protein deposition

Two types of nutrient synchrony were studied in heavy preruminant calves (Figure 1). The first type of nutrient synchrony concerns the matching of total nutrient supply with total nutrient requirements within a day. The second type of nutrient synchrony concerns the matching of the supply of glucose and amino acids.
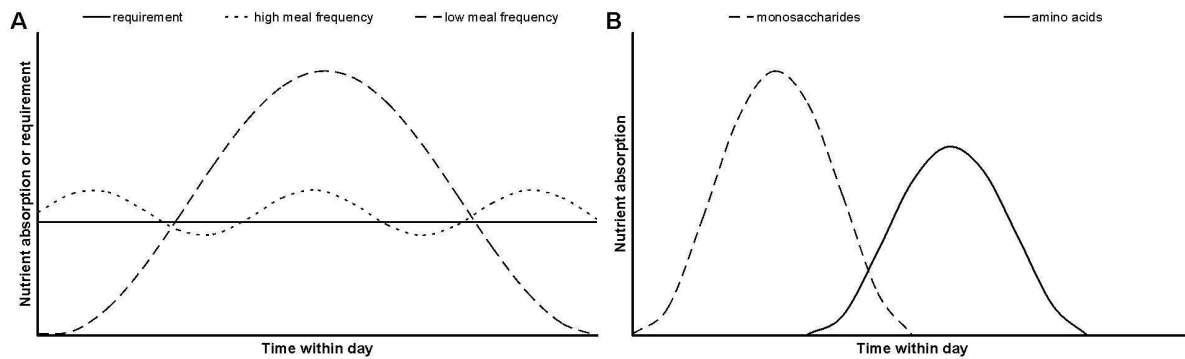
Figure 1. The two types of nutrient synchrony described in this thesis: synchrony of total nutrient supply and total nutrient requirement (type 1; panel A), synchrony of the supply of individual nutrients mutually (type 2; panel B).

For type 1 nutrient synchrony, distributing the daily nutrient supply over an increased number of meals was expected to increase protein retention because of a limited storage capacity relative to the high amount of amino acids absorbed within a short time span (Chapter 3). Furthermore, it was hypothesized that this effect would be more pronounced at a high than at a low feeding level. Increasing the feeding frequency indeed increased protein utilization in heavy preruminant calves, but an interaction between feeding frequency and feeding level was absent. Increasing the feeding frequency from 2 to 4 meals per day at a high feeding level increased the efficiency of digestible protein utilization from 49.1 to 54.5%. This increase indicated that improving type 1 nutrient synchrony increased protein deposition, but explains only a minor portion of the low efficiency of protein utilization in heavy preruminant calves.

For type 2 nutrient synchrony, we expected that separating the protein and carbohydrate intake within a day would decrease protein retention. In pigs, the efficiency of digestible protein retention decreased from 57% to 47% when protein and carbohydrate intake were separated over meals within a day. In preruminant calves, an increasing separation of protein and carbohydrate intake did not affect protein retention. The sequence of the high-protein and high-carbohydrate meals (viz. morning vs. evening) did also not affect protein retention.

**Interactions between type 1 and 2 nutrient synchrony**

Although the concept of two types of nutrient synchrony is useful, it is very difficult to separate the two types and interactions will affect the net result on nutrient utilization. A striking example is the concept of 'slow' and 'fast' dietary proteins (Boirie et al., 1997) to which much attention has been paid during the last decade. A continuous absorption of amino acids can be obtained by using slowly digestible protein sources in the diet. In man, a slower

digestion rate of protein stimulated postprandial protein retention (Dangin *et al.*, 2001; Bos *et al.*, 2003). Although effects of 'slow' and 'fast' proteins on protein turnover and protein synthesis as well as on the fate of exogenous and endogenous amino acids have been extensively studied, the simultaneous (a)synchrony with other nutrients (i.e. type 2) has been ignored, despite its effect on protein (Chapter 5) and fat metabolism (Chapter 6).

In addition, if pigs are fed 4 or 6 meals per day and high-protein and high-carbohydrate meals are alternated, effects on protein retention are expected to be smaller than when they are fed twice daily (as in Chapter 5). Such interactions are inevitable and complicate the interpretation of both types of nutrient synchrony. The second type of nutrient synchrony studied, for example, resulted in a separation of protein and carbohydrate intake, but also changed type 1 nutrient synchrony for each nutrient (i.e. protein and carbohydrate) individually. Very few studies (e.g. Randles, 2001) have deliberately evaluated specific types of nutrient synchrony, and usually only total concepts are described without identifying the underlying mechanisms.

Comparison with other species

The efficiency of protein utilization is lower in calves compared with pigs and preruminant lambs (Chapter 2). Protein deposition decreased in pigs but was unaffected in calves when protein and lactose intakes were separated in time, and potential reasons have been discussed in Chapters 5 and 6. In addition, nutrient synchrony markedly affected the circadian pattern of heat production, the respiratory quotient (RQ) (Chapters 3, 5 and 6) and substrate oxidation (Chapter 4). The postprandial increase in heat production in preruminant calves was compared with that in pigs at an identical feeding level (Figure 2 A). Remarkably, calves reach their maximal activity corrected heat production only at 4.5 hours after feeding, whereas pigs reach their maximal activity corrected heat production already within 30 minutes after feeding. Thus, the postprandial heat production peak is considerably broader in calves than it is in pigs. Potential reasons for this discrepancy include a high dietary fat content, and a slow gastric emptying and nutrient absorption. An increase in the dietary fat content to 10% delayed the maximum heat production in pigs. The time until this maximum was reached in pigs was however still three hours earlier than in calves.
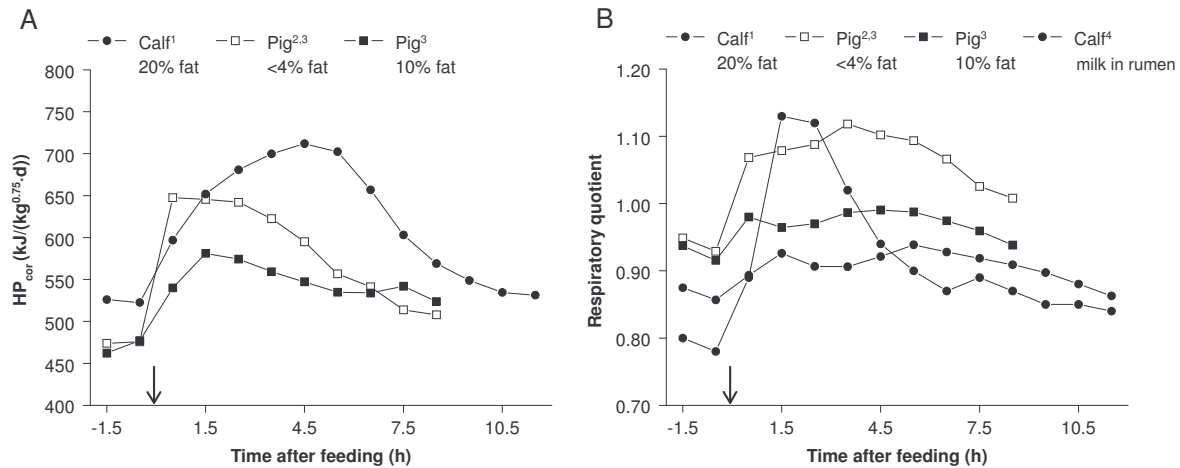
Figure 2. Response of the circadian patterns of heat production corrected for physical activity (panel A) and the respiratory quotient (panel B) to one of two daily meals in individually housed calves and pigs fed at 2.5 times the energy requirements for maintenance. Data were obtained from [1]This thesis (Chapter 3), [2]Rijnen et al. (unpublished), [3]Van Heugten et al. (unpublished), and [4]Van den Borne et al (2004). The arrow ($\downarrow$) indicates the time of feed intake.

Also the circadian pattern of RQ after feeding was compared between calves and pigs (Figure 2 B). The RQ showed larger fluctuations in pigs (approx. 0.20 units) than in preruminant calves (approx. 0.07 units). Again, the high dietary fat intake (resulting in a slow absorption and low RQ when fatty acids are oxidized) and limitations to rapidly oxidize the big quantities of glucose (see next paragraph) may be reasons for the difference between species. The RQ in calves after feeding is characterized by two peaks. Although it is obvious that glucose oxidation increased soon after feed intake, it can also be expected that leakage of milk into the rumen results in anaerobic fermentation and thus in an increased CO_2 production relative to O_2 consumption (i.e. higher RQ) resulting in the first peak in RQ. This expectation was supported by parallel increases in methane production during the first postprandial peak in RQ in almost all calves, and especially in one calf which was clinically ruminal drinking (Figure 7 B; Van den Borne et al., 2004). Recently, Suárez (2006) quantified leakage of milk into the rumen in heavy preruminant calves. Co-EDTA was added as a marker to the milk replacer in the last meal before slaughter. Between 20.5 and 34.9% of the marker was recovered in the rumen, indicating that significant amounts of milk entered the rumen. These calves were not clinically bloating or showing other signs of ruminal drinking, supporting the possibility that sub-clinical ruminal drinking can contribute to the low efficiency of protein deposition in calves (see Chapter 2).

Conclusions

A more equal distribution of nutrient intake over a day, i.e. an increased feeding frequency, increases the efficiency of protein utilization, but explains only a minor proportion of the low efficiency.

An asynchronous availability of amino acids and glucose within a day does not contribute to the low marginal efficiency of protein utilization in heavy preruminant calves, but it decreases protein deposition in pigs.

The postprandial increase in heat production in calves is much slower, takes longer and is associated with a lower RQ compared with pigs.

Effects of nutrient synchrony on glucose metabolism

Heavy preruminant calves can have problems to maintain glucose homeostasis, and they often develop hyperglycemia (i.e. high plasma glucose concentrations) and glucosuria (i.e. glucose excretion in urine) (Doppenberg & Palmquist, 1991; Blum & Hammon, 1999). More than 80 years ago, it was already stated by Rabinowitch et al (1925) that "hyperglycemia and glucosuria result when the combined rates of oxidation and storage do not keep pace with the rate of absorption of glucose from the alimentary canal or its formation in the body". The disposal of glucose is regulated by pancreatic insulin, and hyperglycemia and glucosuria can be a consequence of an insufficient insulin-mediated glucose disposal. Tissues can become less sensitive for insulin and as a result, more insulin will be needed to lower blood glucose concentrations. Insulin resistance is defined as the impaired ability of insulin to lower blood glucose (Garvey & Hermayer, 1998). High plasma insulin concentrations in response to feeding are therefore often observed in calves, and may compensate for insulin resistance, like in type 2 diabetes (Mari et al., 2005; Reaven, 2005).

The entry of glucose originates largely from dietary sources in preruminant calves, in contrary to ruminant calves which depend on gluconeogenesis. Therefore, it was hypothesized that nutrient synchrony could affect the development of insulin resistance, hyperglycemia and glucosuria, resulting in an interference with nutrient utilization. To obtain more insight in the glucose metabolism and its endocrine regulation in preruminant calves, blood samples were taken after and during the balance studies described in Chapter 3 and 6, respectively, and analyzed for glucose and insulin concentrations. Although insulin sensitivity was not directly assessed by means of a glucose tolerance test, the results provide some leads on the regulation of glucose metabolism by insulin and the effects of nutrients on this regulation.

Type 1 nutrient synchrony

An increase in feeding frequency, as well as a decrease in feeding level, resulted in lower responses of plasma glucose and insulin concentrations to a meal. In addition, less time was needed before concentrations were back at baseline (Figure 3 A-D). Glucose concentrations in calves that were fed only once daily were not back to baseline values at 6 h after feeding and insulin concentrations were still very high. Glucose and insulin concentrations showed some (non-significant) increase with increasing age (Figure 3 A and B).

The ratios of insulin to glucose concentrations during an integrated 6 h period after feeding are presented in Figure 3 E. The insulin to glucose ratio increased ($P < 0.05$) with increasing feeding level and decreasing feeding frequency. The urinary glucose excretion rate increased ($P < 0.05$) with increasing feeding level, and tended ($P = 0.066$) to increase with decreasing feeding frequency (Figure 3 F). The glucose excretion in urine ranged from 0 to 91 g/d between individual calves.

In young heifer calves, feeding frequency (once or twice daily) did not affect glucose and insulin concentrations or urinary glucose concentrations (Stanley et al., 2002). In young veal calves, only small effects were observed (Nussbaum et al., 2002). In heavy veal calves, Kaufhold et al (2000) showed that an increased frequency of feeding skimmed milk powder and whole milk improved glucose homeostasis. Effects of frequency were however confounded with the feeding system in that study (bucket or automated feeding system) and plasma glucose and insulin concentrations were much higher in our study. Individual calves even reached maximum glucose concentrations of 14.5 mmol/L and insulin concentrations exceeded 130 μg/L in several animals.

The ratios between postprandial insulin and glucose responses showed good agreement with the urinary glucose excretion, indicating that a high insulin to glucose ratio leads to excretion of high amounts of glucose in urine. This can be explained by an increased insulin resistance in preruminant calves. The renal threshold for glucose excretion in urine is approximately 8.3 mmol/L (Hostettler-Allen et al., 1994), indicating that glucose is cleared during several hours after a meal for most treatments (Figure 2 A-D).
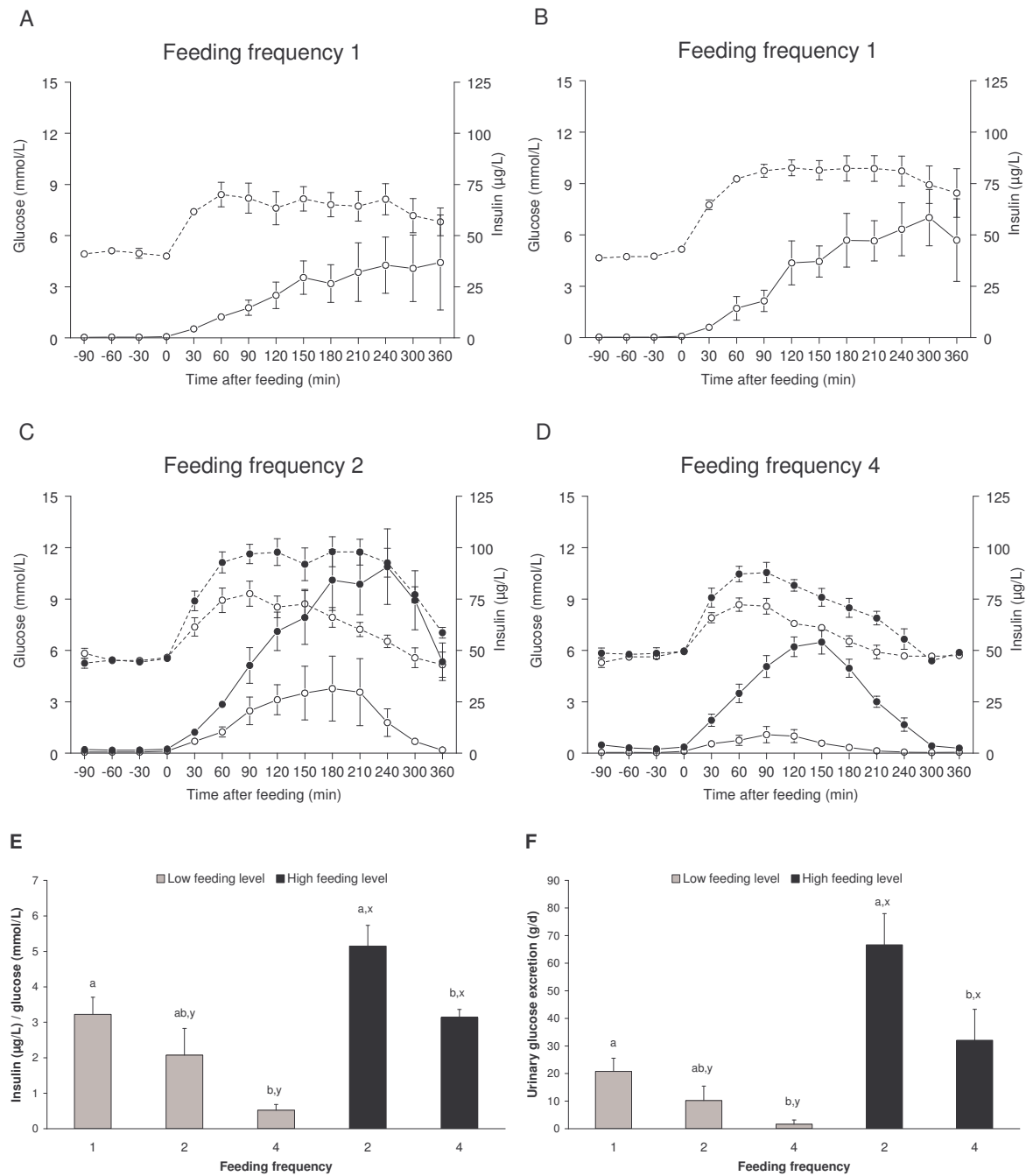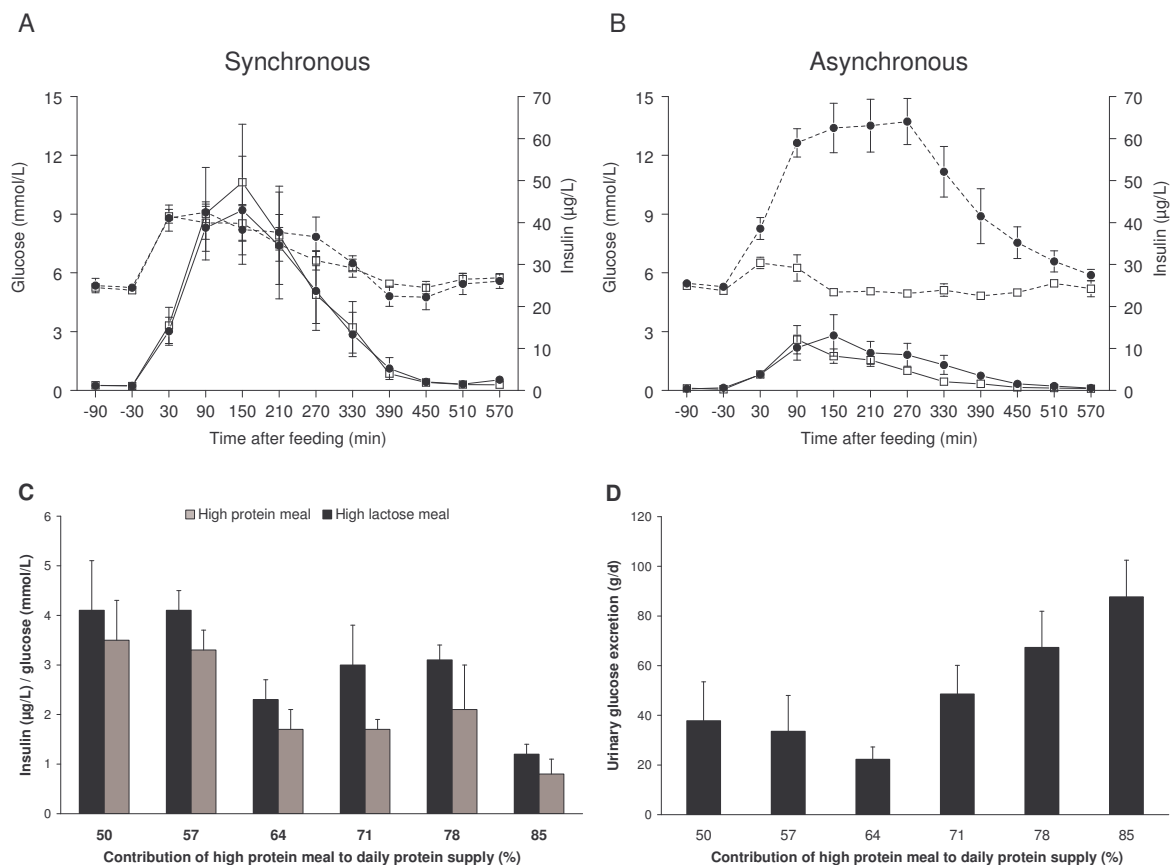
Figure 3. Plasma glucose (-----) and insulin (——) concentrations (panel A-D), insulin to glucose ratios of integrated postprandial 6-h plasma concentrations (from 1200 to 1800 h; panel E), and urinary glucose excretion in preruminant calves fed once (n=8), twice (n=5) or four (n=6) times daily (panel F). Calves were fed at a low feeding level (FL) in the first experimental period (open symbols) and at a high FL in the second experimental period (closed symbols). The low and high FL were 1.5 and 2.5 × metabolizable energy requirements for maintenance, respectively. Calves fed once daily were fed at a low FL in both the first (panel A) and second experimental period (panel B). Data are means ± SEM. [a,b,c] For panel E and F, values with different letters indicate significant differences ($P < 0.05$) between feeding frequencies within a FL. [x,y] For panel E and F, values with different letters indicate significant differences ($P < 0.05$) between FL. Data from Vicari et al. (submitted).

Type 2 nutrient synchrony

Spreading the daily protein and lactose intake equally over two meals (one at 1200 and one at 2400 h) resulted in similar responses of plasma glucose and insulin concentrations after the meal in preruminant calves (Figure 4 A). The plasma glucose concentration increased from 5.3 to approximately 9 mmol/L, whereas the plasma insulin concentration increased from 1 to 50 µg/L after feeding. When 85% of the daily protein and 20% of the daily lactose was fed in one meal, this resulted in a very small increase of both the plasma glucose and insulin concentrations (Figure 4 B). The other meal, which contained 15% of the daily protein and 80% of the daily lactose supply, resulted in a very high response of the plasma glucose concentration (14 mmol/L) but plasma insulin concentrations were very low (maximum of 13 µg/L).

Figure 4. Plasma glucose (-----) and insulin (——) concentrations (panel A and B), insulin to glucose ratios of integrated postprandial 6-h plasma concentrations (panel C), and urinary glucose excretion (panel D) after a high protein (□) and a high lactose (●) meal in synchronously (panel A; 50% of the daily protein and 50% of the daily lactose in each meal) and asynchronously fed preruminant calves (panel B; 85% of the daily protein and 20% of the daily lactose in the high protein meal and the remainder in the high lactose meal). Values are means ± SEM, n = 5 (50-64%) or 6 (71-85%). Data from Vicari et al. (submitted).

Plasma insulin concentration did not respond differently to the high lactose meal compared with the high protein meal in asynchronously fed calves. Mean 24-h glucose concentrations increased ($P < 0.05$) and mean insulin concentrations decreased ($P < 0.05$) with increasing asynchrony. The ratio between insulin and glucose during 6 h after both meals decreased ($P < 0.05$) with increasing separation of protein and lactose in time (Figure 4 C). Urinary glucose excretion increased ($P < 0.01$) with increasing nutrient asynchrony (Figure 4 D) and varied between 0 and 135 g/d for individual calves.

The high glucose concentrations and low insulin concentrations after a high lactose meal (Figure 4 B) were surprising. Usually, a rapid increase of the plasma glucose concentration results in a rapid increase of the plasma insulin concentration (Wolever, 2000), and a high glycemic index correlates well with a high insulinemic index in man (Björck et al., 2000). The discrepancy between the normal response to a high lactose meal, i.e. increased insulin secretion, and the low insulin response in the current study can be the result of several mechanisms including the following.

First, the low insulin to glucose ratios could indicate an enhanced sensitivity to insulin. That may be associated with increased insulin-stimulated fatty acid incorporation into adipose tissue and muscle. This mechanism could explain the increased fat retention in whole body (Chapter 6) and in the *Rectus Abdominis* muscle (Chapter 7). In contrast, urinary glucose excretion increased with increased separation of lactose and protein intake within a day. If the insulin sensitivity is increased, this glucosuria could be an independent result of the temporary high glucose concentrations.

Second, the low insulin response may also be the consequence of reduced insulin secretion from pancreatic β-cells (not sensing glucose or not secreting insulin). These cells normally respond to an increase in plasma glucose concentration by secretion of insulin to maintain glucose homeostasis. The (small) increase in insulin concentrations after a meal at the most asynchronous treatment indicated that the β-cells still react to nutrient availability. Apart from its stimulation of β-cells, glucose also suppresses the release of glucagon from pancreatic α-cells, whereas amino acids stimulate glucagon release (Unger, 1985; Calbet & MacLean, 2002). Plasma glucagon concentrations, however, increased after each meal (i.e. also the meal including 80% of the daily lactose intake; data not shown), which may indicate a defect in the nutrient-regulated signalling of the endocrine pancreas. Postprandial glucagon concentrations

can be associated with type 1 diabetes in man (Fineman et al., 2002). Increases in plasma glucagon were however not affected by an increased separation of protein and lactose intake. Third, the low insulin response after a high lactose meal with decreased nutrient synchrony may not reflect the actual insulin secretion in the portal vein. The liver is a main site of insulin extraction, because ~50% of insulin produced by the pancreas is removed by the liver during the first portal passage before reaching the systemic circulation (Wiesenthal et al., 1999). The real pancreatic insulin secretion can be quantified by measuring the appearance of both insulin and the C-peptide (connecting peptide) in the systemic circulation. Insulin and the C-peptide are produced in equimolar proportions when proinsulin is released from the pancreas into the portal blood. Insulin is intensively extracted by the liver, but C-peptide not (Polonsky & Rubenstein, 1984; Toffolo *et al.*, 2006). An increasing hepatic insulin extraction with decreasing nutrient synchrony can explain the low peripheral insulin concentrations when protein and lactose are separated.

Comparison with other species

A simultaneous ingestion of carbohydrates and protein also increased insulin secretion compared with ingestion of only carbohydrates in man (Rabinowitz et al., 1966; Van Loon et al., 2000), but differences were smaller than in the calf study and plasma glucose concentrations hardly exceeded 7 mmol/L in human subjects. Moreover, plasma glucose concentrations in non-diabetic man are back to baseline values in 2-3 hours after a meal (Rabinowitch *et al.*, 1925; Perley & Kipnis, 1967; Manders *et al.*, 2006), whereas diabetic man may need twice as much time depending on the severity of the insulin resistance (Rabinowitch *et al.*, 1925; Manders *et al.*, 2006). In calves fed once daily, the glucose (and insulin) concentrations had not returned to baseline at 6 h after the meal.

In contrast to the considerable effect of feeding level on plasma glucose concentrations in preruminant calves, effects in pigs are much smaller. Feeding level did not affect plasma glucose concentrations in lactating sows (Van den Brand et al., 2000), but another study with a larger contrast between feeding levels showed greater plasma glucose concentrations with increased feeding level in pigs. However, the variation in plasma glucose concentrations (from 4.1 to 6.5 mmol/L; Ponter et al., 1991) was much smaller than in calves. In addition, feeding frequency did not affect body composition and glucose tolerance in pigs (Romsos et al., 1978). The different response of glucose metabolism to type 2 nutrient asynchrony in pigs compared with calves was confirmed by the absence of glucose in the urine of pigs in Chapter 5, regardless whether protein and starch intake were separated in time or not.

Conclusions

In heavy preruminant calves, an increase in feeding frequency decreased postprandial insulin to glucose ratios and decreased glucose excretion in urine.

Simultaneous ingestion of lactose and protein resulted in lower plasma glucose concentrations, a much larger insulin response and a lower glucose excretion in urine than when ingested separately in heavy preruminant calves.

Crosstalk between glucose metabolism and nutrient utilization

To obtain more insight in the role of glucose metabolism and its regulation by insulin on nutrient utilization, two questions need to be answered: (1) How do nutrients induce the development of insulin resistance in heavy preruminant calves?, and reversely (2) What is the consequence of a decreased insulin sensitivity for the utilization of nutrients?

Interactions between insulin resistance, nutrient availability and nutrient utilization are very complex (Figure 5) and effects of macronutrient intake on the development of insulin resistance have to some extent been studied in preruminant calves (Figure 5; *arrows 1, 3 and 5*). The influence of insulin resistance on nutrient utilization is however less clear (*arrows 2, 4 and 6*). The bi-directional relationships between macronutrients and insulin resistance in preruminant calves are briefly discussed, and, if not available, results in other species are described.
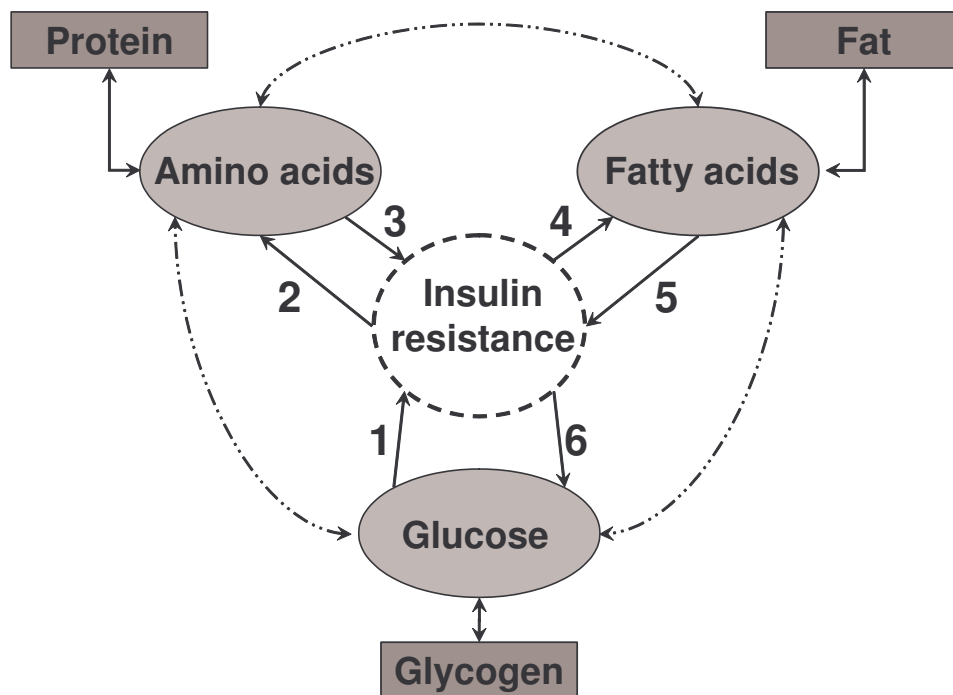
Nutrients and the development of insulin resistance

An increase in lactose intake (*arrow 1*) increases insulin resistance in preruminant calves (Wijayasinghe *et al.*, 1984; Hugi *et al.*, 1997; Hugi *et al.*, 1998). Glucose toxicity (i.e. reducing the sensitivity of glucose transport proteins by high plasma glucose concentrations) may therefore cause insulin resistance in calves, as demonstrated in other species (for review: Yki-Järvinen, 1992).

An increase in digestible protein intake (*arrow 3*) at a constant protein-free energy intake decreased insulin resistance and blood glucose concentrations in heavy preruminant calves (Gerrits & Blum, 1998). Increasing the protein intake in man, on the expense of carbohydrates, resulted in an increased insulin resistance and hyperglycemia (Blazquez & Lopez Quijada, 1970; Rossetti *et al.*, 1989; Krebs *et al.*, 2002). Moreover, individual amino

acids may affect glucose metabolism. A high glutamine (Traxinger & Marshall, 1989) and low arginine intake (Wu et al., 2004) have been suggested to induce insulin resistance.

An increase in fat intake (*arrow 5*) is generally perceived not to affect glucose homeostasis in calves (e.g. Palmquist *et al.*, 1992; Blum & Hammon, 1999). Increasing the fat intake did not provide indications for increased insulin resistance in preruminant calves (Doppenberg & Palmquist, 1991; Palmquist *et al.*, 1992). In these studies, however, the fat-free nutrient intake also changed and resulted in a lower protein and greater lactose content in the low fat (10%) diet than in the high fat (20%) diet (Palmquist et al., 1992) This may have increased insulin resistance due to decreased protein and increased lactose intakes. Therefore, effects of fat intake on glucose metabolism are unclear in calves. In man and rats, fat intake has been positively correlated with the development of insulin resistance (e.g. Storlien et al., 1996).

Figure 5. Schematic presentation of the cross-talk between nutrients and the potential intermediate role of insulin resistance. The numbers (1-6) indicate direct relationships between nutrients and insulin resistance. The dashed lines indicate other interactions between nutrients than via insulin resistance.

Insulin resistance and nutrient utilization

An increased insulin resistance may decrease the efficiency of amino acid utilization (*arrow 2*). In man, this was demonstrated by the loss of body protein during diabetes mellitus (Donkin, 1999). Even obese human subjects, but not diabetic, showed an increased insulin resistance and a concomitantly higher amino acid oxidation than less insulin resistant subjects

(Nair et al., 1987). Also, in type 1 (insulin dependent) diabetes, urea production rates increased approximately 20% compared with healthy controls (Freyse & Knospe, 1998). In calves, quantitative information is lacking.

An increase in insulin resistance (*arrow 4*) increases fatty acid content in liver, muscle and viscera in man (Moller & Flier, 1991; Cline *et al.*, 1999; Frayn, 2000; Blaak & Wagenmakers, 2002; Ravikumar *et al.*, 2005). Fat itself may again increase insulin resistance. The interaction between lipids (in different tissues) and glucose metabolism is an intensive area of research in man, but effects in calves are unknown.

An increased insulin resistance (*arrow 6*) increased glucose excretion in urine in preruminant calves (Gerrits & Blum, 1998; Blum & Hammon, 1999; this thesis). An increased insulin response after exercise was found to increase muscle glycogen content in man (Zawadzki *et al.*, 1992; Van Loon *et al.*, 2000), and insulin also increased glycogen content *in vitro* in pig hepatocytes (Fernández-Fígares et al., 2004). A lower sensitivity for insulin indeed decreased glycogen in muscle and liver of rats and man (Moller & Flier, 1991; Cline *et al.*, 1999; Kusunoki *et al.*, 2002). Glucose oxidation is usually decreased in insulin resistant human subjects (Ravussin *et al.*, 1983; Golay *et al.*, 1988). Independently of insulin, high glucose concentrations can increase glucose oxidation (Yki-Jarvinen et al., 1987). The impact of insulin resistance on glucose utilization in farm animals is however poorly understood.

Conclusions

Insulin resistance in calves increases with extra lactose intake, it decreases with extra protein intake, and the influence of fat intake is unknown.

Effects of insulin resistance on nutrient utilization have not been studied in calves, but human studies indicate that protein deposition may decrease and fat deposition may increase with increasing insulin resistance.

Fat metabolism and *de novo* fatty acid synthesis

Ruminants, but also carnivores like cats, use acetate as principal precursor for *de novo* fatty acid synthesis. In most non-ruminants, like man and pigs, de novo fatty acid synthesis mainly occurs from glucose (Bergen & Mersmann, 2005). For example, adipose tissue accounts for 25-40% of the glucose uptake in pigs (Dunshea et al., 1992), but only for ~1% in ruminants (Pethick, 1984). An important difference between glucose utilizers and acetate utilizers is the pathway via which precursors for fatty acids are transported. In glucose utilizers, carbon flow

is routed through the mitochondria, whereas in acetate utilizers, acetate is directly available in the cytosol (Figure 6). Therefore, ruminants usually do not need high activities of the enzymes citrate lyase (to convert citrate to acetyl CoA and oxaloacetate) and malate dehydrogenase (to convert oxaloacetate to malate and NAD). Nonetheless, ATP citrate lyase and malate dehydrogenase activities were increased in growing steers when large amounts of high-starch concentrates were fed (Schoonmaker et al., 2004). Preruminant calves absorb large amounts of monosaccharides that can serve as a precursor for *de novo* fatty acid synthesis. The activity of ATP citrate lyase in liver and adipose tissue was indeed substantially higher in preruminant calves than in ruminating calves (Roehrig et al., 1988). This indicates that *de novo* fatty acid synthesis from glucose may be increased in preruminant compared with ruminant calves. The extent of de novo fatty acid synthesis from glucose in preruminant calves was however not known.
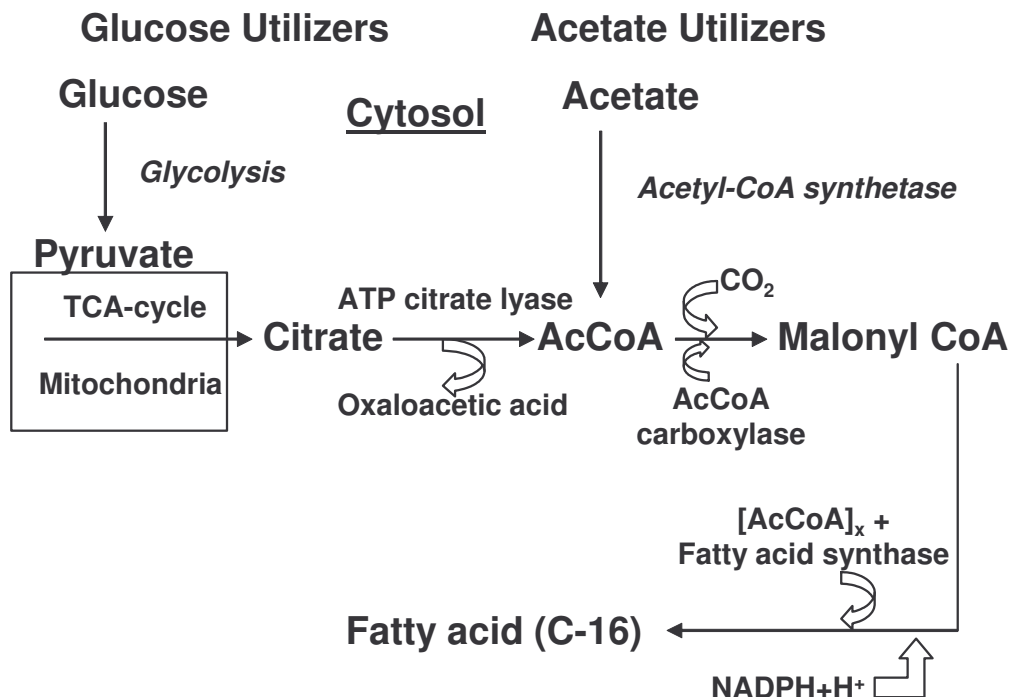

Figure 6. Different pathways for delivery of precursors (glucose or acetate) for *de novo* fatty acid synthesis. From Bergen and Mersmann (2005).

In Chapter 4, we found that dietary glucose was almost completely oxidized in preruminant calves, regardless of the feeding level. The increase in fat deposition with increased feed intake almost exclusively originated from a reduced oxidation of fatty acids, indicating that dietary fatty acids are the predominant precursor of lipid synthesis in calves. Although average glucose oxidation was very high (94% of lactose intake), it slightly decreased from about 100 to 88% of lactose intake with increasing insulin to glucose ratio in the feeding

frequency study. Simultaneously, urinary glucose excretion increased ($P < 0.001$) from 0 to 6% of the carbohydrate intake, and partly compensated for the decreased oxidation.

The increased fat deposition with increased nutrient asynchrony (Chapter 6) corresponded with an increased intramuscular fat content (Chapter 7). In man, there is a strong positive correlation between the accumulation of triacylglycerols and the development of insulin resistance in muscle (Shulman, 2000; McGarry, 2002). But it is still not completely clear if the increased intramuscular triglyceride content is a cause or a consequence of insulin resistance in man (Frayn, 2000; Frayn, 2003) and relationships are not straightforward. For example, endurance athletes have 75% higher intramuscular triglyceride contents than diabetics but are very insulin sensitive (Goodpaster et al., 2000). If the decreased insulin to glucose ratios in calves indicate an increased insulin sensitivity in the nutrient separation study, this contradicts the concept that with increased intramuscular fat content also insulin resistance increased. Moreover, an increase in insulin sensitivity may have increased protein utilization and could therefore explain the absence of an effect of type 2 nutrient synchrony on protein retention.
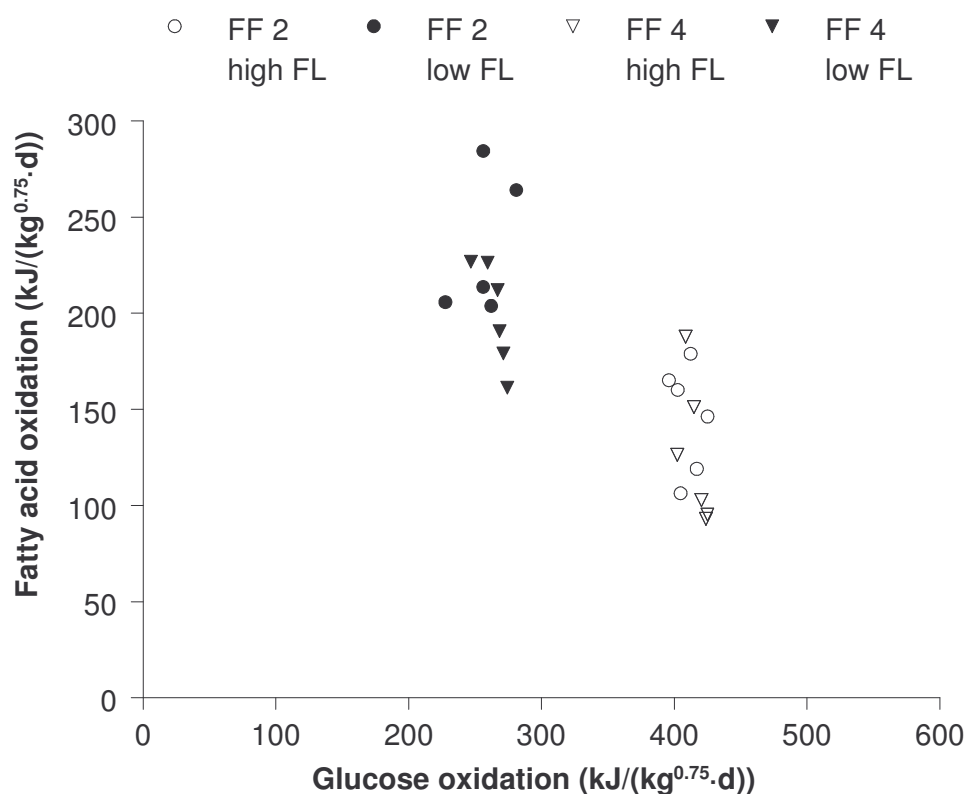
Figure 7. Relationship between glucose and fatty acid oxidation as affected by feeding frequency (FF; 2 and 4 meals/d) and feeding level (FL; $1.5 \times ME_m$ and $2.5 \times ME_m$). Values are means, n = 5 (FF 2, low FL), n = 6 (FF 2, high FL), or n = 6 (FF 4). All observations were used to calculate a regression line, which was $y = -0.53 \cdot x + 353$ (r = -0.79; $P < 0.001$).

In Chapter 4, fatty acid oxidation was high (>70% of intake) at a low feeding level ($1.5 \times ME_m$), but decreased to approximately 30% of fat intake at a high feeding level ($2.5 \times ME_m$). If fatty acids are oxidized at a low feeding level to meet energy requirements for maintenance, it would be expected that an increase in feeding level would decrease fatty acid oxidation, because more glucose is available (the reversed glucose-fatty acid cycle, Sidossis & Wolfe, 1996). The exchange between glucose and fatty acid oxidation was however not iso-energetically: a decrease of 1 kJ from fatty acid oxidation was associated with an increase of 1.9 kJ from glucose oxidation (Figure 7). This may indicate that glucose is oxidized because it is in excess of the capacity for glycogen and *de novo* fatty acid synthesis rather than that fatty acid oxidation is exchanged for glucose oxidation to provide energy.

Comparison with other species

The recovery of [^{13}C]glucose as $^{13}CO_2$ in calves was approximately twofold higher than in exercising man after drinking a [^{13}C]glucose solution (Ruzzin *et al.*, 2003), indicating the high intensity of glucose oxidation in calves. In contrast to preruminant calves, fat deposition in pigs largely depends on *de novo* fatty acid synthesis. The contribution of glucose to fat deposition increases with increasing feeding level and explains about 2/3 of total fat deposition in *ad libitum* fed pigs (calculated from Bikker et al., 1996), whereas its contribution is non-significant in calves (calculated from Gerrits *et al.*, 1996; this thesis). The discrepancy between *de novo* fatty acid synthesis in calves and pigs at a similar feeding level raises the question whether genetic factors (e.g. the preferred precursor pathway) or nutritional factors are responsible for the fate of dietary carbohydrates.

Conclusions

Orally provided carbohydrates are almost exclusively oxidized and fatty acid synthesis from glucose is negligible in heavy preruminant calves. The high contribution of fatty acids to fat deposition may allow dietary manipulation of fatty acid composition in body fat in preruminant calves.

The calf's metabolism – driven by genetics or nutrition?

Heavy preruminant calves are ontogenetically ruminants. The nutrients available for metabolism, however, correspond much more with those available in man than in ruminants.

For the production of veal, calves are predominantly fed milk replacer diets from shortly after birth until approximately 26 weeks of age (i.e. 240 kg body weight). Then, calves are slaughtered. The situation in dairy calves which are used for replacement is different. These calves are only fed milk replacer until 6 to 8 weeks of age. Thereafter, dairy calves are exclusively fed roughage and concentrates. The (gradual) transition from liquid to solid feed results in a completely different supply of digestible nutrients in ruminant compared with non-ruminant cattle. Ruminant calves obtain the majority (~70%) of the digestible energy intake from volatile fatty acids (VFA) originating from fermentation processes in the rumen (Figure 8). The digestible glucose intake is very low and fat intake is usually slightly above 10% of the digestible energy intake.
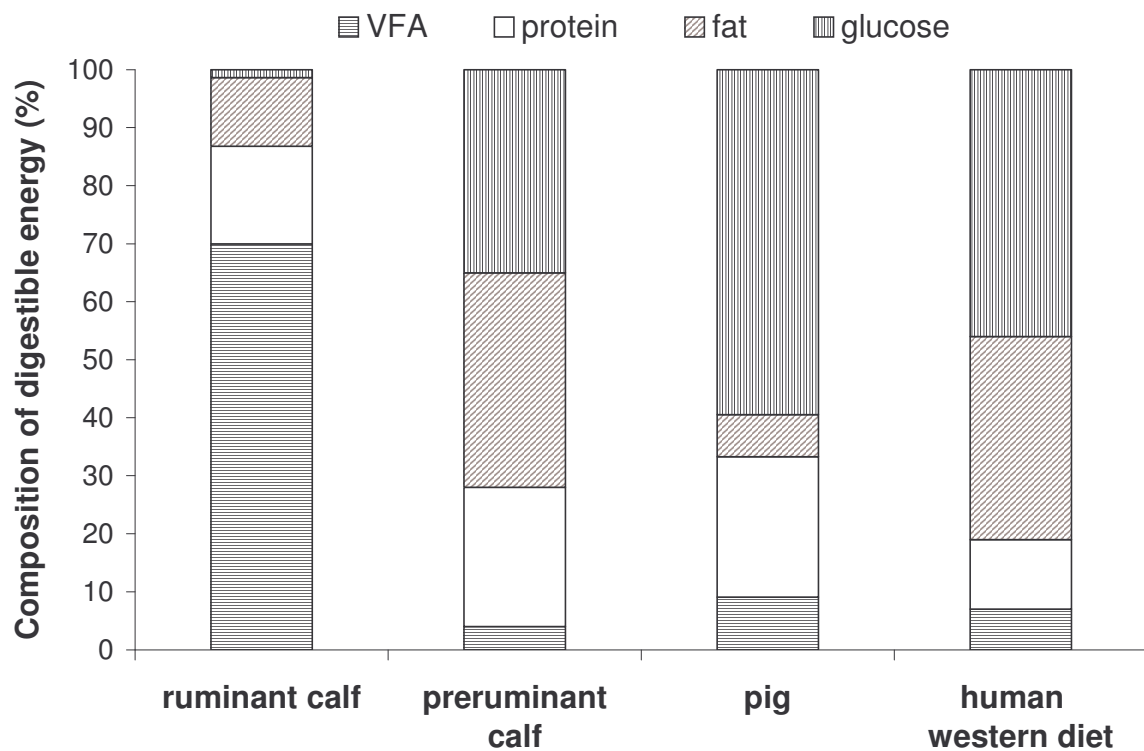
Figure 8. Contribution of volatile fatty acids (VFA), protein, fat and carbohydrates to the digestible energy intake in the ruminant calf, preruminant calf, pig, and man (typical Western diet).

Preruminant calves absorb only small amounts of VFA. Feeding roughage to veal calves (as imposed by law) stimulates rumen development and consequently VFA production and absorption. Intake of VFA was assumed to be 4% of digestible energy intake. Carbohydrates and fat supply together contribute for ~75% to the digestible energy intake. This is quite similar to monogastric animals, like pigs, although pigs obtain ~60% of the digestible energy intake from carbohydrates and only 5-10% from fat. Interestingly, the digestible nutrient intake in the preruminant calf resembles that of the typical Western human very much

(Schwarz et al., 2003). Total nutrient intake is obviously much higher in a quickly growing farm animal than in adult man, but it is remarkable that problems with glucose homeostasis (i.e. insulin resistance, type 2 diabetes) are often observed in both calves and man, but seldom in pigs or ruminants. Throughout the discussion, it was mentioned that the circadian patterns of nutrient oxidation, the responses of plasma glucose and insulin concentrations and *de novo* fatty acid synthesis from glucose in preruminant calves differ from similar those in 'real' monogastrics, such as pigs and man. Subsequently, an obvious question is: can these metabolic differences be attributed to 'feeding a potential ruminant like man'?

Genetic background

Some data indicate that glucose metabolism in potential ruminants is less sensitive to regulation by insulin than in monogastrics. For example, insulin, with or without increased concentrations of branched chain amino acids, failed to stimulate muscle protein synthesis in growing lambs (Wester et al., 2004). This contradicts findings in non-ruminants in which insulin and branched chain amino acids exert an independent, but also a synergetic, stimulation of protein synthesis. Moreover, genetic selection may have increased the insulin resistance in calves. Preruminant calves mostly originate from dairy farms where cows are selected for a high milk production. It has been suggested that selection for a high milk production has implied a simultaneous selection for a decrease in sensitivity of adipose and muscle tissue for insulin (Cronjé, 2000). In the dairy cow, this results in less glucose and amino acids taken up by insulin-dependent tissues, and facilitates a higher nutrient uptake by the mammary glad, in which nutrient uptake depends on the concentration gradient (not insulin-dependent). A decreased insulin sensitivity can therefore lead to an increased partitioning of nutrients to milk production in dairy cows (Cronjé, 2000). Implicitly, this selection can have further decreased insulin sensitivity in calves used for veal production and may have affected both glucose metabolism and protein retention.

Nutrition

On the other hand, the high fat intake in combination with the high carbohydrate intake may confuse regulatory mechanisms of substrate oxidation in preruminant calves. Increasing the fat intake in pigs resulted in more delayed peaks in heat production peaks and RQ (Figure 2; Van Heugten et al, unpublished), thus more similar to patterns in preruminant calves. An iso-energetic replacement of starch for fat (1% fat vs 13% fat) in pig diets reduced the synthesis

of fatty acids from glucose by almost 50% (Allee et al., 1971). Moreover, the classical studies of Himsworth (1935) showed already that increasing the carbohydrate intake iso-energetically at the expense of fat intake resulted in an increase in insulin sensitivity. The carbohydrate content varied from 7.5 to 75% of the dietary energy and effects were most clear when carbohydrates provided more than 30% of the dietary energy (Himsworth, 1935). Unfortunately, to our knowledge, effects of a large range in dietary fat content (e.g. 3-30%) on glucose metabolism have not been studied in pigs. Conversely, human studies cover a large range in dietary fat content, but the relatively low level of nutrient intake complicates comparison with heavy preruminant calves. To our knowledge, studies about effects of a very low fat content on glucose metabolism in calves are also lacking.

Conclusions

It is not clear whether the origin of the metabolic discrepancies between preruminant calves and non-ruminants (circadian patterns of nutrient oxidation, glucose homeostasis and *de novo* fatty acid synthesis) is the genetic background of an ontogenetic ruminant or the typical type of feed: high fat, easily digestible carbohydrates and low fibre.

Current and future work

Type 2 nutrient synchrony and within-day nutrient fluxes

Effects of type 2 nutrient synchrony (i.e. separation of protein and lactose intake) on 24-h glucose oxidation, glucose entry rate and gluconeogenesis are studied. Calves were housed in a respiration chamber and were continuously infused with [U-^{13}C]glucose. Blood samples were collected in 30-min intervals during 24 h, and $^{13}CO_2$ and total CO_2 excretion was measured in 6-min intervals. Plasma samples are analyzed for ^{13}C enrichment of glucose. In addition, a selection of the plasma samples is analyzed for isotopomers of glucose for estimating gluconeogenesis.

Effects of type 2 nutrient synchrony on 24-h urea production and leucine oxidation are studied. Calves were housed in a respiration chamber and were continuously infused with [$^{15}N^{15}N$]urea and L-[1-^{13}C]leucine. Blood samples were collected in 30-min intervals during 24 h, and $^{13}CO_2$ and total CO_2 excretion was measured in 6-min intervals. Plasma samples are analyzed for ^{15}N enrichment of urea and for ^{13}C enrichment of leucine. Isotopomers of urea ($^{14}N^{14}N$, $^{14}N^{15}N$ and $^{15}N^{15}N$) are analyzed in urine to estimate urea recycling to the gut.

Type 2 nutrient synchrony, glucose metabolism and fat deposition

Effects of an asynchronous intake of protein and lactose (treatments 1 and 4 from Chapter 6) on performance and slaughter characteristics in calves were determined under practical conditions. Postprandial plasma glucose and insulin responses are measured. Lipids in various tissues are characterized. Molecular biology and genomics tools may be used to identify essential processes in energy and glucose metabolism in preruminant calves.

Type 3 nutrient synchrony: asynchronous availability of amino acids

The third type of nutrient synchrony (i.e. synchrony of individual amino acids; see Chapter 1) was studied. Calves were fed a milk replacer in which 80% of the crude protein originated from wheat gluten (i.e. strong deficiency in lysine and threonine). Synthetic amino acids were supplemented at various times relative to feeding. Nitrogen balance, urea production and dietary L-[1-^{13}C]leucine oxidation were measured.

Energetic costs of behaviour in calves

Energetic costs of behaviour and posture were quantified in heavy preruminant calves. Each calf (Chapter 3 and 6) was observed for 2 days during each experimental period. An ethogram was used to score its behaviour in 6 min intervals. These ethological data are merged with the simultaneous data on energy metabolism allowing quantification of energetic costs of behaviour for the standing and lying posture separately.

Lysine requirements in heavy preruminant calves

An indirect amino acid oxidation technique is used to quantify lysine requirements in heavy preruminant calves. Previous studies have not demonstrated a response in nitrogen balance to increasing lysine intake at a lysine-deficient diet (see Chapter 2), which may be due to large variation between individual calves and to high amounts of crystalline amino acids in the diet. In the current study, a lysine-deficient protein source (wheat protein) is used. Oxidation of a non-limiting amino acid is measured by oral supplementation of a ^{13}C-labelled amino acid, measurement of ^{13}C enrichment in the precursor pool (blood plasma) and in the expired CO_2. Oxidation of the non-limiting amino acid is expected to decrease with increasing lysine deposition in body protein.

Conclusions

- Heavy preruminant calves utilize protein with a very low marginal efficiency for protein gain compared with other farm animals. A lack of type 1 nutrient synchrony explains however only a minor portion of the low efficiency.

- Increasing the feeding frequency increased the digestible protein utilization for growth in heavy preruminant calves when a non-clotting protein source was used at either a low or a high feeding level.

- Dietary glucose is almost completely oxidized in heavy preruminant calves, and comparison between [U-^{13}C]glucose and [2-^{13}C]glucose indicates that glucose is not deposited as body fat. An increase in feed intake increased glucose but decreased fatty acid oxidation.

- A complete separation of protein and starch intake in time decreased the efficiency of protein retention from 57 to 47% in growing pigs.

- A partial separation of protein and lactose intake in time did not affect protein retention but substantially decreased heat production and increased both fat retention and intramuscular fat content in heavy preruminant calves.

- Oxidative enzyme activities in skeletal muscle were not correlated with average whole body heat production in heavy preruminant calves, but citrate synthase activity in muscle showed a positive correlation with the within-day fluctuations in activity related heat production.

- A decrease in the feeding level and/or increase in the feeding frequency decreased postprandial insulin/glucose ratios and urinary glucose excretion in heavy preruminant calves.

- Synchronizing protein and lactose intake within a day increased postprandial insulin to glucose ratios, but decreased urinary glucose excretion.

- Separating protein and lactose intake within a day strongly increased the response of plasma glucose concentration to the high lactose meal, but decreased the response of plasma insulin concentration.

References

Allee GL, Baker DH & Leveille GA (1971) Fat utilization and lipogenesis in the young pig. *J Nutr* **101**, 1415-1422.

Bergen WG & Mersmann HJ (2005) Comparative aspects of lipid metabolis: impact on contemporary research and use of animal models. *J Nutr* **135**, 2499-2502.

Bikker P, Verstegen MWA & Campbell RG (1996) Performance and body composition of finishing gilts (45 to 85 kilograms) as affected by energy intake and nutrition in earlier life: II. Protein and lipid accretion in body components. *J Anim Sci* **74**, 817-826.

Björck I, Liljeberg H & Östman E (2000) Low glycaemic-index foods. *Br J Nutr* **83**, S149-S155.

Blaak EE & Wagenmakers AJM (2002) The fate of [U-^{13}C]palmitate extracted by skeletal muscle in subjects with type 2 diabetes and control subjects. *Diabetes* **51**, 784-789.

Blazquez R & Lopez Quijada C (1970) The effect of a high-protein diet on plasma glucose concentration, insulin sensitivity and plasma insulin in rats. *J Endocrinol* **46**, 445-451.

Blum JW & Hammon HM (1999) Endocrine and metabolic aspects in milk-fed calves. *Domest Anim Endocrinol* **17**, 219-230.

Boirie Y, Dangin M, Gachon P, Vasson MP, Maubois JL & Beaufrère B (1997) Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proc Natl Acad Sci* **94**, 14930-14935.

Bos C, Metges CC, Gaudichon C, Petzke KJ, Pueyo ME, Morens C, Everwand J, Benamouzig R & Tomé D (2003) Postprandial kinetics of dietary amino acids are the main determinant of their metabolism after soy or milk protein ingestion in humans. *J Nutr* **133**, 1308-1315.

Calbet JAL & MacLean DA (2002) Plasma glucagon and insulin responses depend on the rate of appearance of amino acids after ingestion of different protein solutions in humans. *J Nutr* **132**, 2174-2182.

Cline GW, Petersen KF, Krssak M, Shen J, Hundal RS, Trajanoski Z, Inzucchi S, Dresner A, Rothman DL & Shulman GI (1999) Impaired glucose transport as a cause of decreased insulin-stimulated muscle glycogen synthesis in type 2 diabetes. *N Engl J Med* **341**, 240-246.

Cronjé PB (2000) Nutrient-gene interactions: future potential and applications. In *Ruminant Physiology: digestion, metabolism, growth and reproduction*, pp. 409-422 [PB Cronjé, editor]. Oxon, UK: CAB International.

Dangin M, Boirie Y, Garcia-Rodenas C, Gachon P, Fauquant J, Callier P, Ballèvre O & Beaufrère B (2001) The digestion rate of protein is an independent regulating factor of postprandial protein retention. *Am J Physiol* **280**, E340-E348.

Donkin SS (1999) Role of the endocrine pancreas in animal metabolism, growth and performance. In *Biology of the pancreas in growing animals*, pp. 315-328 [SG Pierzynowski and R Zabielski, editors]. Amsterdam: Elsevier Science BV.

Doppenberg J & Palmquist DL (1991) Effect of dietary fat level on feed intake, growth, plasma metabolites and hormones of calves fed dry or liquid diets. *Livest Prod Sci* **29**, 151-158.

Dunshea FR, Harris DM, Bauman DE, Boyd RD & Bell AW (1992) Effect of somatotropin on in vivo glucose kinetics and lipogenesis in growing pigs. *J Anim Sci* **70**, 141-151.

Fernández-Fígares I, Shannon AE, Wray-Cahen D & Caperna TJ (2004) The role of insulin, glucagon, dexamethasone, and leptin in the regulation of ketogenesis and glycogen storage in primary cultures of porcine hepatocytes prepared from 60 kg pigs. *Domest Anim Endocrinol* **27**, 125-140.

Fineman MS, Koda JE, Shen LZ, Strobel SA, Maggs DG, Weyer C & Kolterman OG (2002) The human amylin analog, pramlintide, corrects postprandial hyperglucagonemia in patients with type 1 diabetes. *Metabolism* **51**, 636-641.

Frayn KN (2000) Visceral fat and insulin resistance - causative or correlative. *Br J Nutr* **83**, S71-S77.

Frayn KN (2003) The glucose-fatty acid cycle: a physiological perspective. *Biochem Soc Transact* **31**, 1115-1119.

Freyse EJ & Knospe S (1998) Estimation of urea production rate with [^{15}N$_2$]urea and [^{13}C]urea to measure catabolic rates in diabetes mellitus. *Isotopes Environ Health Stud* **34**, 107-118.

Garvey WT & Hermayer KL (1998) Clinical implications of the insulin resistance syndrome. *Clin Cornerstone* **1**, 13-26.

Gerrits WJJ & Blum JW (1998) A role of protein intake in the development of insulin resistance in preruminant calves. In *Symposium on Growth in Ruminants: Basic Aspects, Theory and Practice for the Future*, pp. 310 (Abstr) [JW Blum, T Elsasser and P Guilloteau, editors]. Berne, Switzerland.

Gerrits WJJ, Tolman GH, Schrama JW, Tamminga S, Bosch MW & Verstegen MWA (1996) Effect of protein and protein-free energy intake on protein and fat deposition rates in preruminant calves of 80 to 240 kg live weight. *J Anim Sci* **74**, 2129-2139.

Golay A, DeFronzo RA, Ferrannini E, Simonson DC, Thorin D, Acheson K, Thiébaud D, Curchod B, Jéquier E & Felber JP (1988) Oxidative and non-oxidative glucose metabolism in non-obese Type 2 (non-insulin-dependent) diabetic patients. *Diabetologica* **31**, 585-591.

Goodpaster BH, He J, Watkins S & Kelley DE (2000) Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. *J Clin Endocrinol Metab* **86**, 5755-5761.

Himsworth HP (1935) The dietetic factor determining the glucose tolerance and sensitivity to insulin of healthy men. *Clin Sci* **2**, 67-94.

Hostettler-Allen RL, Tappy L & Blum JW (1994) Insulin resistance, hyperglycemia, and glucosuria in intensively milk-fed calves. *J Anim Sci* **75**, 160-173.

Hugi D, Bruckmaier RM & Blum JW (1997) Insulin resistance, hyperglycemia, glucosuria, and galactosuria in intensively milk-fed calves: dependency on age and effects of high lactose intake. *J Anim Sci* **75**, 469-482.

Hugi D, Tappy L, Sauerwein H, Bruckmaier RM & Blum JW (1998) Insulin-dependent glucose utilization in intensively milk-fed calves is modulated by supplemental lactose in an age-dependent manner. *J Nutr* **128**, 1023-1030.

Kaufhold JN, Hammon HM, Bruckmaier RM, Breier BH & Blum JW (2000) Postprandial metabolism and endocrine status in veal calves fed at different fequencies. *J Dairy Sci* **83**, 2480-2490.

Krebs M, Krssak M, Bernroider E, Anderwald C, Brehm A, Meyerspeer M, Nowotny P, Roth E, Waldhäusl W & Roden M (2002) Mechanism of amino acid-induced skeletal muscle insulin resistance in humans. *Diabetes* **51**, 599-605.

Kusunoki M, Tsutsumi K, Hara T, Ogawa H, Nakamura T, Miyata T, Sakakibara F, Fukuzawa Y, Suga T, Kakumu S & Nakaya Y (2002) Correlation between lipid and glycogen contents in liver and insulin resistance in high-fat-fed rats treated with the lipoprotein lipase activator NO-1886. *Metabolism* **51**, 792-795.

Manders RJ, Koopman R, Sluijsmans WE, Van den Berg R, Verbeek K, Saris WH, Wagenmakers AJ & Van Loon LJ (2006) Co-ingestion of a protein hydrolysate with or without additional leucine effectively reduces postprandial blood glucose excursions in type 2 diabetic men. *J Nutr* **136**, 1294-1299.

Mari A, Ahren B & Pacini G (2005) Assessment of insulin secretion in relation to insulin resistance. *Curr Opin Clin Nutr Metab Care* **8**, 529-533.

McGarry JD (2002) Dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. *Diabetes* **51**, 7-18.

Moller DE & Flier JS (1991) Insulin resistance - Mechanisms, syndromes, and implications. *N Engl J Med* **325**, 938-948.

Nair KS, Halliday D, Ford GC, Heels S & Garrow JS (1987) Failure of carbohydrate to spare leucine oxidation in obese subjects. *Int J Obes* **11**, 537-544.

Nussbaum A, Schiessler G, Hammon HM & Blum JW (2002) Growth performance and metabolic and endocrine traits in calves pair-fed by bucket or by automate starting in the neonatal period. *J Anim Sci* **80**, 1545-1555.

Palmquist DL, Doppenberg J, Roehrig KL & Kinsey DJ (1992) Glucose and insulin metabolism in ruminating and veal calves fed high and low fat diets. *Domest Anim Endocrinol* **9**, 233-241.

Perley MJ & Kipnis DM (1967) Plasma insulin responses to oral and intravenous glucose: studies in normal and diabetic subjects. *J Clin Invest* **46**, 1954-1962.

Pethick DW (1984) Energy metabolism of skeletal muscle. *Ruminant Physiology Concepts and Consequences*, 277-287.

Polonsky KS & Rubenstein AH (1984) C-peptide as a measure of the secretion and hepatic extraction of insulin. Pitfalls and limitations. *Diabetes* **33**, 486-494.

Ponter AA, Salter DN, Morgan LM & Flatt PR (1991) The effect of energy source and feeding level on the hormones of the entero-insular axis and plasma glucose in the growing pig. *Br J Nutr* **66**, 187-197.

Rabinowitch IM, Frith AB & Bzain EV (1925) Simultaneous respiratory exchange and blood sugar time curves obtained in diabetic and non diabetic individuals following ingestion of glucose. *J Clin Invest* **2**, 143–156.

Rabinowitz D, Merimee TJ, Maffezzoli R & Burgess JA (1966) Patterns of hormonal release after glucose, protein, and glucose plus protein. *Lancet* **2**, 454-456.

Randles WG (2001) The effects of different temporal patterns of post-ruminal energy and protein supply on nitrogen metabolism in growing lambs. PhD thesis, University of Aberdeen, UK.

Ravikumar B, Carey PE, Snaar JEM, Deelchand DK, Cook DB, Neely RDG, English PT, Firbank MJ, Morris PG & Taylor R (2005) Real-time assessment of postprandial fat storage in liver and skeletal muscle in health and type 2 diabetes. *Am J Physiol* **288**, E789-E797.

Ravussin E, Bogardus C, Schwartz RS, Robbins DC, Wolfe RR, Horton ES, Danforth E & Sims EAH (1983) Thermic effect of infused glucose and insulin in man. *J Clin Invest* **72**, 893-902.

Reaven GM (2005) The insulin resistant syndrome: definition and dietary approaches in treatment. *Ann Rev Nutr* **25**, 391-406.

Roehrig K, Nestor KE & Palmquist DL (1988) ATP citrate lyase activity in liver and adipose tissue of veal or ruminating calves (*Bos Taurus*). *Comp Biochem Physiol* **90B**, 147-149.

Romsos DR, Miller ER & Leveille GA (1978) Influence of feeding frequency on body weight and glucose tolerance in the pig. *Proc Soc Exp Biol Med* **157**, 528-530.

Rossetti L, Rothman DL, DeFronzo RA & Shulman GI (1989) Effect of dietary protein on in vivo insulin action and liver glycogen repletion. *Am J Physiol* **257**, E212-219.

Ruzzin J, Péronnet F, Tremblay J, Massicotte D & Lavoie C (2003) Breath [$^{13}CO_2$] recovery from an oral glucose load during exercise: comparison between [U-^{13}C] and [2-^{13}C]glucose. *J Appl Physiol* **95**, 477-482.

Schoonmaker JP, Fluharty FL & Loerch SC (2004) Effect of source and amount of energy and rate of growth in the growing phase on adipocyte cellularity and lipogenic enzyme activity in the intramuscular and subcutaneous fat depots of Holstein steers. *J Anim Sci* **82**, 137-148.

Schwarz J-M, Linfoot P, Dare D & Aghajanian K (2003) Hepatic de novo lipogenesis in normoinsulinemic and hyperinsulinemic subjects consuming high-fat, low-carbohydrate and low-fat, high carbohydrate isoenergetic diets. *Am J Clin Nutr* **77**, 43-50.

Shulman GI (2000) Cellular mechanisms of insulin resistance. *J Clin Invest* **106**, 171-176.

Sidossis LS & Wolfe RR (1996) Glucose and insulin-induced inhibition of fatty acid oxidation: the glucose-fatty acid cycle reversed. *Am J Physiol* **270**, E733-E738.

Stanley CC, Williams CC, Jenny BF, Fernandez JM, Bateman II HG, Nipper WA, Lovejoy JC, Gantt DT & Goodier GE (2002) Effects of feeding milk replacer once versus twice daily on glucose metabolism in Holstein and Jersey calves. *J Dairy Sci* **85**, 2335-2343.

Storlien LH, Baur LA, Kriketos AD, Pan DA, Cooney GJ, Jenkins AB, Calvert GD & Campbell LV (1996) Dietary fats and insulin action. *Diabetologica* **39**, 621-631.

Suárez BJ (2006) Rumen development in veal calves. PhD thesis, Wageningen University.

Toffolo G, Campioni M, Basu R, Rizza RA & Cobelli C (2006) A minimal model of insulin secretion and kinetics to assess hepatic insulin extraction. *Am J Physiol* **290**, E169-176.

Traxinger RR & Marshall S (1989) Role of amino acids in modulating glucose-induced desensitization of the glucose transport system. *J Biol Chem* **264**, 20910-20916.

Unger RH (1985) Glucagon physiology and pathophysiology in the light of new advances. *Diabetologica* **8**, 574-578.

Van den Borne JJGC, Alferink SJJ & Gerrits WJJ (2004) Identifying ruminal drinking by measurement of respiratory quotient and methane production in preruminant calves. *J Anim Sci* **82, Suppl. 1**, 365 (Abstr.).

Van den Brand H, Dieleman SJ, Soede NM & Kemp B (2000) Dietary energy source at two feeding levels during lactation of primiparous sows: I. Effects on glucose, insulin, and luteinizing hormone and on follicle dvelopment, weaning-to-estrus interval, and ovulation rate. *J Anim Sci* **78**, 396-404.

Van Loon LJC, Saris WHM, Kruijshoop M & Wagenmakers AJM (2000) Maximizing postexercise muscle glycogen synthesis: carbohydrate supplementation and the application of amino acid or protein hydrolysate mixtures. *Am J Clin Nutr* **72**, 106-111.

Vicari T, Van den Borne JJGC, Gerrits WJJ, Zbinden Y & Blum JW Postprandial blood hormone and metabolite concentrations are differentially influenced by feeding frequency and feeding level in veal calves. *Domest Anim Endocrinol*, submitted.

Vicari T, Van den Borne JJGC, Gerrits WJJ, Zbinden Y & Blum JW Synchronizing protein and lactose intake increases the postprandial insulin response in veal calves. *Domest Anim Endocrinol,* submitted.

Wester TJ, Lobley GE, Birnie LM, Crompton LA, Brown S, Buchan V, Calder AG, Milne E & Lomax MA (2004) Effect of plasma insulin and branched-chain amino acids on skeletal muscle protein synthesis in fasted lambs. *Br J Nutr* **92**, 401-409.

Wiesenthal SR, Sandhu H, McCall RH, Tchipashvili V, Yoshii H, Polonsky K, Shi ZQ, Lewis GF, Mari A & Giacca A (1999) Free fatty acids impair hepatic insulin extraction in vivo. *Diabetes* **48**, 766-774.

Wijayasinghe MS, Smith NE & Baldwin RL (1984) Growth, health and blood glucose concentrations of calves fed high-glucose or high-fat milk replacers. *J Dairy Sci* **67**, 2949-2956.

Wolever TMS (2000) Dietary carbohydrates and insulin action in humans. *Br J Nutr* **83**, S97-S102.

Wu G, Knabe DA & Kim SW (2004) Arginine nutrition in neonatal pigs. *J Nutr* **134**, 2783S-2790S.

Yki-Järvinen H (1992) Glucose toxicity. *Endocr Rev* **13**, 415-431.

Yki-Jarvinen H, Bogardus C & Howard BV (1987) Hyperglycemia stimulates carbohydrate oxidation in humans. *Am J Physiol* **253**, E376-E382.

Zawadzki KM, Yaspelkis BB, 3rd & Ivy JL (1992) Carbohydrate-protein complex increases the rate of muscle glycogen storage after exercise. *J Appl Physiol* **72**, 1854-1859.

Summary

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Samenvatting

Summary

Introduction

In animal nutrition, feedstuffs are classically characterized by their chemical composition (i.e. protein, fat and carbohydrates), and average values are adopted in feed evaluation tables. These tabulated values are used to compose diets and to match dietary nutrient supply with nutrient requirements on a daily basis. Nonetheless, there may be periods within a day during which the supply and requirements are not in harmony due to circadian variation in both. Fluctuations in the nutrient availability within the day are known to be large. These are induced by the feeding pattern and by the characteristics of feed ingredients. For example, animals that are fed only once or twice daily will usually have larger fluctuations in nutrient availability than *ad libitum* fed animals, and using 'slow' protein sources (such as casein) results in a more constant amino acid availability than using 'fast' protein sources (such as vegetable proteins or whey). The time at which different nutrients (e.g. protein and carbohydrates) become available may also be different.

We defined three types of nutrient synchrony (Chapter 1) of which two were studied in this thesis (i.e. type 1 and 2 nutrient synchrony). Type 1 nutrient synchrony concerns the matching of nutrient availability with requirements within a day, whereas type 2 nutrient synchrony concerns the simultaneity of protein (i.e. amino acids) and glucose availability.

The heavy preruminant calf (i.e. exceeding 100 kg of body weight) was used as a model to study these two types of nutrient synchrony for two reasons. Firstly, heavy preruminant calves utilize protein with a very low efficiency for protein deposition compared with young calves. This efficiency is also low when compared with other farm animal species, such as pigs and milk-fed lambs, at a similar stage of maturity (Chapter 2). An asynchronous nutrient supply was identified as one of the potential reasons for the low efficiency (Chapter 2). Secondly, calves are commonly fed twice daily and nutrient absorption is expected to be peak-wise. The replacement of skimmed milk protein (a slow, clotting protein source) by vegetable proteins and whey (fast, non-clotting protein sources) during the past decades has probably amplified these within-day fluctuations in nutrient availability (especially for amino acids).

Hypotheses

Type 1 nutrient synchrony

Effects of feeding frequency on nitrogen and energy retention (Chapter 3) and on urea production and within-day nutrient oxidation (Chapter 4) were studied at two feeding levels in heavy preruminant calves. In Chapter 3, it was hypothesized that an increased feeding

frequency would increase protein retention by an improved synchrony between the supply of and requirements for protein during the day. The effects on protein utilization were expected to be more pronounced at a high than at a low feeding level. Moreover, it was hypothesized that substantial amounts of glucose were used for de novo fatty acid synthesis and that feeding frequency and feeding level would affect fractional glucose oxidation in heavy preruminant calves (Chapter 4). Increasing the feeding frequency was expected to improve glucose homeostasis and decrease urinary glucose excretion (glucosuria) in heavy preruminant calves (Chapter 8).

Type 2 nutrient synchrony

Separating protein and carbohydrate intake within a day was hypothesized to decrease protein retention both in growing pigs (Chapter 5) and in preruminant calves (Chapter 6). A reduced protein retention was expected to result in either an increase in fat deposition or an increase in heat production. In addition, it was hypothesized that oxidative enzyme activities would decrease and intramuscular fat content would increase with decreasing nutrient synchrony in preruminant calves.

Methodology and results

Type 1 nutrient synchrony

In Chapter 3, 18 Holstein Friesian calves of were assigned to a feeding frequency (1, 2, or 4 meals daily) at two feeding levels (1.5 or 2.5 times the metabolizable energy requirements for maintenance), except for calves fed once daily (only at a low feeding level). Calves were individually housed in respiration chambers during 2 experimental periods of 10 d. Whey protein, which is a quickly hydrolysed protein source, was used as only protein source in the diet. Apparent faecal nutrient digestibility was determined and nitrogen and energy balances were measured. Increasing feeding frequency increased the efficiency of digestible protein utilization in calves. The increase was greater at a high feeding level (+11% from 2 to 4 meals/d) than at a low feeding level (+5% from 2 to 4 meals/d), but no significant interaction occurred. An increased feeding frequency and a higher feeding level enhanced fat deposition. Heat production was not affected by feeding frequency, but its circadian rhythm differed considerably between feeding frequencies.

In Chapter 4, using the same animals as in Chapter 3, fractional oxidation of orally provided 1-[1-^{13}C]leucine, [U-^{13}C]glucose and [2-^{13}C]glucose was studied to assess the pattern of dietary substrate oxidation and to estimate *de novo* fatty acid synthesis from glucose. $^{13}CO_2$

excretion in breath was measured after oral supply of the stable isotopes, and gas exchange were measured by indirect calorimetry during the two experimental periods in respiration chambers. After each period, urea production was measured by dilution of a primed, continuous infusion of $[^{13}C]$urea. Diurnal kinetics of urea production, but not total urea production, were affected by feeding frequency in heavy preruminant calves (Chapter 4). Postprandial urea production increased with increasing feeding level, but 12 h production rates did not differ between treatments. Daily production rates correlated well with urinary N excretion. The $^{13}CO_2$ recovery from orally supplied L-$[1-^{13}C]$leucine did not correspond with whole body protein utilization and was not correlated with urinary N excretion (as proportion of N intake). Orally supplied glucose was almost completely oxidized (80% from $[^{13}C]$glucose and 94% from indirect calorimetry), regardless of the feeding level. In addition, because recoveries of $[U-^{13}C]$glucose and $[2-^{13}C]$glucose as CO_2 did not differ, *de novo* fatty acid synthesis from glucose was considered negligible in preruminant calves.

In addition, an increased feeding frequency decreased postprandial plasma glucose and insulin concentrations, insulin to glucose ratios and glucose excretion in urine in calves (Chapter 8).

Type 2 nutrient synchrony

In Chapter 5, 10 pigs were assigned to each of two dietary treatments (synchronous vs. asynchronous nutrient supply) in a change-over design. At the synchronous treatment, pigs received two balanced meals: one at 08.00 h and one at 16.00 h. At the asynchronous treatment, pigs received virtually all protein at 08.00 h and all carbohydrates at 16.00 h. Daily intakes of all nutrients and dietary ingredients were similar for both treatments. Pigs were housed individually in respiration chambers. Apparent faecal nutrient digestibility was determined and nitrogen and energy balances were measured. The efficiency of utilization of digestible protein for protein gain was higher for synchronously (56.7%) than for a synchronously fed pigs (47.1%). Activity related heat production tended to be lower for a synchronously than for synchronously fed pigs. Amino acid oxidation appeared to be increased after the high protein meal for asynchronously compared with synchronously fed pigs.

In Chapter 6, 38 preruminant calves were assigned to one of six degrees of nutrient synchrony and to one of two meal sequences (i.e. the high protein meal in the morning or in the evening). Calves at the synchronous treatment received two balanced meals: one at 06.00 h and one at 18.00 h. Nutrient synchrony decreased stepwise to the most asynchronous treatment in which calves received 85% of the daily protein supply in one meal. The

digestible energy intakes at 06.00 h and 18.00 h were equal between treatments. Daily intakes of all nutrients and dietary ingredients were similar for all treatments. Calves were housed individually in respiration chambers. Apparent faecal nutrient digestibility and nitrogen and energy balances were measured. Apparent nutrient digestibility decreased when more than 71% of the dietary protein was fed in one meal. Nutrient synchrony did not affect the efficiency of digestible protein utilization in calves at a similar digestible nutrient intake. Heat production decreased from 691 to 629 kJ/(kg$^{0.75}$·d) and energy retained as fat increased from 116 to 184 kJ/(kg$^{0.75}$·d) with decreasing nutrient synchrony.

In Chapter 7, using the same animals as in Chapter 6, the effects of asynchronous availability of amino acids and glucose on muscle composition and enzyme activities in skeletal muscle were studied. Activities of citrate synthase, cytochrome-*c*-oxidase and lactate dehydrogenase were determined in an oxidative muscle, *M. Rectus Abdominis*, and in a glycolytic muscle, *M. Semitendinosus*. Intramuscular fat and glycogen content were measured in the same muscles. The correlation between oxidative enzyme activities and whole-body heat production (Chapter 6) was studied. Oxidative enzyme activities and fat content increased with decreasing nutrient synchrony in *M. Rectus Abdominis*, but not in *M. Semitendinosus* of preruminant calves (Chapter 7). Oxidative enzyme activities in both muscles were not correlated with average daily heat production, but citrate synthase activity in *M. Rectus Abdominis* was positively correlated with the circadian amplitude and maximum of heat production related to physical activity.

In addition, simultaneous ingestion of lactose and protein resulted in a much larger insulin response in heavy preruminant calves than when ingested separately (Chapter 8). Despite very high plasma glucose concentrations after the high lactose meal in asynchronously fed calves, insulin concentrations increased only slightly and not more than after the high protein meal.

Main conclusions

Type 1 nutrient synchrony

Increasing feeding frequency improved the efficiency with which protein was utilized for deposition in heavy preruminant calves.

Despite the high lactose intakes, it appeared that fatty acid synthesis *de novo* from glucose is negligible in heavy preruminant calves. Therefore, increased fat deposition rates observed at higher levels of feed intake were mainly caused by a reduced oxidation of fatty acids.

Postprandial insulin to glucose ratios and decreased glucose excretion in urine indicated that insulin sensitivity and glucose homeostasis improved with increasing feeding frequency.

Type 2 nutrient synchrony

In pigs, an asynchronous availability of glucose and amino acids within a day increased amino acid oxidation, resulting in a substantial reduction in protein utilization but with virtually no effect on fat retention.

Unlike in pigs, in preruminant calves, an asynchronous availability of amino acids and glucose within a day did not decrease the efficiency of protein utilization, but substantially increased fat retention. Separating the intake of protein and lactose over meals inhibited postprandial plasma insulin responses, and increased the glucose excretion in urine. In addition, intramuscular fat and oxidative enzyme activities increased with decreasing nutrient synchrony in an oxidative muscle in calves.

Samenvatting

Inleiding

In de diervoeding worden grondstoffen gekenmerkt door de gehalten aan voedingsstoffen (eiwit, vet en koolhydraten). De gemiddelde gehalten worden weergegeven in voederwaarderingstabellen en worden gebruikt om voeders samen te stellen, zodat het aanbod aan voedingsstoffen overeenkomt met de dagelijkse behoefte van dieren. Binnen een dag kunnen er echter perioden optreden dat het aanbod van en de vraag naar voedingsstoffen niet overeenstemmen vanwege variatie in zowel vraag als aanbod. Met name fluctuaties in de beschikbaarheid van voedingsstoffen binnen een dag kunnen groot zijn. Deze worden beïnvloed door de voederstrategie en de kenmerken van de gebruikte grondstoffen. Bijvoorbeeld, dieren die een- of tweemaal daags worden gevoerd zullen grotere veranderingen in de beschikbaarheid van voedingsstoffen binnen een dag ondervinden dan dieren die *ad libitum* (onbeperkt) worden gevoerd. Tevens kunnen langzame eiwitbronnen (zoals caseine) resulteren in een meer constante beschikbaarheid van aminozuren dan snelle eiwitbronnen (zoals plantaardig eiwit of wei-eiwit). Hierdoor kunnen er ook verschillen ontstaan tussen de tijdstippen waarop de voedingsstoffen (bijv. eiwit en koolhydraten) ten opzichte van elkaar beschikbaar komen voor de stofwisseling van het dier. We hebben drie typen nutriëntsynchronisatie gedefinieerd (Hoofdstuk 1) waarvan er twee zijn bestudeerd in dit proefschrift (type 1 en 2 nutriëntsynchronisatie). Type 1 nutriëntsynchronisatie betreft de overeenstemming tussen de beschikbaarheid van nutriënten met de behoefte aan nutriënten binnen een dag, terwijl type 2 nutriëntsynchronisatie de afstemming tussen individuele voedingsstoffen betreft (in dit proefschrift eiwit vs. koolhydraten).

Er waren twee redenen om het zware vleeskalf (> 100 kg lichaamsgewicht) als modeldier te gebruiken om deze twee typen nutriëntsynchronisatie te bestuderen. Ten eerste is de efficiëntie waarmee voedingseiwit wordt gebruikt voor eiwitaanzet erg laag in zware vleeskalveren vergeleken met jonge (lichtere) kalveren. Deze efficiëntie is ook erg laag vergeleken met andere landbouwhuisdieren, zoals varkens en melkgevoerde lammeren, in eenzelfde ontwikkelingsstadium (Hoofdstuk 2). Een asynchroon aanbod van voedingsstoffen zou één van de mogelijke redenen voor deze lage efficiëntie kunnen zijn (Hoofdstuk 2). Ten tweede worden kalveren vaak tweemaal daags gevoerd waardoor de absorptie van voedingsstoffen pieksgewijs is binnen een dag. De vervanging van magere melkpoeder (een langzame, stremmende eiwitbron) door plantaardige eiwitten en wei-eiwit (snelle, niet-stremmende eiwitbronnen) gedurende de afgelopen decennia heeft deze variatie in aanbod binnen een dag waarschijnlijk versterkt (vooral voor aminozuren).

Hypotheses

Type 1 nutriëntsynchronisatie

Effecten van voerfrequentie in zware vleeskalveren op de eiwit- en energiebalans (Hoofdstuk 2) en op de ureumproductie en binnendag oxidatie van voedingsstoffen (Hoofdstuk 4) werden bestudeerd op twee voerniveaus. In Hoofdstuk 3 werd de hypothese getest dat een verhoging van de voerfrequentie de eiwitaanzet zou verhogen vanwege een betere synchronisatie tussen het aanbod van en de vraag naar voedingsstoffen gedurende een dag. We verwachtten dat de effecten groter zouden zijn op een hoog dan op een laag voerniveau. Bovendien was de verwachting dat aanzienlijke hoeveelheden glucose zouden worden gebruikt voor vetzuursynthese en dat de voerfrequentie en voerniveau de fractionele oxidatie van glucose in zware vleeskalveren zou beïnvloeden (Hoofdstuk 4). Tevens werd verondersteld dat een verhoging van de voerfrequentie zou leiden tot een betere homeostase van de glucosestofwisseling en minder uitscheiding van glucose in de urine (glucosurie) in zware vleeskalveren (Hoofdstuk 8).

Type 2 nutriëntsynchronisatie

De hypothese was dat een scheiding van de opname van eiwit en koolhydraten zou resulteren in een verlaagde eiwitaanzet in zowel varkens (Hoofdstuk 5) als vleeskalveren (Hoofdstuk 6). We verwachtten dat de verlaagde eiwitaanzet gepaard zou gaan met hetzij een verhoogde warmteproductie of een verhoogde vetaanzet. Ook werd verwacht dat de oxidatieve enzymactiviteit zou afnemen en het intramusculair vetgehalte zou toenemen met verminderde nutriëntsynchronisatie in vleeskalveren.

Methodologie en resultaten

Type 1 nutriëntsynchronisatie

In Hoofdstuk 3 werden 18 Holstein-Friesian kalveren toegewezen aan een voerfrequentie (1, 2 of 4 voerbeurten per dag) op twee voerniveaus (1.5 of 2.5 maal de metaboliseerbare energiebehoefte voor onderhoud), behalve kalveren die eenmaal daags warden gevoerd (alleen op het lage voerniveau). De kalveren werden individueel gehuisvest in klimaatrespiratiecellen gedurende twee experimentele perioden van elk 10 d. Wei-eiwit, een eiwit dat snel wordt gehydrolyseerd, werd als enige eiwitbron in de voeding gebruikt. De schijnbare fecale verteerbaarheden van voedingsstoffen werden bepaald en stikstof- en energiebalansen werden gemeten. Een verhoging van de voerfrequentie verhoogde de efficiëntie waarmee kalveren verteerbaar eiwit gebruikten om aan te zetten als lichaamseiwit.

Deze toename in efficiëntie was groter op een hoog voerniveau (+11% van 2 naar 4 voerbeurten per dag) dan op een laag voerniveau (+5% van 2 naar 4 voerbeurten per dag), maar een interactie tussen voerfrequentie en –niveau kon niet worden aangetoond. Een verhoogde voerfrequentie en een hoger voerniveau verhoogden de vetaanzet. De warmteproductie werd niet beïnvloed door de voerfrequentie, maar de variatie in warmteproductie binnen een dag verschilde sterk tussen voerfrequenties.

In Hoofdstuk 4 werd de fractionele oxidatie van met de voeding opgenomen L-[1-^{13}C]leucine, [U-^{13}C]glucose en [2-^{13}C]glucose bestudeerd om het patroon van de oxidatie van substraten uit de voeding vast te stellen en om *de novo* vetzuursynthese uit glucose te bepalen. $^{13}CO_2$ uitscheiding in ademlucht werd gemeten na de orale toediening van deze stabiele isotopen en de gaswisseling werd gemeten via indirecte calorimetrie gedurende de twee experimentele perioden in respiratiecellen. Na elke periode werd de ureumproductie gemeten via verdunning van een continue infusie van [^{13}C]ureum. Voerfrequentie had effect op de binnendagpatronen van de ureumproductie, maar niet de totale ureumproductie in zware vleeskalveren. De ureumproductie na een voerbeurt nam toe met een toename in voerniveau, maar de 12 u productie verschilde niet tussen behandelingen. De dagelijkse ureumproductie vertoonde een goede correlatie met de stikstofuitscheiding in urine. De recovery van oraal gesupplementeerd L-[1-^{13}C]leucine als $^{13}CO_2$ stemde niet overeen met de eiwitbenutting en was niet gecorreleerd aan de stikstofuitscheiding in urine (als percentage van stikstofopname). Koolhydraten uit de voeding werden nagenoeg volledig geoxideerd (80% via [^{13}C]glucose en 94% via indirect calorimetry), ongeacht het voerniveau. Aangezien de recovery van [U-^{13}C]glucose als CO_2 niet verschilde van die van [2-^{13}C]glucose, werd geconcludeerd dat *de novo* vetzuursynthese uit glucose kwantitatief niet belangrijk was in deze vleeskalveren.

Een verhoging van de voerfrequentie resulteerde in een afname van de glucose- en insulineconcentraties in bloedplasma, lagere insuline:glucose ratios en minder uitscheiding van glucose in urine in kalveren (Hoofdstuk 8).

Type 2 nutriëntsynchronisatie

In Hoofdstuk 5 werden 10 varkens toegewezen aan elk van de twee behandelingen (synchroon vs. asynchroon aanbod van voedingsstoffen) in een change-over experiment. Op de synchrone behandeling werd aan de varkens tweemaal (om 08.00 u en 16.00 u) eenzelfde, gebalanceerd voer verstrekt. Op de asynchrone behandeling werd nagenoeg de gehele dagelijkse hoeveelheid eiwit om 08.00 u en de gehele dagelijkse hoeveelheid koolhydraten om 16.00 u verstrekt. De dagelijkse opname van alle voedingsstoffen en grondstoffen was

gelijk tussen de behandelingen. Varkens werden individueel gehuisvest in klimaatrespiratiecellen. De schijnbare fecale verteerbaarheden van voedingsstoffen werden bepaald en stikstof- en energiebalansen werden gemeten. De efficiëntie waarmee eiwit werd benut voor eiwitaanzet was hoger voor synchroon (56.7%) dan voor asynchroon gevoerde varkens (47.1%). De activiteitgerelateerde warmteproductie neigde lager te zijn voor synchroon dan voor asynchroon gevoerde varkens. Oxidatie van aminozuren was hoger na een eiwitrijke maaltijd in asynchroon gevoerde dieren dan na een maaltijd in synchroon gevoerde dieren.

In Hoofdstuk 6 werden 38 vleeskalveren toegewezen aan één van de zes gradaties van nutriëntsynchronisatie en aan één van de twee volgorden van de voerbeurten (d.w.z. hoog eiwitvoerbeurt in de ochtend of in de avond). Kalveren op de synchrone behandeling kregen twee gebalanceerde voerbeurten: de eerste om 06.00 u en de tweede om 18.00 u. De mate van nutriëntsynchronisatie nam stapsgewijs af tot de meest asynchrone behandeling waarin 85% van de dagelijkse eiwitgift in één voerbeurt werd verstrekt. De verteerbare energieopname was gelijk om 06.00 u en 18.00 u en identiek tussen behandelingen. De dagelijkse opname van alle voedingsstoffen en grondstoffen was identiek tussen behandelingen. De kalveren werden individueel gehuisvest in klimaatrespiratiecellen. De schijnbare fecale verteerbaarheden van voedingsstoffen werden bepaald en stikstof- en energiebalansen werden gemeten. De schijnbare verteerbaarheid nam af indien meer dan 71% van de dagelijkse hoeveelheid eiwit in één voerbeurt werd verstrekt. Nutriëntsynchronisatie had geen invloed op de efficiëntie waarmee verteerbaar eiwit werd gebruikt voor eiwitaanzet in kalveren op een gelijke verteerbare nutriëntopname. De warmteproductie nam af van 691 tot 629 kJ/(kg$^{0.75}$·d) en de energieaanzet als vet nam toe van 116 tot 184 kJ/(kg$^{0.75}$·d) met afnemende nutriëntsynchronisatie.

In Hoofdstuk 7, werden de effecten van een asynchrone beschikbaarheid van aminozuren en glucose op de samenstelling van spieren en enzymactiviteit in spieren bestudeerd. De activiteit van de enzymen citraat synthase, cytochrome-*c*-oxidase en lactaat dehydrogenase werd bepaald in een oxidatieve spier, *M. Rectus Abdominis*, en in een glycolytische spier, *M. Semitendinosus*. Het intramusculair vet- en glycogeengehalte werd gemeten in dezelfde spieren. Teven werd de correlatie tussen de activiteit van oxidatieve enzymen op spierniveau en warmteproductie op dierniveau bestudeerd. De activiteit van oxidatieve enzymen en het intramusculair vetgehalte nam toe met afnemende nutriëntsynchronisatie in *M. Rectus Abdominis*, maar niet in *M. Semitendinosus* van vleeskalveren. De activiteit van oxidatieve enzymen in beide spieren was niet gecorreleerd aan de gemiddelde dagelijkse

warmteproductie, maar de activiteit van citraat synthase in *M. Rectus Abdominis* vertoonde een positieve correlatie met de amplitude en het maximum van de activiteitgerelateerde warmteproductie binnen een dag.

Een gelijktijdige opname van lactose en eiwit resulteerde in een veel hogere insulinerespons dan wanneer beide voedingsstoffen gescheiden werden opgenomen in zware vleeskalveren (Hoofdstuk 8). Ondanks de erg hoge glucoseconcentratie in het bloedplasma na de hoog lactosevoerbeurt was er slechts een geringe toename in de insulineconcentratie in asynchroon gevoerde kalveren.

Conclusies

Type 1 nutriëntsynchronisatie

Een verhoging van de voerfrequentie leidde tot een verhoogde efficiëntie waarmee verteerbaar eiwit werd gebruikt voor eiwitaanzet in zware vleeskalveren.

Ondanks de hoge lactoseopname in zware vleeskalveren, bleek dat de vetzuursynthese uit glucose nihil was. De toename in vetaanzet bij een verhoging van het voerniveau is daardoor vooral toe te schrijven aan een vermindering van de vetzuuroxidatie.

De lagere insuline:glucose ratios en verminderde uitscheiding van glucose in urine geeft aan dat de insulinegevoeligheid en homeostase van de glucosestofwisseling verbetert met een verhoging van de voerfrequentie.

Type 2 nutriëntsynchronisatie

In varkens leidde een asynchrone beschikbaarheid van glucose en aminozuren binnen een dag tot een verhoogde oxidatie van aminozuren. Daardoor nam de eiwitbenutting af, maar werd de vetaanzet niet beïnvloed.

In vleeskalveren leidde een asynchrone beschikbaarheid van glucose en aminozuren niet tot een afname in de efficiëntie van de eiwitbenutting, maar resulteerde in een aanzienlijke verhoging van de vetaanzet. Een scheiding van de eiwit- en lactoseopname remde de insulinerespons na een voerbeurt en zorgde voor een toename in uitscheiding van glucose in de urine. Het intramusculair vetgehalte nam toe naarmate de opname van eiwit in lactose verder gescheiden werden. Bovendien bleek de oxidatieve enzymactiviteit in een oxidatieve spier gerelateerd te zijn aan de binnendagvariatie in activiteitgerelateerde warmteproductie in vleeskalveren.

Slotwoord

Een groot aantal mensen heeft ervoor gezorgd dat de opzet en uitvoering van die rare, arbeidsintensieve proeven mogelijk is geweest. Dankzij de inzet en het enthousiasme van begeleiders, collega's en studenten heb ik van elke dag van mijn promotietijd genoten.

Dank aan NWO/STW voor de financiering van dit onderzoeksproject. Tevens wil ik het Productschap Diervoeder en ORFFA Additives BV bedanken voor de cofinanciering en de positief kritische houding tijdens de projectbijeenkomsten. Ook de bijdrage Tate & Lyle in de laatste fase van het project wordt erg op prijs gesteld.

Mijn promotor-in-z'n-vrije-tijd, prof. dr. ir. Martin Verstegen, ben ik dankbaar voor de discussies over fysiologie van varkens en kalveren en andere wijze levenslessen. De geboden vrijheid in mijn project heb ik erg gewaardeerd. Mariet, bedankt voor het lenen van Martin.

Voordat ik met het aio-project begon had ik nog nooit een witvleeskalf gezien. Het idee dat zo'n kalf meer op een varken lijkt dan op een koe (Gerrits, persoonlijke mededeling in de maalkamer, 2001) wist me echter te overtuigen om vier jaar van mijn leven aan rundvee te besteden. Walter, bedankt voor je overredingskracht en de perfecte dagelijkse begeleiding tijdens deze periode. Ik heb veel geleerd van je kritische benadering en van onze discussies over energie- en eiwitstofwisseling bij kalveren. De overige leden van de begeleidingscommissie, Johan Schrama, Victor Schreurs en Han Verdonk, wil ik hartelijk bedanken voor hun waardevolle bijdragen aan de opzet en uitwerking van de proeven.

Zonder de technische ondersteuning van Sven Alferink was het project niet mogelijk geweest. Sven, de gezamenlijke uurtjes bij de respiratiecellen, de duizenden bloedmonsters en de begeleiding van studenten was intensief, maar ik kijk met erg veel plezier terug op onze samenwerking. Ik heb vaak en dankbaar gebruik gemaakt van je kalverkennis. Ook wil ik de overige collega's bij de cellen bedanken voor hun hulp. Ik voel me vereerd dat één van mijn kalverproeven het begin (Sven en Ilona) en einde (Koos) van jullie carrière bij de respiratiecellen hebben gemarkeerd. Marcel, mooi dat we de online $^{13}CO_2$ meting op de rit hebben gekregen! Tamme, dank voor je hulp bij de bloedmonster- en balansdagen.

Het personeel van de proefaccommodatie (André, Ben, Marleen, Peter, Ries, Roel en Willem), dank voor jullie medewerking en goede zorgen voor de dieren. De proefvoeders werden gemaakt dankzij de prima medewerking van Gerard en Piet (Reseach Diet Services).

Na verzameling moesten de monsters natuurlijk nog geanalyseerd worden. Dick, Huug, Jane-Martine, Léon, Meijke, Saskia en Truus, dankjewel voor jullie hulp en begeleiding van studenten op het lab. Jane-Martine, als beginnend massaspectrometrist is het niet altijd even

makkelijk. Zeker niet als we dan ook nog snel resultaten willen hebben. Hartelijk dank voor je inzet en hopelijk kunnen we, samen met Dick, de komende tijd nog enkele stapjes voorwaarts maken met de GC-C-IRMS (er liggen namelijk nog een paar duizend monsters te wachten; sorry…). De fijne werkomgeving bij Diervoeding heb ik erg gewaardeerd; alle collega's bedankt.

Bernardo, my roommate, thanks for our inspiring talks about veal calves and women (not only a gender difference!) and many other topics. I would like to thank all students who have participated in the project: Annemarie, Antoon, Frank, Gijsbert, Hilde, Jessica, Marjoleine, Olga, Paulien, Ria, Roland, Sharon, Stefano, Suzan en Suzanne. I have enjoyed working with you and receiving valuable and unexpected input from your side (such as quantifying energetic costs of behaviour in calves, Suzanne).

Dr. Lobley, dear Gerald, thanks a lot for having me at the Rowett. Although I was used to eliminate rats with the backside of a shovel before going to Scotland, you persuaded me to use these rodents for scientific purposes such as studying homocysteine metabolism: a very instructive experience! Dr. Hocquette, dear Jean-François, I am very grateful for our collaboration which allowed integration of whole body and muscle energy metabolism in calves. Thanks for your enthusiasm and patience with me! Prof. dr. Blum and dr. (!) Vicari, dear Jürg and Till, I have gratefully borrowed some of our results for the general discussion. I have very much appreciated your willingness to cooperate and to share your knowledge with us, but I still do not understand the glucose metabolism of veal calves.

Ongetwijfeld was de synchronisatie met vrienden en familie de afgelopen jaren wel eens wat minder optimaal. Marleen, bedankt voor je steun tijdens mijn promotiejaren. Mannen van Epididymis en vrienden uit Mierd, bedankt voor de support en vriendschap. Pap, mam, Marieke en Noortje, fijn dat jullie altijd interesse hebben getoond in mijn onderzoek.

Joost

Publications

Refereed scientific journals

Van den Borne JJGC, Hocquette JF, Verstegen MWA & Gerrits WJJ Whole body and muscle energy metabolism in preruminant calves: effects of nutrient synchrony and physical activity. *Submitted to British Journal of Nutrition*.

Van den Borne JJGC, Lobley GE, Verstegen MWA, Muijlaert JM, Alferink SJJ & Gerrits WJJ Effects of feeding frequency and feeding level on within-day nutrient oxidation in heavy preruminant calves. *To be submitted*.

Van den Borne JJGC, Schrama JW, Heetkamp MJW, Verstegen MWA & Gerrits WJJ Synchronizing the availability of amino acids and glucose increases protein retention in pigs. *Submitted to Animal*.

Van den Borne JJGC, Verdonk JMAJ, Schrama JW & Gerrits WJJ (2006) Reviewing the low efficiency of protein utilization in heavy preruminant calves - a reductionist approach. *Reproduction Nutrition Development* **46**, 121-137.

Van den Borne JJGC, Verstegen MWA, Alferink SJJ, Giebels RMM & Gerrits WJJ (2006) Effects of feeding frequency and feeding level on nutrient utilization in heavy preruminant calves. *Journal of Dairy Science* **89**, 3578-3586.

Van den Borne JJGC, Verstegen MWA, Alferink SJJ, Van Ass FHM & Gerrits WJJ (2006) Synchronizing the availability of amino acids and glucose decreases fat retention in heavy preruminant calves. *Journal of Nutrition* **136**, 2181-2187.

Van den Borne JJGC, Weström BR, Kruszewska D, Botermans JAM, Svendsen J, Wolinski J & Pierzynowski SG Exocrine pancreatic secretion in pigs fed sow milk and milk replacer and the relationship to growth performance. *Accepted by Journal of Animal Science*.

Vicari T, Van den Borne JJGC, Gerrits WJJ, Zbinden Y & Blum JW Postprandial blood hormone and metabolite concentrations are differentially influenced by feeding frequency and feeding level in veal calves. *Submitted to Domestic Animal Endocrinology*.

Vicari T, Van den Borne JJGC, Gerrits WJJ, Zbinden Y & Blum JW Synchronizing protein and lactose intake increases the postprandial insulin response in veal calves. *Submitted to Domestic Animal Endocrinology*.

Conference proceedings

Alferink SJJ, Van den Borne JJGC, Gerrits WJJ, Lammers-Wienhoven SCW & Heetkamp MJW (2003) On-line, continuous determination of $^{13}CO_2/^{12}CO_2$ ratios by non-dispersive infrared absorption in indirect calorimetry facilities. *Progress in research on energy and protein metabolism*, 465-468.

Rijnen MMJA, Van den Borne JJGC, Schrama JW & Gerrits WJJ (2003) Housing conditions and carbohydrate source affect within-day variation of energy metabolism in growing pigs. *Progress in research on energy and protein metabolism*, 367 - 370.

Van den Borne JJGC, Alferink SJJ & Gerrits WJJ (2004) Identifying ruminal drinking by measurement of respiratory quotient and methane production in preruminant calves. *Journal of Animal Science* **82,** Suppl. 1, 365.

Van den Borne JJGC, Botermans JAM, Svendsen J, Weström BR & Pierzynowski SG (2005) The effect of milk replacer on exocrine pancreatic secretion in pigs: a comparison with sow milk. *Meeting on milk-fed farm and companion animals: basic aspects and practice for the future*, 19.

Van den Borne JJGC, Giebels RMM, Alferink SJJ & Gerrits WJJ (2005b) Effect of feeding frequency on protein and energy utilization in veal calves. *Meeting on milk-fed farm and companion animals: basic aspects and practice for the future*, 25.

Van den Borne JJGC, Giebels RMM, Alferink SJJ & Gerrits WJJ (2005) Effects of meal frequency on nutrient utilization in heavy preruminant calves. *Proceedings 30ste Studiedag Nederlandstalige Voedingsonderzoekers*, 55-56.

Van den Borne JJGC, Heetkamp MJW, Alferink SJJ, Muijlaert JM, Verstegen MWA & Gerrits WJJ (2006) Substrate oxidation in farm animals; the use of stable isotopes and indirect calorimetry. *7th Meeting of the Benelux Isotope Group*.

Van den Borne JJGC, Van der Heijden SJFM, Oorsprong H, Bokkers EAM, Bolhuis JE & Gerrits WJJ (2004) Energetische kosten van stereotype gedragingen in vleeskalveren. *Proceedings 29ste Studiedag Nederlandstalige Voedingsonderzoekers*, 27.

Van den Borne JJGC, Van der Heijden SJFM, Oorsprong H, Bokkers EAM, Bolhuis JE & Gerrits WJJ (2004) High energetic costs of stereotyped behaviour in preruminant calves. *Journal of Animal Science* **82,** Suppl. 1, 251.

Vicari T, Van den Borne JJGC, Gerrits WJJ, Zbinden Y & Blum JW (2006) Feeding frequency and feeding level affect blood hormones and metabolites in veal calves. *Proceedings of the Bi-annual Meeting of the Fachgruppe für Physiologie und Biochemie, Deutsche Gesellschaft für Veterinärmedizin*, 42.

Reports

Rijnen MMJA, Schrama JW, Van den Borne JJGC, Gerrits WJJ, Cone JW & Van Gelder AH (2004) Effecten van fermenteerbare grondstoffen op de fysieke activiteit in relatie tot energiebenutting bij varkens. Fase 2: Invloed van fermentatiesnelheid van fermenteerbare koolhydraten, pp. 43: Wageningen Universiteit.

Training and supervision plan	**Graduate school WIAS**	
Name: Joost van den Borne		
Group: Animal Nutrition		
Period: 2002-2006		
Daily supervisor: Dr. ir. W.J.J. Gerrits		
Supervisor: Prof. dr. ir. M.W.A. Verstegen		
	Year	Credits[1]
The basic package		
WIAS introduction course	2003	1.5
Course of philosophy of science and ethics	2002	1.5
International conferences		
Symposium on energy and protein metabolism, Rostock, Germany	2003	1.5
Symposium ADSA/ASAS/PSA, St. Louis, USA	2004	3.5
Symposium milk-fed farm and companion animals, Bern, Switzerland	2005	2.0
Seminars and workshops		
WIAS seminar Studies on stress and metabolic adaptation	2001	0.3
WIAS seminar Integrated management systems for pigs using visual imaging analyses	2002	0.1
WIAS seminar Gut integrity of piglets around weaning	2002	0.1
WIAS seminar Animal Reproduction	2003	0.1
WIAS seminar Methane emissions in cattle	2005	0.1
WIAS seminar Farewell seminar prof. dr. ir. Seerp Tamminga	2005	0.1
WIAS seminar Feeding strategies: potentials and constraints for metabolism	2006	0.1
WIAS Science day (5×)	2002-2006	1.5
Presentations (non-symposia)		
Dag voor Nederlandstalige Voedingsonderzoekers, Wageningen (2×)	2004	2.0
Dag voor Nederlandstalige Voedingsonderzoekers, Gent	2005	1.0
WIAS seminar Feeding strategies: potentials and constraints for metabolism, Wageningen	2006	1.0
WIAS Science day, Wageningen	2006	1.0
In-depth studies		
Stable isotopes in studies of nutrient dynamics, Wageningen	2001	0.7
User's meeting isotope ratio mass spectrometry, Groningen	2002	0.6
Ecophysiology of the gastrointestinal tract, Wageningen	2003	1.5
Course in tracer methodology in metabolism, Maastricht	2004	0.9
Modelling practicals of Nutrient Dynamics	2004	0.8
New developments in feed evaluation	2005	1.5
Experimental design	2002	0.9
Professional skills support courses		
Use of laboratory animals (article 9 authorization)	2001	4.3
Techniques for scientific writing	2002	1.1
Laboratory use of isotopes (article 5b authorization)	2003	1.5
Debating course Broaden your horizon	2004	1.5
Research skills training		
External training period for 5 months at the Rowett Research Institute, Aberdeen, UK	2004-2005	2.0
Didactic skills: teaching and supervising		
Practical Biologie van de dierlijke productie	2002	1.0
Practical Inleiding Dierwetenschappen	2003	1.0
Practical Principles of Animal Nutrition	2005	0.4
MSc-theses: 7 students	2002-2006	10.5
Internships: 7 students	2002-2006	5.6
Total		53.2

[1] One ECTS (European Credit Transfer System) credit equals a study load of approximately 28 hours

Curriculum Vitae

Joost (doopnamen: Joost Josephus Gerardus Cornelis) van den Borne werd geboren op 1 november 1977 te Hooge Mierde en is opgegroeid op het ouderlijke varkens- en akkerbouwbedrijf. In 1996 behaalde hij zijn VWO diploma aan het Pius X College te Bladel. In datzelfde jaar begon hij met de studie Zoötechniek aan de toenmalige Landbouwuniversiteit in Wageningen. Afstudeervakken over fermenteerbare koolhydraten en het energiemetabolisme bij vleesvarkens (Diervoeding) en een bedrijfseconomische vergelijking van verwerkingssystemen voor varkensmest (Agrarische Bedrijfseconomie) werden afgerond. Een stage van zes maanden werd uitgevoerd aan Lund Universiteit in Zweden. In november 2001 haalde hij zijn doctoraal diploma Zoötechniek met Diervoeding als hoofdvak. Na een periode van vier maanden als toegevoegd onderzoeker varkensvoeding bij de leerstoelgroep Diervoeding, begon hij in maart 2002 met een promotieonderzoek bij diezelfde leerstoelgroep. Tijdens zijn promotietijd verbleef hij gedurende vijf maanden aan het Rowett Research Institute in Aberdeen, Verenigd Koninkrijk. Sinds april 2006 is hij werkzaam als onderzoeker bij de leerstoelgroep Diervoeding van Wageningen Universiteit.

Joost (baptism names: Joost Josephus Gerardus Cornelis) van den Borne was born on 1 November 1977 in Hooge Mierde and has grown up on the parental farm. In 1996, he graduated from the secondary grammar school Pius X College in Bladel. In the same year he started the study Animal Science at the former Agricultural University in Wageningen. Theses concerning fermentable carbohydrates in relation to the energy metabolism in pigs (Animal Nutrition) and an economical comparison of processing systems for pig manure (Business Economics) were completed. A training period of six months was carried out at Lund University in Sweden. In November 2001 he obtained his Master's diploma Animal Science with animal nutrition as a specialization. After a period of four months as researcher on pig nutrition at the Animal Nutrition Group, he started with a PhD project at the same group in March 2002. During his PhD period, he stayed for five months at the Rowett Research Institute in Aberdeen, UK. Since April 2006 he has been working as a researcher at the Animal Nutrition Group of Wageningen University.