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PROPIONATE REGULATION OF FEED INTAKE

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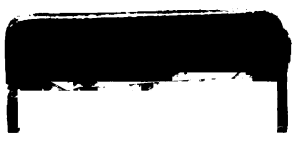

PROPIONATE REGULATION OF FEED INTAKE

By

Barry Joseph Bradford

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ABSTRACT

PROPIONATE REGULATION OF FEED INTAKE

By

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Ruminant diets that include large amounts of readily-fermentable starch are known to limit feed intake. Experiments conducted over the past 20 years have indicated that physiological changes in rumen osmolality, rumen pH, or plasma insulin concentration do not consistently explain hypophagic responses to highly fermentable diets. Meanwhile, evidence has accumulated in a number of species that hepatic oxidation of fuels stimulates satiety, and the rapid increase in propionate flux to the liver during meals likely results in oxidation of some propionate. Six studies were conducted to evaluate the hypothesis that hepatic oxidation of propionate limits feed intake in lactating dairy cattle. In a crossover experiment including 32 cows with a wide range of production levels, increased dietary starch fermentability depressed dry matter intake (DMI) 8% but did not alter digestible DMI. Although consistent with the hepatic oxidation hypothesis, these results could also be explained by other proposed mechanisms for regulation of intake. Evidence from both ruminant and non-ruminant studies has suggested that propionate may directly stimulate leptin secretion. Therefore, 2 experiments were conducted utilizing pulse-dose and intermediate-term propionate infusions to evaluate whether this mechanism is important in lactating cows. Based on the small, non-linear effects of propionate administration on

leptin secretion that were observed, we concluded that leptin does not likely mediate intake depression by propionate. We then conducted a set of experiments using the glucose analog phlorizin to increase glucose demand and gluconeogenic capacity. By increasing the potential for glucose production from absorbed propionate, we expected to decrease the proportion of propionate that was oxidized, and in turn, increase feed intake. Evidence from 3 experiments suggested that glucose demand was increased by phlorizin treatment, and measurement of transcript abundance for potentially rate-limiting gluconeogenic enzymes indicated that hepatic gluconeogenic capacity was increased by phlorizin treatment. However, phlorizin treatment also consistently stimulated lipolysis, which likely increased hepatic fatty acid oxidation. This confounding effect probably played a role in the lack of an effect on feed intake, and prevented assessment of the hepatic oxidation hypothesis. Finally, we tested the hypothesis that more rapid infusion of propionate during meals would overwhelm hepatic gluconeogenic capacity to a greater extent, resulting in quicker satiety and smaller meals. However, we did not find differences in feeding behavior in response to infusion of 1.2 mol of Na propionate over the course of 5 or 15 minutes during meals. In conclusion, these experiments have not added to the existing evidence for the hepatic oxidation hypothesis for ruminants. Nevertheless, progress has been made toward understanding the mechanism for propionate regulation of feed intake, because leptin has been ruled out as a necessary mediator of this response, and because glucose demand has been shown to have little influence on DMI in the short-term.

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CHAPTER 1

LITERATURE REVIEW

Feed intake of ruminants is regulated by an extremely complex system which integrates disparate signals from various organs (Forbes, 1996). Although homeorhetic mechanisms can alter the feed intake set point over the long-term, daily intake is determined only by meal size and meal frequency. From a physiological perspective, gastrointestinal distention and chemosensory feedback are the most important determinants of meal patterns. While physical fill limitations to feed intake have been well characterized (Allen, 2000), the effects of diet on chemosensory regulation of intake, and the mechanisms involved, are poorly understood in ruminants. Recent work has focused on explaining feed intake depression by propionate and on providing a relatively comprehensive mechanism for feed intake regulation by metabolites. This review of the literature will highlight the determinants of propionate production, its role in ruminant metabolism, effects of propionate on feed intake, and potential mechanisms for these responses.

Starch in ruminant diets

The rates of ruminal starch digestion and passage vary greatly across grains fed to ruminants and depend upon the type of cereal grain, conservation method, and processing (National Research Council, 2001). Pure starch is readily degraded by many ruminal microbes; however, starch found in feed grains is

surrounded by a protein matrix. Degradation of this protein matrix is typically the rate-limiting step in ruminal starch digestion (Kotarski et al., 1992). Differences in fermentability between types of grains and varieties within a grain type (eg. vitreous vs. floury corn varieties) are primarily due to differences in the protein matrices within the grain endosperm. Likewise, degrading the protein matrix through ensiling, or increasing the surface area available for enzymatic degradation by processing, can increase the ruminal fermentability of a grain source.

Ruminal digestion kinetics determine the site and extent of nutrient digestion, which can greatly affect the type and temporal pattern of fuels absorbed. While ruminal starch digestion results in the production of volatile fatty acids (VFAs), starch that escapes ruminal digestion can be degraded by pancreatic amylase in the duodenum. Although little glucose appears in the portal circulation in ruminants, glucose is efficiently absorbed in the small intestine (Huntington and Reynolds, 1986). Gastrointestinal epithelial cells apparently metabolize most absorbed glucose to lactate, and a relative high net flux of lactate has been measured across the splanchnic bed of lactating cows (Reynolds et al., 2003). Therefore, diets with similar concentrations of starch can provide the animal with VFAs and lactate in different proportions depending on the physical characteristics of the starch source.

Increasing the amount of grain in the diet, or the fermentability of grain, results in increased ruminal VFA production primarily because of greater propionate production (Bauman et al., 1971; Sutton et al., 2003). Ruminal starch-digesting microbes have a competitive advantage when high-grain rations are fed because of their ability to rapidly utilize starch, and these species produce more propionate than cellulolytic bacteria (Russell, 1998). Furthermore, increased ruminal fermentation rate is typically associated with a decrease in ruminal pH, and bacteria that tend to produce propionate are favored in this situation because of their tolerance for low pH (Russell, 1991).

In addition to effects on nutrient absorption and rumen pH, variation in ruminal starch digestion alters animal productivity and behavior. Compared to feeding lactating cows forage alone, inclusion of grain can increase dry matter intake (DMI) by removing physical fill constraints on intake. Because grains also provide more metabolizable energy than forages, this can result in dramatically increased energy intake and milk production (Conrad et al., 1964). Not surprisingly, grain feeding became popular in the mid-1900's and most diets fed to lactating cows now contain at least 40% concentrate. However, it is now known that cereal grains that are highly digestible in the rumen can depress feed intake of lactating cows. Dry matter intake (DMI) was depressed by nearly 3 kg DM / d (~13%) when more fermentable grains were substituted in diets of lactating cows in several studies reported in the literature (Allen, 2000). Oba and Allen (2003b) demonstrated that a more rapidly fermented starch source reduced

meal size 17%, causing an 8% reduction in food intake despite a 10% decrease in intermeal interval. The more fermentable treatment nearly doubled the fractional rate of starch digestion in the rumen, increasing the contribution of VFAs, especially propionate, as fuels at the expense of lactate. The evidence that propionate mediates feed intake depression by highly fermentable starch sources is discussed below.

Propionate and its role in ruminant metabolism

Propionate is a 3-carbon VFA that plays a central role in ruminant metabolism. Propionate is produced by microbial degradation of carbohydrates, and to a lesser extent, amino acids (Bergman, 1990). Work in the late 1800's led to the assumption that VFA were an important source of energy for ruminants, but propionate was first identified as a glucose precursor by Ringer (1912), who showed that injecting phlorizin-treated dogs with propionate resulted in increased glucose excretion. A number of investigators, using similar protocols, later showed that infusion of either propionate or butyrate in ruminants led to increased plasma glucose concentrations (Kronfeld, 1957; Potter, 1952). However, Leng and Annison (1963) demonstrated that propionate, but not butyrate, contributed labeled carbon to glucose during incubations with sheep liver slices. Ash and colleagues (1964) also showed that infused butyrate did not contribute labeled carbon to plasma glucose, and did not increase glucose production by isolated hepatocytes. Propionate is now recognized as the primary glucogenic precursor in ruminants, and estimates of its contribution to glucose

requirements are typically in the range of 50 – 75% (Bergman, 1990). One reason that propionate is such an important glucose precursor is that hepatic uptake exceeds 70% of supply, and more than 90% of absorbed propionate is taken up by the liver (Reynolds, et al., 2003). Propionate can be used quite efficiently for glucose production (Steinhour and Bauman, 1988), but direct assessment of propionate oxidation demonstrated that approximately 13% of propionate taken up by the liver is oxidized (Black et al., 1966). Propionate not taken up by the liver can be used by peripheral tissue for oxidative metabolism or by adipose and mammary tissues for de novo lipogenesis, leading to synthesis of odd-chain fatty acids.

Propionate production rates vary greatly across diets fed to lactating cows. Mean calculated production rates were reported in the range of 13.4 to 16.9 mol/d in lactating Holstein cows fed diets containing 30 – 33% starch on a DM basis (Oba and Allen, 2003b; Sutton, et al., 2003). However, calculated production was 9.3 mol/d from a diet containing 21% starch (Oba and Allen, 2003b), and Sutton et al. (2003) measured a mean production rate of 36.2 mol/d while feeding a 48% starch diet. Therefore, within similar cows, dietary differences can alter propionate production rates by at least 4-fold. Diet-induced differences in propionate production are further amplified if one considers temporal variation in propionate production. Starch that is more completely digested in the rumen is also more rapidly fermented, resulting in greater differences in propionate production rates during and immediately after meals.

Propionate absorption may be more limiting than production during times of rapid fermentation. However, propionate and other VFAs act within the rumen to increase blood flow to ruminal papillae. Small amounts of VFA applied to the ruminal epithelium can stimulate an increase in local blood flow (Thorlacius, 1972), and portal blood flow increased with increasing rate of intraruminal propionate infusion (Weeks and Webster, 1975). Absorption of VFAs is thought to be primarily a passive process; therefore, greater blood flow to the ruminal epithelium clears nutrients from intracellular pools more quickly, resulting in a larger cross-membrane concentration gradients and increased absorption rates. The combination of increased blood flow and rapid production of VFAs during meals results in a rapid increase in propionate delivery to the liver during the course of a meal. Benson et al. (2002) reported that net appearance of propionate from the portal-drained viscera can increase by more than 40% within an hour of feeding.

Propionate's central role in ruminant metabolism suggests that regulation of feed intake by propionate is feasible from an evolutionary perspective. Although most wild ruminants consume little grain, propionate is also produced during ruminal digestion of forages. The production of adequate propionate is especially important in lactating ruminants consuming only forages, because little lactate is absorbed and amino acids are ideally conserved for milk protein synthesis. Therefore, within the constraints imposed by the volume of the gastrointestinal

tract, feed intake should increase until sufficient propionate is available for glucose production. The mechanism proposed below provides the additional benefit of responding to changes in glucose demand rather than simply responding to propionate concentrations *per se*.

Hypophagic effects of propionate

Early in the study of feed intake regulation, ruminants were recognized as a valuable model for investigating feed intake depression by metabolites. Following the suggestion by Mayer (1953) that feed intake is regulated by changes in blood glucose concentration, a number of investigators began assessing the possibility that acetate, propionate, or butyrate might play a role in regulating feed intake of ruminants. Dowden and Jacobsen (1960) first reported that 8-h intravenous infusions of propionic acid or acetic acid significantly depressed 24-h feed intake of dairy calves, but that infusions of butyric acid, lactic acid, and glucose did not influence feeding behavior. Later work from Baumgardt's group confirmed that VFA, but not glucose, were capable of decreasing feed intake of mature dairy cows (Montgomery et al., 1963; Simkins et al., 1965). Baile and Pfander (1966) reported that pulse-dose intraruminal infusion of acetate (0.25 mol) prior to feeding depressed feed intake of sheep by 40%, but also showed that infusion of isomolar NaCl decreased feed intake by 25%, suggesting that ruminal osmolality is involved in intake modulation by VFAs. This led Baile and Mayer (1968) to investigate the relative effects of intraruminal and intravenous infusions of acetate administered during

spontaneous meals. The hypophagic response to intraruminal acetate infusion was not observed when acetate was infused intravenously (Baile and Mayer, 1968), supporting the hypothesis that intraruminal sensors are involved in regulation of feed intake by acetate. At that point in time, most investigators had focused on acetate as a potential feedback signal because its concentration in peripheral blood is higher than that of other VFAs. However, using intraruminal infusion of VFAs at spontaneous meals, Baile and Mayer (1969) demonstrated that propionate is at least as effective as acetate and more effective than butyrate at eliciting feed intake depression in sheep.

In the last 30 years, hypophagic effects of propionate infusions have been documented extensively for ruminants (Anil and Forbes, 1980; Elliot et al., 1985; Farningham and Whyte, 1993; Hurtaud et al., 1993; Leuvenink et al., 1997; Mbanya et al., 1993; Sheperd and Combs, 1998). Propionate was more hypophagic than acetate or butyrate when infused into the portal vein of sheep (Anil and Forbes, 1980), and infusion of propionate into the mesenteric vein of steers reduced feed intake while acetate infused at similar rates did not (Elliot et al., 1985). Although propionate might be expected to decrease food intake compared to acetate because it has higher energy content, propionate linearly decreased metabolizable energy intake compared to acetate in lactating cows when infused intra-ruminally as iso-osmotic mixtures (Oba and Allen, 2003d). As the proportion of propionate increased, the reduction in ME intake from the diet exceeded that supplied from the infusate. Food intake was reduced primarily

from a linear reduction in meal size from 2.5 to 1.5 kg DM as propionate increased from 0 to 100% of infusate. Meal frequency also tended to decrease linearly (P = 0.08) from 7.4 to 6.1 meals during the 12 h monitoring period as propionate was increased (Oba and Allen, 2003d). Therefore, propionate decreased energy intake compared to acetate by increasing satiety and possibly by decreasing hunger. Propionate also decreased food intake of dairy cows compared to iso-energetic infusions of VFA mixtures (Hurtaud, et al., 1993) or acetate (Sheperd and Combs, 1998). These studies suggest that hypophagic effects of propionate cannot be explained simply by the additional energy supplied as propionate. It is unlikely that animals consume feed to meet their energy requirements *per se* but rather have fuel-specific mechanisms regulating satiety and hunger.

Proposed mechanisms for depression of feed intake by propionate

The mechanism by which propionate regulates satiety is not fully understood. A wide array of potential mechanisms has been proposed, reflecting the myriad metabolic and physiological responses to this single metabolite.

Osmotic effects. One possibility is that increased ruminal VFA concentration causes satiety by increasing the osmolality of rumen fluid during meals. Osmolality may stimulate satiety through osmoreceptors in the rumen wall (Carter and Grovum, 1990; Leek and Harding, 1975) or by stimulating release of vasopressin, a hormone that can affect satiety (Langhans et al., 1991), in

response to water efflux from the blood to the rumen (Allen, 2000). While mechanisms associated with osmolality likely contribute to satiety, changes in osmolality *per se* do not affect food intake. Choi and Allen (1999) compared iso-osmotic infusions of NaCl, Na acetate, and Na propionate administered intraruminally at spontaneous meals for 12 h. Although NaCl decreased meal size 27%, it did not affect daily food intake because intermeal interval decreased 31% compared to sham infusion. In that experiment, both Na acetate and Na propionate decreased food intake compared to NaCl, primarily because of greater intermeal interval, indicating delayed hunger (Choi and Allen, 1999). This makes the important point that regulatory mechanisms must be investigated for time courses beyond the first meal after treatment, because feed intake is determined by both satiety and hunger. Feed intake depression by propionate infusion cannot be explained by osmotic effects alone.

Insulin signaling. One of propionate's physiological effects is to increase insulin secretion. Peripheral infusion of propionate dose-dependently increases plasma insulin concentrations, and the peak insulin response precedes the peak in plasma glucose (Bradford et al., 2006a). This is not simply a pharmacological effect, because portal infusion of propionate at physiological rates also increased plasma insulin concentration (Leuvenink, et al., 1997). Propionate's role as an insulin secretagogue led Grovum (1995) to suggest that hypophagic effects of propionate are mediated by insulin (Allen, 2000). Grovum's hypothesis is supported by the broad consensus that insulin is an anorexic agent in

monogastrics (Benoit et al., 2004; Porte et al., 2005), and by evidence that intraventricular administration of insulin depressed feed intake of sheep (Foster et al., 1991). However, hypophagic effects of propionate infusions have been observed without an increase in plasma insulin concentration (Farningham and Whyte, 1993; Frobish and Davis, 1977), and hyperinsulinemic-euglycemic clamps do not depress energy intake of dairy cattle when energy provided by infused glucose is considered (Griinari et al., 1997; Mackle et al., 1999; McGuire et al., 1995). Insulin can likely influence feed intake through its effects on metabolism, nutrient partitioning, and possibly by influencing hypothalamic satiety centers; however, there is little evidence that it is directly responsible for the depression of feed intake by propionate (Allen, 2000).

Central nervous system sensors. According to the glucostatic hypothesis of Mayer (1953), neurons in the central nervous system (CNS) alter feeding behavior in response to changes in plasma glucose concentration. Indeed, neurons in the hypothalamus have long been known to alter their firing rates in response to hyperglycemia and hypoglycemia (Anand et al., 1964). While few investigators today believe that glucosensors play an important role in normal energy homeostasis, glucosensors can likely stimulate eating in cases of glucoprivation (Routh, 2002). Likewise, a sensory system in the CNS must be considered as a potential mechanism for regulation of feed intake by propionate. First-pass extraction of propionate by the liver exceeds 90% (Reynolds, et al., 2003), resulting in much lower concentrations of propionate reaching the brain

compared to that reaching the liver. However, increasing rate of intraruminal propionate infusion linearly increased peripheral plasma propionate concentrations (Oba and Allen, 2003a), suggesting that the CNS can potentially monitor propionate concentrations directly. Several important findings make a CNS sensory system unlikely, however.

Over three decades ago, Baile (1971) found that propionate injections into the ruminal vein during spontaneous meals decreased feed intake of sheep, yet injections of larger amounts into the jugular vein did not alter feed intake. Anil and Forbes (1980) confirmed this finding, showing that infusion of propionate into the portal vein of sheep depressed feed intake while jugular infusion of the same dose of propionate had no effect. If the CNS were involved in regulation of feed intake by propionate, the opposite responses would be expected, because infusion of propionate into the ruminal or portal veins results in efficient clearance by the liver and limited delivery of propionate to the brain. In a follow-up experiment, sheep underwent surgery to denervate the liver prior to infusion of propionate, and in sheep which were successfully denervated, subsequent propionate infusions did not depress feed intake (Anil and Forbes, 1980). Later work showed that intake responses to portal infusion of propionate were eliminated by sectioning the hepatic branch of the vagus nerve or by anaesthetizing the splanchnic nerve (Anil and Forbes, 1988). An important shortcoming of these approaches is that interrupting efferent communication from the CNS to the liver and pancreas can have multiple physiological consequences

(Berthoud, 2004), and the elimination of hepatic afferent signaling alone has not been accomplished in ruminants. Nevertheless, in combination with the infusion experiments discussed above, these results strongly suggest that signaling from a peripheral sensory system to the CNS mediates propionate's effects on feed intake, rather direct sensing within the CNS.

Peripheral sensory system. In light of his findings regarding the effects of propionate infusion into different veins, Baile (1971) proposed that propionate receptors in the ruminal region of sheep might regulate food intake. His hypothesis was supported in part by evidence that intraruminal administration of VFA decreased gastric motility (Ash, 1959), presumably by activating chemosensors in the rumen wall or in the veins draining the rumen. However, later work showed strong feed intake responses to portal infusion of propionate (Anil and Forbes, 1980; Farningham and Whyte, 1993), shifting the focus from the rumen or ruminal vein to the region of the portal vein and liver. The "propionate sensor" hypothesis continues to be supported by some investigators (Leuvenink, et al., 1997).

An alternative way to explain hepatic involvement in feed intake depression by propionate has arisen from the monogastric literature. The liver is in a unique position to monitor changes in fuel metabolism to control eating behavior because of its central role in energy metabolism of animals (Friedman and Stricker, 1976). Langhans and Scharrer (1987) suggested that hepatic oxidation

of a variety of fuels contributes to regulation of food intake by altering signals to brain satiety centers via the hepatic vagus, and a large body of evidence has accumulated to support this hypothesis. Portal glucose infusion decreased discharge rates of hepatic vagal afferents in guinea pigs (Niijima, 1983) and portal infusion of 2-deoxy-D-glucose, which reduced glucose utilization, increased the firing rate of hepatic vagal afferents and increased feed intake in rabbits (Novin et al., 1973). Administration of the fructose analog 2,5-anhydro-D-mannitol (2,5-AM) decreased hepatic ATP concentration by trapping inorganic phosphate and elicited an eating response in rats (Koch et al., 1998; Rawson et al., 1994; Tordoff et al., 1988). Furthermore, phosphate loading prevented both the decrease in hepatic ATP and the stimulation of feeding by 2,5-AM (Rawson and Friedman, 1994). The hepatic oxidation hypothesis, then, proposes that meals can be terminated by a signal carried from the liver to the brain via afferents in the vagus nerve that are affected by hepatic oxidation of fuels and generation of ATP (Friedman, 1995; Langhans and Scharrer, 1992).

The mechanism by which propionate may alter the firing rate of the hepatic vagus in ruminants is unknown. However, regulation of food intake by propionate in ruminants is consistent with the hepatic oxidation hypothesis; propionate might decrease food intake of ruminants by stimulating oxidative metabolism in the liver (Allen, 2000). Of fuels metabolized by the ruminant liver, propionate is likely a primary satiety signal because its flux to the liver increases greatly during meals (Benson, et al., 2002). While propionate is extensively

metabolized by the ruminant liver, there is little net metabolism of acetate (Reynolds, 1995) because ruminant liver has high activity of propionyl CoA synthetase but not acetyl CoA synthetase (Demigne et al., 1986; Ricks and Cook, 1981), thus explaining differences in hypophagic effects of infusions of propionate and acetate in ruminants.

Evidence currently available in the ruminant literature cannot delineate whether feed intake responses to propionate infusion are mediated by portal propionate sensors or by hepatic oxidation of propionate. For the purpose of debating the likelihood of each proposed mechanism, comparisons with the (proposed) analogous portal glucosensors are useful. Much of the data supporting the presence of glucosensors in the portal vein of monogastrics relies on the use of 2-deoxy-D-glucose (Novin, et al., 1973; Rezek and Kroeger, 1976; Rossi et al., 1996), which has its effects by blocking metabolism of glucose. Therefore, both proposed mechanisms rely on glucose metabolism to initiate a signaling cascade. Although many ruminant tissues use little glucose, ruminant neural tissue apparently utilizes glucose as extensively as that of monogastrics (Lindsay and Setchell, 1976). If one makes the common assumption that mechanisms regulating feed intake are well conserved across species, it follows that portal glucosensors should be present in ruminants as well as in monogastrics. While glucose can clearly alter feeding behavior of monogastrics (Forbes, 1988), it has been shown to have no effect on feed intake in ruminants (Baile and Forbes, 1974; Dowden and Jacobson, 1960; Frobish and Davis, 1977; Simkins, et al.,

1965). To rely on receptors to explain the observed effects of nutrient infusions on feed intake of ruminants, one must propose that the ability to metabolize glucose in sensory receptors was lost, while the ability to metabolize propionate was gained during the evolutionary divergence of ruminants. This seems especially unlikely given that ruminant neural tissue is known to utilize glucose (Lindsay and Setchell, 1976). Rather, differences in hypophagic effects of glucose infusion observed between ruminants and monogastrics are likely because of differences in hepatic oxidation of glucose; liver hexokinase activity is low in ruminants compared to nonruminants (Ballard, 1965), and in mature ruminants, hepatic removal of glucose appears to be negligible (Stangassinger and Giesecke, 1986). Although results of propionate infusion experiments could be explained by the presence of propionate sensors, the hepatic oxidation hypothesis is more attractive because of its "broad explanatory power" (Friedman, 1995) across different fuels and species.

Potential mechanisms linking hepatic oxidation and feeding behavior

Hepatic ATP is clearly involved in the hypophagic response to fuels in rats; however, the mechanism by which intracellular ATP concentration affects the firing rate of the hepatic vagus has not been determined. All mechanisms that have been proposed involve depolarization of the plasma membrane of the hepatocyte (Boutellier et al., 1999). Hepatocytes, like nerve cells, contain voltage-gated ion channels that can rapidly alter membrane potential, and this depolarization can trigger a number of physiological responses.

Perhaps the best-described mechanism for regulating membrane potential by cellular ATP concentration is through ATP-sensitive K^+ (K_{ATP}) channels (Miki and Seino, 2005). Because of the large K^+ ion gradient across the plasma membrane, K^+ ions move out of the cell when channels are open, driving the negative resting potential even lower (hyperpolarization). However, when ATP concentrations increase, ATP allosterically alters the conformation of K_{ATP} channels to stop ion flow. This results in accumulation of K^+, potentially leading to membrane depolarization. This mechanism is involved in insulin secretion by pancreatic beta cells (Nichols and Koster, 2002), and K_{ATP} channels mediate the increased firing rate of some glucose-sensitive neurons in the brain (Miki et al., 2001). Although K_{ATP} channels are probably found in hepatocytes (Malhi et al., 2000), they do not serve as an important component of the regulation of feeding behavior by hepatic oxidation. All evidence regarding this hypothesis indicates that transmembrane voltage becomes more positive (depolarizes) as hepatic ATP concentrations decrease (Boutellier, et al., 1999; Friedman et al., 1999); the opposite relationship would be expected if K_{ATP} channels played a primary role in linking cellular metabolism to membrane potential in hepatocytes.

Another link between cellular energy status and membrane potential is the Na^+/K^+ ATPase pump (Langhans, 1996). This pump drives a net movement of 1 cation out of the cell, and is the primary mechanism responsible for maintaining the negative resting membrane potential of most cells. Like other catalytic proteins, activity of Na^+/K^+ ATPase is affected by substrate concentration, and

Niijima (1983) proposed that changes in cytosolic ATP concentration could influence activity of this pump sufficiently to alter resting membrane potential. Strong evidence supports the conclusion that glucose utilization in the liver of guinea pigs reduces the firing rate of hepatic vagal afferents by activation of Na^+/K^+ ATPase; portal infusion of ouabain, which blocks activation of Na^+/K^+ ATPase, prevented effects of glucose on the firing rate of vagal hepatic afferents (Niijima, 1983). More recently, it was reported that 2,5-AM not only decreased ATP concentration in hepatocytes, but also increased intracellular Na^+ concentration, consistent with decreased Na^+/K^+ ATPase activity and a less negative membrane potential (Friedman et al., 2003). Therefore, information regarding hepatic ATP concentration may be encoded as membrane potential through substrate-level effects of ATP on Na^+/K^+ ATPase activity.

Another emerging candidate is AMP-activated protein kinase (AMPK). AMPK is activated in response to high AMP:ATP ratios, and direct activation of AMPK in the hypothalamus increased food intake of rats (Andersson et al., 2004). The ability of AMPK to phosphorylate ion channels and other proteins involved in signal transduction pathways provides a broad range of possible mechanisms for communicating energy status to sensory neurons. For example, AMPK is known to alter the function of voltage-gated sodium channels, increasing the length of time that the channels are in open state following depolarization (Light et al., 2003). AMPK also affects the function of certain Cl^- (Hallows et al., 2003) and Na^+ (Carattino et al., 2005) channels; changing the flow of these ions through the

membrane could cause membrane depolarization. In addition, AMPK could interact with any number of intracellular signaling cascades to amplify secretory responses to membrane depolarization. Continued characterization of the downstream targets of AMPK phosphorylation should shed further light on its potential role in signaling hepatic energy status to the nervous system.

The most tenuous component of the proposed mechanism for hepatic oxidation control of feeding behavior is the communication between hepatocytes and neighboring vagal afferent nerve endings (Langhans, 1996). Although little work has been done to investigate potential modes of communication, several possibilities are feasible. Hepatocytes contain gap junctions that allow intercellular movement of ions, including Ca^{2+} (Gaspers and Thomas, 2005), which can cause membrane depolarization. Rawson et al. (2003) reported that 2,5-AM caused a phopholipase C –mediated release of intracellular calcium stores in hepatocytes in a time frame that coincided with decreases in cellular ATP concentration. However, gap junctions between hepatocytes and afferent receptor nerves have not been found (Berthoud, 2004), making it unlikely that direct transport of ions between hepatocytes and neurons occurs. It may be more important that increased intracellular Ca^{2+} can stimulate exocytosis of signaling molecules. Hepatocytes are known to utilize ATP as an autocrine/paracrine signaling molecule (Wang et al., 1996), and some afferent nerves are activated by ATP (Kirkup et al., 1999). Depolarization of a hepatocyte could trigger release of intracellular Ca^{2+} stores (Weiss and Burgoyne, 2002),

leading to exocytosis of ATP. ATP could then move through the extracellular space to nearby parasympathetic nerves, binding purinergic receptors and leading to the generation of action potentials.

However communication between hepatocytes and afferent vagal nerves takes place, the response is an alteration in the number of action potentials that are generated in the sensory neurons. Action potentials are propagated along the vagus nerve, and these vagal afferents terminate in the dorsal vagal complex of the hindbrain; more specifically, they innervate the nucleus of the solitary tract, or NTS (Berthoud, 2004). Information received at the NTS can initiate the classic autonomic reflex responses, which include control over digestive processes (Luckman and Lawrence, 2003). Projections from the NTS also innervate the hypothalamus, including the paraventricular and arcuate nuclei (Ricardo and Koh, 1978), which are important sites for regulation of feeding behavior. Inhibitors of hepatic oxidative metabolism have been shown to activate neurons throughout this pathway, clearly indicating that vagal-derived signals can influence behaviors that are under the control of the forebrain (Horn et al., 1999).

Although certain components of the hepatic oxidation hypothesis remain tentative, sufficient evidence has accumulated to indicate that these proposed mechanisms are feasible (**Figure 1.1**). Redundancy has been a common theme in the study of feed intake regulation (Inui, 2000), and multiple, overlapping

mechanisms likely play a role in communicating the energy status of the liver to the hypothalamus.

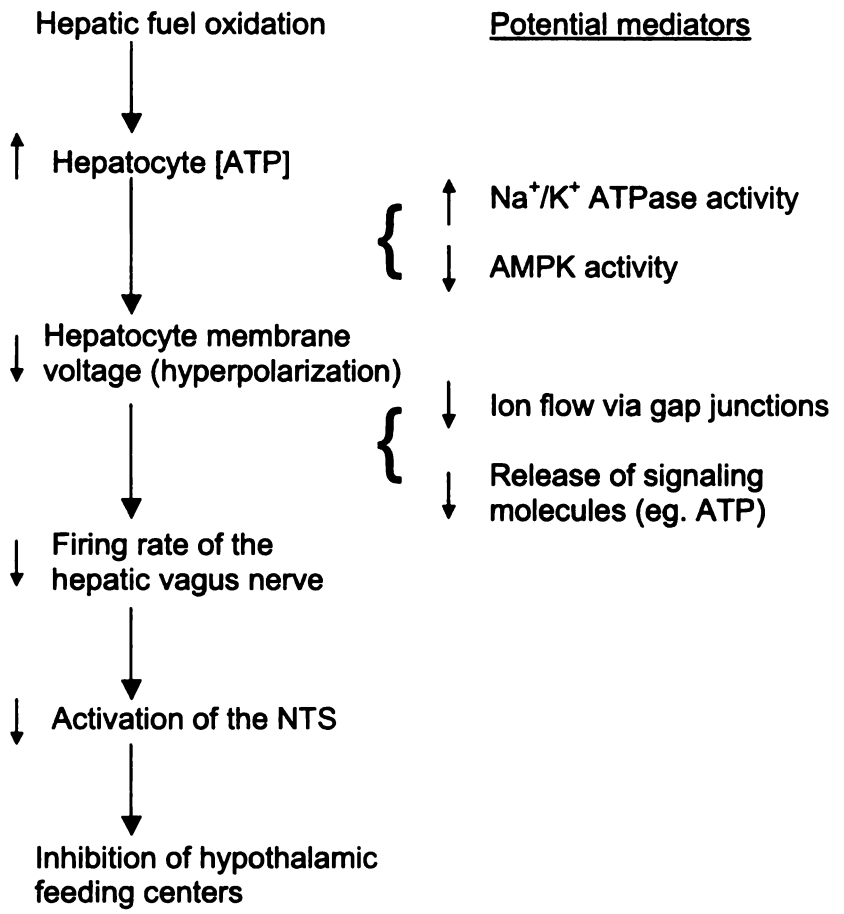

Figure 1.1 Proposed mechanism for regulation of feed intake by hepatic oxidation. Pathways that are involved signal transduction from the liver to the forebrain are shown. Potential mediators are listed for components of the mechanism that remain unresolved.

Implications of the hepatic oxidation hypothesis for ruminants

Inconsistent effects of propionate infusion on food intake have led to doubts that VFA *per se* are signals of satiety for ruminants (Grovum, 1995). However, the partitioning of fuels among different tissues and between metabolic pathways affects food intake substantially (Friedman, 1998). Propionate can be used for gluconeogenesis, which consumes ATP, or oxidized in the tricarboxylic acid (TCA) cycle as acetyl CoA, generating ATP. Therefore, the temporal pattern of oxidative metabolism in the liver is greatly altered by both propionate flux to the liver and fate of propionate within the liver (Allen, et al., 2005; **Figure 1.2**).

Insulin and glucagon are important for directing fuel partitioning, especially during meals. Decreased rate of gluconeogenesis when plasma glucose and insulin are high is expected to speed oxidation of propionate in the liver and cause satiety sooner. In support of this, the extent of hypophagia caused by propionate infusion increased linearly with plasma glucose concentration in dairy cows (Oba and Allen, 2003c). On the other hand, increased plasma insulin concentrations may also contribute to increased hunger between meals by speeding clearance of fuels from the blood, especially following relatively smaller meals. This might explain inconsistent intake effects of peripheral insulin administration reported in the literature (Hayirli et al., 2002); while increased hepatic oxidation of fuels likely causes satiety, increased clearance of fuels from the blood is expected to cause hunger (Oba and Allen, 2000).

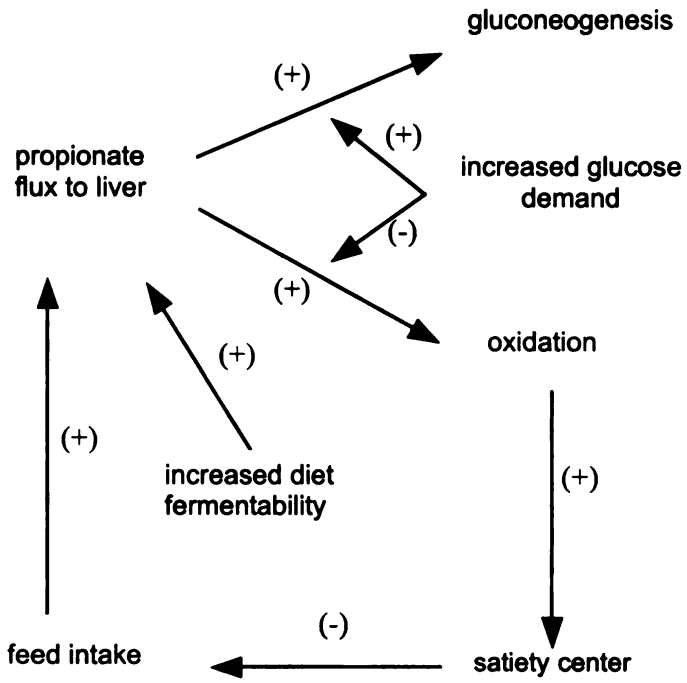

Figure 1.2 Model by which propionate might affect satiety in ruminants. Propionate flux to the liver is affected by feed intake and diet fermentability and increases greatly during meals. Propionate can be used for gluconeogenesis, which consumes ATP, or oxidized in the tricarboxylic acid cycle as acetyl CoA, generating ATP. Supply of propionate to the liver relative to glucose demand should affect the temporal pattern of ATP concentration in the liver and is expected to affect satiety. When glucose demand is high, gluconeogenesis increases and less propionate is oxidized, resulting in greater meal size. When glucose demand is low, a greater fraction of propionate is oxidized, resulting in satiety and smaller meal size (Allen et al., 2005).

Formulation of diets to shift starch digestion to the small intestine not only decreases propionate production, but also increases lactate absorption. This shift often results in greater DMI, which is inconsistent with the hypothesis that feed intake is regulated to meet energy requirements (NRC, 2001). In contrast, the hepatic oxidation hypothesis predicts increased DMI in response to shifting the site of starch digestion. Stimulation of hepatic oxidation by lactate is much less than propionate, especially during meals (Allen, et al., 2005), because of the greater lag before lactate absorption, and because hepatic extraction of lactate is much less than propionate (Reynolds, et al., 2003). Hepatic extraction of lactate is probably lower because metabolism of lactate to pyruvate is thermodynamically unfavorable when cellular NAD/NADH is low (Forman, 1988). Therefore, because of differences in metabolism, the hepatic oxidation hypothesis predicts that more energy can be absorbed in the form of lactate than propionate.

Feed intake regulation by hepatic oxidation may also help explain other phenomena observed in ruminants. Administration of somatotropin to lactating dairy cows consistently leads to greater DMI, yet this increase lags behind the increase in milk production by several weeks (Dohoo et al., 2003). This lag may be caused by a short-term increase in fatty acid mobilization and oxidation in the liver (Baldwin and Knapp, 1993). After cows deplete their most readily-available energy stores, a gradual deficit of hepatic fuels may result in lower mean ATP concentrations in the liver and increased DMI. Another unresolved issue is the

elusive mechanism for depressed feed intake in the periparturient period (Ingvartsen and Andersen, 2000). Plasma non-esterified fatty acid (NEFA) concentrations can be up to tenfold higher in early lactation than during gestation (Ingvartsen and Andersen, 2000) as a result of decreased plasma insulin concentration, combined with lower insulin sensitivity of adipose tissue during the transition to lactation (Doepel et al., 2002). This hyperlipidemia likely contributes to depressed feed intake rather than the reverse because plasma NEFA concentrations increase preceding periparturient hypophagia (Vazquez-Anon et al., 1994). Uptake of NEFA by the liver increases with increasing plasma NEFA concentration, resulting in greater hepatic fatty acid oxidation (Drackley and Andersen, 2006). Hepatic fatty acid oxidation likely contributes to satiety by generating ATP (Ji et al., 2000), and negative energy balance is further exacerbated because intake depression limits insulin secretion and promotes continued lipolysis.

The hepatic oxidation hypothesis, therefore, provides a unifying mechanism for explaining behavioral responses to changes in both nutrient digestion and metabolism. However, despite the strength of the evidence supporting the hepatic oxidation hypothesis, no mechanistic studies have been conducted to evaluate the hypothesis in ruminants. To assess its potential explanatory power for regulation of feed intake by lactating dairy cows, we designed and conducted 5 experiments; the results of these experiments are reported and discussed in the remaining chapters of this dissertation.

CHAPTER 2

INCREASING DIETARY STARCH FERMENTABILITY DECREASES FEED INTAKE OF LACTATING DAIRY COWS

ABSTRACT

The effects of dietary starch fermentability on feed intake, nutrient digestibility, milk production, and plasma metabolites and hormones were evaluated in a crossover study. Thirty-two multiparous Holstein cows [121 ± 48 d in milk, 41 ± 9 kg/d 3.5% fat-corrected milk (FCM); mean ± SD] were randomly assigned to treatment sequence and were fed a diet intermediate to the treatments during an initial 21-d period. Treatments were dry ground corn grain (DG) and high moisture corn (HM) harvested from the same field. Treatment periods were 14 d, with the final 4 d used for data and sample collection. Diets included corn silage and alfalfa haylage at a 2:1 ratio and were ~26% neutral detergent fiber, 17% crude protein, 32% starch, and 3.5% fatty acids. HM increased plasma glucose, non-esterified fatty acid, and triglyceride concentrations, but treatment had no effect on yield of milk or FCM. HM decreased dry matter intake (DMI) by 8%, but did not significantly alter digestible DMI. Individual DMI responses were highly variable, and variables from pre-trial propionate challenge tests, glucose tolerance tests, and hepatic mRNA analysis were assessed as potential predictors of DMI depression from increased dietary starch fermentability. Of the covariates tested, only pre-trial plasma insulin concentration and insulin response to glucose tolerance test were significant predictors of DMI depression. High

pre-trial plasma insulin concentration was correlated with greater depression in DMI with increased fermentability; conversely, greater insulin secretion in response to glucose infusion was associated with minimal depression in DMI. Consistent with past results, increased dietary starch fermentability decreased DMI. Significant correlations between insulin variables and individual DMI response may warrant further investigation.

INTRODUCTION

High-producing dairy cows require high levels of dietary energy to meet requirements for maintenance, milk synthesis, and reproduction. In the United States, these energy requirements are often met by adding corn grain to the diet. While corn grain in general is a highly digestible energy source, its ruminal fermentability may vary greatly due to differences in preservation method. Several studies have shown increases in ruminal starch digestibility between 19% and 24% for high moisture corn diets (HM) relative to ground dry corn diets (DG) (Knowlton et al., 1998; Oba and Allen, 2003e; Ying et al., 1998). These large differences in starch digestibility are expected to result in very different rates of propionate production and absorption. While propionate is the primary glucose precursor in dairy cattle, it may also serve as the primary metabolic limitation to feed intake (Allen, 2000). Ruminal infusions of propionate decreased NE intake in lactating dairy cows relative to iso-osmotic infusions of acetate (Oba and Allen, 2003d).

Oba and Allen (2003b) compared a HM diet to a DG diet, both including approximately 32% starch. The HM diet caused a 1.7 kg/d depression in DMI relative to the DG diet without affecting mean or variance of ruminal pH. However, responses to the change in starch fermentability were highly variable across cows. The objective of this experiment was to determine if intake and production responses to a change in diet fermentability could be predicted by production level or by measures of gluconeogenic capacity.

MATERIALS AND METHODS

Thirty-two multiparous Holstein cows (121 ± 48 DIM; mean ± SD) from the Michigan State University Dairy Cattle Teaching and Research Center were assigned randomly to sequence in a crossover experiment. At the beginning of the experiment, BW of cows was 675 ± 69 kg and FCM yield was 41.3 ± 9.6 kg/d (mean ± SD). Prior to the initial treatment period, all cows were fed a single diet intermediate in composition to the two treatment diets for 28 d. The purpose of this covariate period (COV) was to obtain baseline values for DMI, milk yield, and hepatic transcript abundance, and to conduct glucose and propionate challenge tests, all independent of dietary treatments.

Treatments were conservation method of corn grain (HM vs. DG). Treatment periods were 14 d, with the final 4 d used to collect samples and data. One corn hybrid (Great Lakes 4526; Great Lakes Hybrids, Ovid, MI) was grown in 2002, and half of the field was harvested as HM at 69% DM, ground, and ensiled in a 2.4 x 30.0-m silage bag (Ag Bagger, Ag Bag Corp., Blair, NE). The remaining half of the field was harvested as DG at 83% DM, dried to 86% DM by a high-temperature forced-air dryer, and finely ground. Experimental diets contained either HM or DG, corn silage (50% of forage DM), alfalfa silage (50% of forage DM), a premix of protein supplements, a premix of minerals and vitamins, and liquid protein supplement (**Table 2.1**). A diet intermediate to the experimental

diets was fed during the COV period. All diets were formulated for 18% dietary CP concentration and fed as a TMR.

Table 2.1 Ingredients and nutrient composition of experimental diets[1].

Item	COV	DG	HM
Diet ingredients			
DG	14.7	30.3	-
HM	15.8	-	32.1
Alfalfa haylage	15.4	14.9	14.5
Corn silage	30.7	30.8	30.0
Protein mix[2]	15.2	15.6	15.2
Mineral and vitamin mix[3]	5.3	5.4	5.3
Liquid protein supplement[4]	2.9	3.0	2.9
Nutrient composition			
DM	46.1	49.1	47.8
OM	93.3	93.5	93.6
Starch	31.5	31.7	32.8
NDF	25.1	26.2	25.3
Indigestible NDF[5]	7.0	6.8	6.7
CP	15.3	16.5	16.3

[1] COV: intermediate covariate diet, DG: dry ground corn diet, HM: high moisture corn diet. Values other than DM are expressed as a percent of dietary DM.

[2] Protein mix contained 75% soybean meal, 20% bloodmeal, and 5% SoyPlus (West Central Soy, Ralston, IA) on a DM basis.

[3] Mineral and vitamin mix contained 58.5% DG, 17.0% limestone, 10.5% dicalcium phosphate, 7.9% sodium bicarbonate, 2.1% magnesium oxide, 1.7% trace mineral premix, 1.6% trace mineral salt, and 0.8% vitamin A, D, and E premix.

[4] QLF Dairy TMR 20.

[5] Estimated after 120 h of in vitro ruminal fermentation.

Data and Sample Collection

Throughout the experiment, cows were housed in tie stalls and fed once daily (1000 h) at 115% of expected intake. One cow was removed from the experiment prior to its completion due to health problems unrelated to the experimental

treatments. Cows were blocked from feed between 900 h and 1000 h daily, and the amount of feed offered and orts were weighed for each cow during collection periods. Samples of all dietary ingredients (0.5 kg) and orts (12.5%) were collected daily and composited into one sample per cow, per period. Fecal samples were collected 3 times at 8 h intervals on d 14 of each experimental period and were composited into one sample per cow period. Cows were milked twice daily in a milking parlor, and milk yield was measured daily during the collection period and was averaged over the collection period. Milk was sampled at every milking on d 11-14 of each period and 3.5% fat-corrected milk (FCM) and energy-corrected milk (ECM; Tyrrell and Reid, 1965) yield were calculated. Body weight and BCS were measured on d 1 of each period and at the conclusion of the experiment. Body condition was scored by three trained investigators on five-point scale where 1 = thin and 5 = fat, as described by Wildman et al. (1982).

Blood samples were collected every 9 hours during d 12-14 of each period to represent every 3 h of a 24-hour period. Blood was sampled from coccygeal vessels and collected into 2 evacuated tubes, one containing potassium EDTA and the other containing potassium oxalate with sodium fluoride as a glycolytic inhibitor. Both were centrifuged at 2000 x g for 15 min immediately after sample collection, and plasma was harvested and frozen at -20°C until analysis. Samples containing K_3EDTA were preserved with benzamidine (0.05 M final

concentration), a proteolytic inhibitor that prevents glucagon degradation (Ensinck et al., 1972; Matsunaga et al., 1997).

Glucose tolerance and propionate challenge tests. Beginning on d 19 of the COV period, glucose tolerance tests (GTTs) and propionate challenge tests (PCTs) were conducted. Infusion tests were administered to blocks of 16 cows on each day; blocks corresponded to treatment sequence in the crossover portion of the experiment. All cows were fitted with a single jugular catheter 2 d prior to the GTT. Indwelling polypropylene catheters (0.24 cm o.d. x 0.17 cm i.d. tubing, MRE 095, Braintree Scientific, Braintree, MA) were inserted through a 10-gauge needle until approximately 30 cm of tubing was inside the jugular vein. Catheter patency was checked daily throughout the experiment. On the day of the GTT, cows were blocked from feed at 800 h and were not allowed access until GTTs were completed. Sterile solutions of 50% dextrose (wt/vol) were administered by intrajugular bolus at a dose of 1.67 mmol glucose/kg BW over the course of 5 – 10 min. Plasma samples were collected from the jugular vein 10 min prior to infusion, immediately before infusion, and every 10 min through 120 min post-infusion. All time points denoted are relative to the beginning of infusion. Catheters were flushed with 5 mL of sterile 4.2% Na citrate after infusion and after each blood sample collection. Propionate challenge tests were conducted after 1 or 2 d of rest. Sodium propionate (USP grade, Spectrum Chemical, New Brunswick, NJ) was dissolved in distilled, deionized water at a concentration of 4.5 mol/L, adjusted to pH 7.4 with NaOH, and filtered (#4 filter,

Whatman International, Maidstone, UK). On test days, Na propionate was infused over a period of 5 - 10 min at a dose of 1.04 mmol/kg BW. Infusion, sampling, and catheter maintenance were completed as for the GTT, and blood samples were processed and stored as described for the tailbleeding protocol. One catheter was lost during both the GTT and PCT, so 31 response curves were determined for each infusion test.

Liver biopsies. On d 15 (block 1) and 16 (block 2) of the COV period, liver biopsies were collected for measurement of transcript abundance for several proteins involved in gluconeogenesis. After local anesthetization with 2% lidocaine hydrochloride, biopsy instruments (14-gauge Vet-Core biopsy needles, Global Veterinary Products, New Buffalo, MI) were inserted between the eleventh and twelfth ribs on a line between the olecranon and the tuber coxae on the right side. Ten samples of approximately 20 mg were collected, immediately placed in RNA*later* (Ambion Inc., Austin, TX) to inhibit endogenous RNAse activity, and stored at -80°C until further processing.

Sample and Statistical Analysis

Diet ingredients and orts were dried in a 55°C forced-air oven for 72 h and analyzed for DM concentration. All samples were ground with a Wiley mill (1-mm screen; Arthur H. Thomas, Philadelphia, PA). Samples were analyzed for ash, NDF, indigestible NDF, CP, and starch. Ash concentration was determined after 5 h of oxidation at 500°C in a muffle furnace. Concentrations of NDF were

determined (Van Soest et al., 1991, method A). Crude protein was analyzed according to Hach et al. (1987). Starch was measured by an enzymatic method (Karkalas, 1985) after samples were gelatinized with sodium hydroxide; glucose concentration was measured with a glucose oxidase method (Sigma Chemical Co., St. Louis, MO). Indigestible NDF was estimated as NDF residue after 120 h of in vitro fermentation (Goering and Van Soest, 1970). Ruminal fluid for the in vitro incubations was collected from a nonpregnant dry cow fed alfalfa hay only. Indigestible NDF was used as an internal marker to calculate apparent digestibilities of DM, OM, starch, and NDF (Cochran et al., 1986). Concentrations of all nutrients except for DM were expressed as percentages of DM determined by drying at 105°C in a forced-air oven for more than 8 h.

Milk samples were analyzed for fat, true protein, and lactose with infrared spectroscopy by Michigan DHIA (East Lansing, MI). Plasma samples were analyzed using commercial kits to determine the plasma concentrations of glucose (Glucose kit #510; Sigma Chemical Co., St. Louis, MO), non-esterified fatty acids (NEFA C-kit; Wako Chemicals USA, Richmond, VA), triglycerides (kit TR0100, Sigma Chemical Co.), beta-hydroxybutyrate (BHBA; procedure #2440, Stanbio Laboratory, Boerne, TX), insulin (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA), and glucagon (Glucagon kit #GL-32K, Linco Research Inc., St. Charles, MO).

Transcript abundance. RNA was isolated from liver samples according to a method modified from Chomczynski and Sacchi (1987). Approximately 40 mg of tissue was homogenized in 1 mL Trizol reagent (Invitrogen, Carlsbad, CA), 200 µL of chloroform was added to the homogenate, and the mixture was centrifuged. The aqueous layer was collected and 500 µL of isopropanol was used to precipitate RNA. RNA isolates were treated with DNAse (Ambion) to remove any DNA contamination, and quality was verified by analysis with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Messenger RNA abundance for phosphoenolpyruvate carboxykinase (cytosolic form: PCK1), glucose-6-phosphatase (catalytic subunit: G6PC), pyruvate carboxylase (PC), and pyruvate dehydrogenase kinase 4 (PDK4) was analyzed by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) using a commercial kit (Superscript III Platinum Two-Step qRT-PCR Kit, Invitrogen). Reverse transcription was conducted using oligo-dT primers with 1 µg total RNA added as template. Following RNase H treatment, the cDNA product was quantified by spectrophotometer. Real-time PCR was carried out in duplicate using 1 µg cDNA and was monitored using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Labeled LUX primers (FAM label) and complementary unlabeled primers (Invitrogen) were designed as previously described (Bradford and Allen, 2005). Melting point analysis confirmed that only the transcripts of interest were amplified during PCR. Copy numbers for each gene were measured with 2 separate qRT-PCR

analyses using standard curves to allow for absolute quantification (Whelan et al., 2003). Clones used for standards were described previously (Bradford and Allen, 2005). Messenger RNA abundance was normalized using the geometric mean of copy numbers of cyclophilin, β-actin, and phosphoglycerate kinase 1 in each sample (Vandesompele et al., 2002). These control genes were selected from genes used for normalization in the literature, based on consistent expression patterns in a preliminary study (unpublished data).

Statistical analysis. Area under the curve (AUC) was calculated by the trapezoidal rule for infusion test variables by using the mean of the -10 and 0 min time points as the baseline value for each test. Insulin sensitivity was calculated from GTT responses using the ratio of maximum insulin : maximum glucose. Main effects of treatment were analyzed using the fit model procedure of JMP (version 5.0, SAS Institute, Cary, NC) according to the following model:

$$Y_{ijkl} = \mu + P_i + T_j + S_k + C_l(S_k) + e_{ijkl}$$

Where Y_{ijkl} = dependent variable, μ = overall mean, P_i = fixed effect of period (i = 1 to 2), T_j = fixed effect of treatment (j = 1 to 2), S_k = fixed effect of treatment sequence (k = 1 to 2), $C_l(S_k)$ = random effect of cow (l = 1 to 16) within sequence, e_{ijkl} = residual. Residual distributions were visually checked for normality. Treatment effects were declared significant at $P < 0.05$, and tendencies for treatment effects were declared at $P < 0.10$.

Individual differences in response to treatments were of interest in this study, and regression analyses were used to assess potential predictors of individual response. To quantify the hypophagic effects of increasing dietary starch fermentability, the following formula was used: DMI depression = DMI during HM treatment – DMI during DG treatment. There was a significant effect of period on DMI, so the parameter estimate for the period term was added to the DMI depression variable for all cows in one sequence and subtracted from all cows in the other sequence. For regression analysis, the distribution of Cook's D statistics was visually checked and outliers were removed from the analysis. No more than 3 data points were removed from any regression, and quadratic regressions were tested for each relationship. To control experiment-wise Type I error rate, significance was declared at $P < 0.01$ and tendencies were declared at $P < 0.02$ for regression analyses.

RESULTS AND DISCUSSION

The HM diet depressed DMI relative to the DG diet ($P < 0.001$, **Table 2.2**). The depression in DMI of 2.0 kg/d for the more highly fermentable diet was similar to the 1.7 kg/d depression in DMI found by Oba and Allen (2003b). However, total-tract starch and DM digestibilities were decreased by DG ($P < 0.001$), and as a result, digestible DMI was not significantly altered by treatment ($P = 0.35$). Consistent with the lack of effect on digestible DMI, neither ECM yield nor BCS change differed by treatment (Table 2.2).

Table 2.2 Effects of corn grain conservation method on intake, digestibility, and productivity of lactating cows.

Item	DG	HM	SEM	*P* value
DMI (kg/d)	25.6	23.6	0.6	< 0.001
Total-tract digestibility (% of intake)				
DM	74.7	77.4	0.5	< 0.001
OM	75.9	78.8	0.4	< 0.001
Starch	94.0	98.0	0.3	< 0.001
NDF	53.6	52.4	0.9	0.24
DM apparently digested (kg/d)	18.3	17.9	0.4	0.35
Yield (kg/d)				
Milk	38.5	38.3	1.5	0.57
FCM (3.5%)	38.3	36.5	1.7	0.17
ECM	38.2	36.6	1.6	0.20
Milk fat	1.34	1.24	0.08	0.15
Milk protein	1.19	1.20	0.05	0.94
Milk lactose	1.80	1.82	0.08	0.78
BW change (kg/d)	0.34	0.40	0.19	0.81
BCS change (/14 d)	0.05	0.09	0.04	0.52

A number of studies have shown increased total-tract starch digestibility with HM relative to DG (Huntington, 1997; Knowlton, et al., 1998; Oba and Allen, 2003e). Earlier harvest of HM can result in decreased vitreousness of starch granules and increased digestibility relative to DG (Correa et al., 2002). In addition, ensiling leads to microbial degradation of the protein matrix surrounding starch granules, increasing fermentability of the starch (Kotarski et al., 1992). Differences in ruminal fermentability of HM and DG are typically greater than differences in whole-tract digestibility (Huntington, 1997; Oba and Allen, 2003e), and HM likely caused substantially greater propionate production than the DG treatment in this experiment.

Several mechanisms have been proposed to explain depression of feed intake by inclusion of highly digestible starch sources in ruminant diets. Propionate has been implicated in feed intake regulation in ruminants because intraruminal propionate infusion depresses energy intake (Oba and Allen, 2003d), and portal infusion decreases DMI (Anil and Forbes, 1980). The expected increase in propionate production by HM in this experiment may have caused the observed decrease in DMI. However, other hypotheses have been advanced which point to reactive oxygen species (Illius et al., 2002) or implicitly identify total energy requirements (NRC, 2001) as factors limiting feed intake. These hypotheses have a number of shortcomings; there is currently no proposed mechanism by which whole-body oxidative stress can be relayed to the brain to influence satiety or hunger, and models which propose that animals eat to their energy requirements ignore the fact that diet can alter energy requirements (Harvatine and Allen, 2006). Nevertheless, these hypotheses, like the propionate oxidation hypothesis, predict a decrease in DMI with HM. Therefore, these results do not provide strong support for any proposed mechanism of feed intake regulation.

Plasma Metabolites and Hormones

The HM treatment significantly increased plasma glucose concentration ($P < 0.01$, **Table 2.3**). Plasma NEFA ($P < 0.001$) and triglyceride ($P = 0.02$) concentrations were also increased by HM, despite the fact that plasma insulin concentration was not significantly altered by treatment ($P = 0.39$). The reason

for this unusual result is not obvious. Plasma BHBA ($P = 0.58$) and glucagon ($P = 0.55$) concentrations were not different between treatments.

Table 2.3 Effects of corn grain conservation method on blood plasma metabolites and hormones.

Item	DG	HM	SEM	*P* value
Glucose (mg/dL)	58.7	60.6	0.6	< 0.01
NEFA (μEq/L)	45.5	54.1	2.1	< 0.001
Triglyceride (mg/dL)	2.9	3.3	0.1	0.02
BHBA (mg/dL)	7.6	7.8	0.3	0.58
Insulin (μIU/mL)	9.5	10.5	0.8	0.39
Glucagon (pg/mL)	189	185	9	0.55

Variability in DMI Responses to Altered Starch Fermentability

In addition to assessing the effects of dietary starch fermentability across animals, this experiment was designed to find animal characteristics that predict individual DMI response to increased starch fermentability. Individual variability in DMI response to starch fermentability was great, ranging from an increase of 0.9 kg/d to a decrease of 6.0 kg/d. DMI depression variables were compared to independent pre-trial (COV) variables, including plasma hormone and metabolite concentrations, production characteristics, infusion test responses, and hepatic transcript abundance. Most COV variables were not related to DMI depression, however, significant relationships with 2 insulin variables were observed (**Table 2.4**). Plasma insulin concentration during the COV period was inversely correlated with DMI depression ($r = -0.53$, $P < 0.01$), indicating that cows with high plasma insulin concentrations prior to treatment initiation experienced greater depression of DMI when starch fermentability was increased (**Figure 2.1**). Insulin response index following glucose infusion was positively related to

DMI depression (r = 0.57, $P < 0.01$), suggesting that cows with greater insulin secretory response to glucose were better able to maintain feed intake on highly fermentable diets (**Figure 2.2**).

Table 2.4 Pearson correlation coefficients for dry matter intake depression and potential pre-trial response predictors.[1]

Variable	r	*P*
Milk production (3.5% FCM)	0.24	0.19
Days in milk	-0.25	0.17
Body condition score	-0.01	0.95
Plasma glucose concentration	-0.04	0.82
Plasma NEFA concentration	0.05	0.78
Plasma BHBA concentration	-0.17	0.37
Plasma insulin concentration	**-0.53**	**< 0.01**
Insulin : glucagon ratio	-0.24	0.21
Plasma leptin concentration	-0.10	0.62
GTT glucose AUC	0.01	0.95
GTT maximum glucose	-0.36	0.06
GTT insulin AUC	0.45	0.03
GTT maximum insulin	0.37	0.07
GTT insulin response index	**0.57**	**< 0.01**
PCT glucose AUC	-0.13	0.49
PCT maximum glucose	-0.25	0.19
PCT insulin AUC	0.12	0.54
PCT maximum insulin	0.14	0.47
Relative PCK1 mRNA abundance	0.08	0.69
Relative PC mRNA abundance	0.18	0.35
Relative G6PC mRNA abundance	0.10	0.58
Relative PDK4 mRNA abundance	-0.10	0.60

[1] DMI depression data is corrected for differences in treatment sequence.

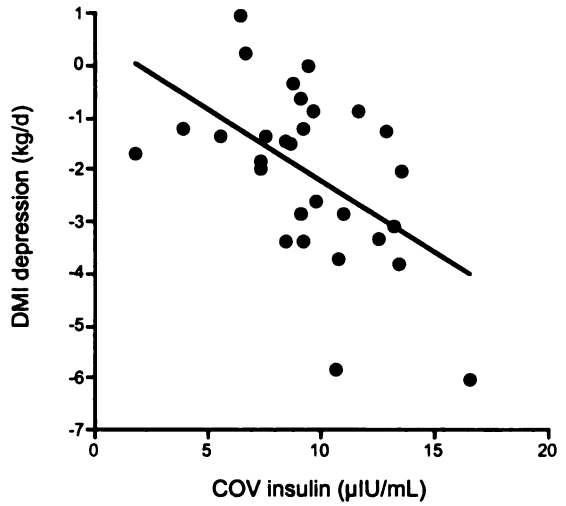

Figure 2.1 Pre-trial plasma insulin concentration (COV insulin) predicts DMI depression resulting from an increase in dietary starch fermentability. DMI depression is calculated as (HM – DG) DMI, corrected for period effect. Two outliers were removed prior to regression analysis. $R^2 = 0.28$, $P < 0.01$, n = 29.

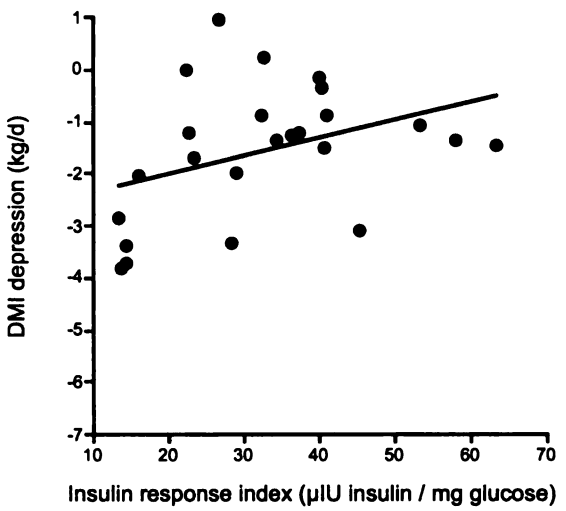

Figure 2.2 Insulin response index from glucose tolerance tests predicts DMI depression in response to increased dietary starch fermentability. DMI depression is calculated as (HM – DG) DMI, corrected for period effect. Insulin response is quantified as the ratio of maximum insulin : maximum glucose. Two outliers were removed prior to regression analysis; other individuals are not included because of missing GTT time points. $R^2 = 0.33$, $P < 0.01$, n = 24.

High plasma insulin concentrations in the COV period may be indicative of adequate nutritional status and may provide negative feedback on hepatic gluconeogenesis. This relationship is consistent with our hypothesis that decreased use of propionate for glucose production leads to greater propionate oxidation and decreased DMI. Individual cows with an adequate supply of glucogenic precursors may respond to a further increase in supply by decreasing DMI.

However, the positive relationship between GTT insulin response index and DMI depression seems to refute this proposed explanation. Cows that responded to increased plasma glucose concentration with greater insulin secretion (at 10 - 20 min post-infusion) were better able to maintain DMI on the more fermentable diet. If negative feedback on gluconeogenesis by insulin were involved in intake depression by HM, an inverse correlation would be expected. However, the multiple, complicated effects of insulin on nutrient homeostasis make this conclusion less clear. It is possible that cows with strong responses to increased plasma glucose concentration are able to clear nutrients from the bloodstream more quickly after meals, potentially decreasing intermeal interval (Oba and Allen, 2000). Although mean daily plasma insulin concentration and insulin response to glucose infusion are clearly unique measures (correlation: $r = 0.06$, $P = 0.78$), the reason for their respective relationships with DMI response is less obvious.

Despite indications that measures related to insulin predicted responses to diet fermentability, the status of insulin as a long-term regulator of feed intake in lactating ruminants is questionable. Although strong relationships between adiposity and insulin concentrations have been reported in the biomedical literature (Bagdade et al., 1967; Polonsky et al., 1988), we have found no evidence of a similar relationship in lactating dairy cattle. Body condition score measured during the COV period was highly correlated with plasma leptin concentration (Bradford et al., 2006b), but was unrelated to plasma insulin concentration (insulin [pmol/L] = 47.6 + 11.0 • BCS, R^2 = 0.03, P = 0.31). These and other findings (Meikle et al., 2004) suggest that in mature cows, insulin does not serve as an adiposity signal for long-term control of feed intake. Nevertheless, insulin may impact feed intake in the short-term through its influences on hepatic metabolism and nutrient partitioning.

There are likely a number of reasons that so few relationships with DMI depression were found. One drawback to the use of dietary treatments is that diet adaptation periods are required; as a result, COV variables were determined 35 - 42 d prior to period 2 data collection. Variables such as COV plasma NEFA concentration or hepatic transcript abundance may have had no relationship with metabolic state 42 d later. The length of the experiment also prevented the use of early lactation cows, because their responses tend to change dramatically by period; this may have contributed to the lack of a relationship with days in milk. More surprising, perhaps, is the fact that measures of glucose and insulin

responses from the propionate challenge test were not related to DMI depression; Oba and Allen (2003c) found a clear relationship between plasma glucose concentration and DMI response to intraruminal propionate infusion. However, we recently reported that jugular administration of propionate stimulates glycogenolysis, indicating that changes in plasma glucose concentration during the PCT do not reflect increased gluconeogenesis exclusively (Bradford et al., 2006a). In addition, insulin responds to changes in both propionate and glucose concentrations during the propionate challenge test, adding noise that may mask differences that were observed during the GTT. Nevertheless, the fact that simple measures such as body condition score, production level, and plasma glucose concentration did not predict DMI response suggests that the mechanisms causing DMI depression on highly fermentable diets may involve interactions of metabolic factors.

CONCLUSIONS

Increasing the fermentability of starch in a high-concentrate dairy ration depressed DMI but did not have significant effects on digestible DMI or yield of milk or milk components. Pre-trial plasma insulin concentration was correlated with the depression of DMI induced by increasing dietary starch fermentability.

CHAPTER 3

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Propionate is not an important regulator of plasma leptin concentration in dairy cattle☆

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Abstract

Propionate was recently shown to increase leptin synthesis in rodents. To determine if a similar effect occurs in ruminants, propionate was administered to lactating dairy cows. In experiment 1, 31 cows were given an intrajugular Na propionate bolus (1040 μmol/kg body weight), increasing plasma propionate from 160 to 5680 μM and plasma insulin from 6.8 to 77.8 μIU/mL. Plasma leptin concentration decreased from 2.11 ng/mL before bolus to 1.99 ng/mL after dosing ($P<0.05$) with no differences in leptin concentrations at 20, 50, and 100 min post-bolus ($P>0.10$). In experiment 2, 12 cows were used in a duplicated 6 × 6 Latin square experiment to assess the dose–response effect of ruminal propionate infusion on plasma leptin concentration. Sodium propionate was infused at rates of 0, 260, 520, 780, 1040, or 1300 mmol/h, while total short-chain fatty acid infusion rate was held constant at 1300 mmol/h by addition of Na acetate to the infusate. Coccygeal blood was sampled following 18 h of infusion. Increasing the rate of propionate infusion linearly increased plasma propionate concentration from 180 to 330 μM ($P<0.001$) and plasma insulin concentration from 6.7 to 9.1 μIU/mL ($P<0.05$). There was a quadratic response in plasma leptin concentration ($P=0.04$) with a maximum at 780 mmol/h propionate, but leptin concentrations increased by no more than 8% relative to the 0 mmol/h propionate infusion. Leptin concentrations were correlated

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with insulin concentrations but not with propionate concentrations in plasma. Propionate is not a physiological regulator of leptin secretion in lactating dairy cows.

Keywords: Leptin; Propionate; Dairy cow; Adipose tissue

1. Introduction

Leptin, a peptide hormone produced primarily by adipocytes, plays a central role in the regulation of food intake. Leptin was originally proposed to be the long-sought feedback signal from adipose tissue to hypothalamic nuclei regulating long-term energy intake [1]. Subsequent research has largely validated this hypothesis, providing strong evidence that leptin can alter energy intake by modulating the hypothalamic production of orexigenic (NPY and AGRP) and anorexigenic (POMC and CART) peptides [2]. However, leptin's proposed roles have now expanded to the regulation of metabolism, growth, and reproduction [3]. Indeed, recent evidence has called into question leptin's role as a purely lipostatic signal. The ability to modulate leptin secretion through short-term nutritional [4] and environmental changes [5] suggests that leptin can also convey metabolic information to the central nervous system [6].

Short-chain fatty acids (SCFA) are derived primarily from microbial fermentation and provide roughly 70% of the energy requirements in ruminants [7]. While SCFA represent only 6–10% of caloric intake in humans, there is evidence that SCFA may be responsible for some of the health benefits of high-fiber diets [8], possibly through modification of gene expression patterns [9]. Recently, several groups have suggested that SCFA may enhance leptin expression and secretion in anterior pituitary cells [10] and adipocytes [11]. Furthermore, jugular infusion of propionate increased leptin mRNA abundance in ovine adipose tissue [12] and oral administration of propionate in mice increased plasma leptin concentration [13].

The objective of this study was to determine if an intrajugular sodium propionate bolus or continuous intraruminal infusion of Na propionate would alter plasma leptin concentrations in lactating dairy cattle.

2. Materials and methods

Experimental procedures were approved by the All-University Committee on Animal Use and Care at Michigan State University and cows were selected from the Michigan State University Dairy Teaching and Research Center herd.

2.1. Experiment 1

Animals, experimental design, and diets for experiment 1 have been described [14]. Thirty-two multiparous lactating Holstein cows (121 ± 48 d in lactation, 41.5 ± 7.8 kg/d milk yield, 675 ± 69 kg body weight; mean $\pm$ S.D.) were offered a single diet (Table 1)

Table 1
Nutrient composition of experimental diets (percent of dietary dry matter)

	Experiment 1	Experiment 2
Organic matter	93.3	93.9
Starch	31.5	27.4
Neutral detergent fiber	25.1	30.0
Crude protein	15.3	18.1

for 22 d, and propionate challenge tests (PCT) were conducted over 2 d. At least 3 d prior to the PCT, indwelling polyurethane catheters (MRE 095, Braintree Scientific, Braintree, MA) were inserted in a single jugular vein of each animal. On each infusion day, feed was removed at 08:00 h, and PCT were initiated for 16 cows over 2 h. Catheter patency was lost for one cow prior to the PCT, so a total of 31 PCT were conducted. Sodium propionate (USP grade, Spectrum Chemical, New Brunswick, NJ) was dissolved in distilled, deionized water at a concentration of 4.5 mol/L, adjusted to pH 7.4 with NaOH, and filtered (#4 filter, Whatman International, Maidstone, UK). Sodium propionate was infused into the jugular vein (1040 μmol/kg body weight) over the course of approximately 8 min. Jugular blood samples were collected 10 min prior to infusion, immediately before infusion, and every 10 min after the initiation of infusion until 120 min post-infusion. Samples were collected by an automated blood collection system [15], and catheters were flushed with a solution of 4.2% Na citrate following infusion and after each sampling.

2.2. *Experiment 2*

Experiment 2 has been described previously [16]. Briefly, 12 multiparous lactating Holstein cows (36.4 ± 6.5 kg/d milk yield, 640 ± 63 kg body weight) with ruminal cannulas were assigned to early lactation (9 ± 6 d in lactation) and mid lactation (192 ± 17 d in lactation) blocks in a duplicated 6 × 6 Latin square design. Treatments were intraruminal infusions of 0, 260, 520, 780, 1040, or 1300 mmol Na propionate/h beginning 6 h before feeding and continuing for 18 h. Rate of total SCFA administration was held constant at 1300 mmol/h by adding required amounts of Na acetate. One cow in each block was removed from the experiment due to adverse reactions to the treatments, resulting in a total of 66 infusions.

For each stage of lactation, cows were adapted to the experimental diet (Table 1) for 14 d. Blood samples were then collected from coccygeal vessels every 9 h for 3 d and composited for determination of plasma leptin concentrations prior to treatment initiation. Treatments were administered on alternate days for the final 11 d of the experiment. On treatment days, cows were blocked from feed for 4 h prior to feeding, but had access to feed during the rest of the infusion. Blood samples were collected from coccygeal vessels at the conclusion of each 18-h infusion.

2.3. *Analytical procedures*

Blood samples were placed on ice immediately after collection and plasma was harvested and frozen at −20 °C until analysis. Plasma leptin concentrations were

determined by a radioimmunoassay specific to bovine leptin [17]. The intra-assay coefficient of variation was 4.1% and the inter-assay CV was 2.6% for leptin analysis. Insulin concentrations were determined by commercial radioimmunoassay (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA). Intra- and inter-assay CV's for insulin analysis were 7.8 and 10.1%, respectively. Plasma propionate and acetate concentrations were determined by HPLC according to the method described for ruminal fluid [18].

For experiment 1, plasma propionate and insulin values were log-transformed to normalize the distribution of the data and were subsequently analyzed by the mixed model procedure of SAS (version 8.0, SAS Institute, Cary, NC). The mixed model included fixed effects of infusion day and sample time and the random effect of cow nested within day. An autroregressive (AR [1]) covariance structure was assigned to the time variable. Orthogonal contrasts were used to determine whether differences from pre-infusion values were significant, and propionate and insulin values were back-transformed following analysis.

Leptin data from experiment 1 and all data from experiment 2 were analyzed by the fit model procedure of JMP (version 5.0, SAS Institute, Cary, NC) using the REML method. The mixed model for experiment 1 included fixed effects of infusion day and sample time and the random effect of cow within day; differences between sample times were tested by Tukey's HSD. The mixed model for experiment 2 included the fixed effects of treatment and period and the random effect of cow. Linear and quadratic effects of propionate infusion rate were tested for each dependent variable. Stage of lactation was included in the original model, but was dropped after establishing the absence of a significant main effect and interaction effects. In addition to these analyses, correlations among several dependent variables were investigated. For these correlations, the distribution of Cook's D statistic was plotted and as many as three outliers were removed from the analysis before the final correlation was calculated.

3. Results

3.1. Experiment 1

Intrajugular bolus of Na propionate significantly increased plasma propionate concentration 10 and 20 min after the initiation of infusion (both $P < 0.001$), but not at subsequent sampling times. Jugular propionate concentrations reached a mean of 5680 μM at 10 min post-infusion compared to 160 μM prior to infusion (Fig. 1). Propionate administration also stimulated insulin secretion, increasing venous insulin concentration from 6.8 μIU/mL pre-infusion to a peak of 77.8 μIU/mL at 10 min post-infusion. Plasma insulin concentrations remained higher than pre-infusion values through the 40 min sampling time (all $P < 0.001$, Fig. 1). Plasma leptin concentrations averaged 2.11 ng/mL immediately prior to infusion, decreased to 1.99 ng/mL at 20 min post-infusion, and remained lower than baseline concentrations at 50 and 100 min post-infusion (S.E.M. = 0.09, $P < 0.05$, Fig. 1). Pre-infusion leptin concentrations were positively correlated with body condition score ($r = 0.61$, $P < 0.001$, Fig. 2), as reported previously [17].

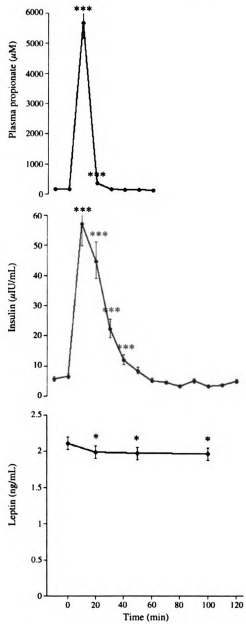

Fig. 1. Responses to intrajugular bolus of Na propionate. Propionate was infused at the rate of 1040 μmol/kg body weight over approximately 8 min beginning at 0 min. $^{*}P<0.05$ compared to pre-infusion values; $^{***}P<0.001$ compared to pre-infusion values.

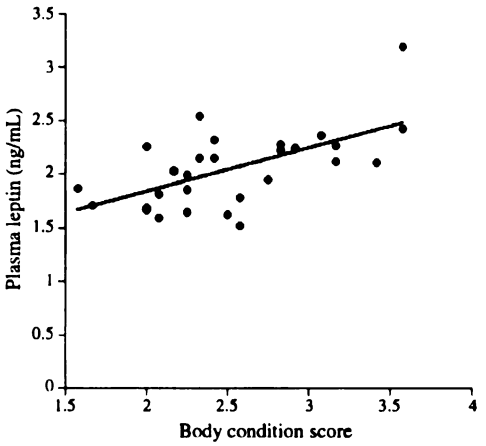

Fig. 2. Pre-infusion leptin concentrations are positively correlated with body condition score ($r = 0.61$, $P < 0.001$, experiment 1).

3.2. Experiment 2

Plasma acetate concentration decreased quadratically with increasing propionate infusion rate (and decreasing acetate infusion rate) from 6.3 to 0.9 mM in early lactation cows and from 2.1 to 0.8 mM in mid-lactation cows. Therefore, it was necessary to verify that Na acetate was an appropriate control for this experiment. Pre-treatment leptin concentrations were compared to leptin concentrations following the 0 mmol/h propionate treatment (1300 mmol/h acetate), and no differences were found ($P = 0.99$). We conclude that Na acetate infusion does not influence leptin production.

Plasma propionate concentrations increased linearly with the amount of propionate infused ($P < 0.001$), increasing from a baseline of 180 μM to a maximum of 330 μM at the highest propionate infusion rate (Fig. 3). Plasma insulin concentrations also increased linearly with propionate infusion rate ($P < 0.05$) from 6.7 to 9.1 μIU/mL. While the rate of propionate infusion had no linear effect on plasma leptin concentrations ($P > 0.15$), there was a significant quadratic effect ($P = 0.04$, Fig. 3; $Y = 2.95 + 9.55 \times 10^{-5}X - 3.86 \times 10^{-7}(X - 650)^2$). Plasma leptin concentration was not related to plasma propionate concentration ($P > 0.15$), but was positively correlated with plasma insulin concentration ($r = 0.45$, $P < 0.001$). To determine if this relationship simply represented long-term regulation of leptin by insulin, the changes in plasma insulin, leptin, and propionate concentrations from baseline values were calculated for each cow period. The change in plasma leptin concentration was positively correlated with the change in plasma insulin concentration across infusions ($r = 0.30$, $P = 0.02$), but not with change in plasma propionate concentration.

4. Discussion

Recent evidence has suggested that propionate can alter gene expression in adipose tissue. Lee and Hossner [12] administered 1920 μmol propionate/kg body weight in sheep

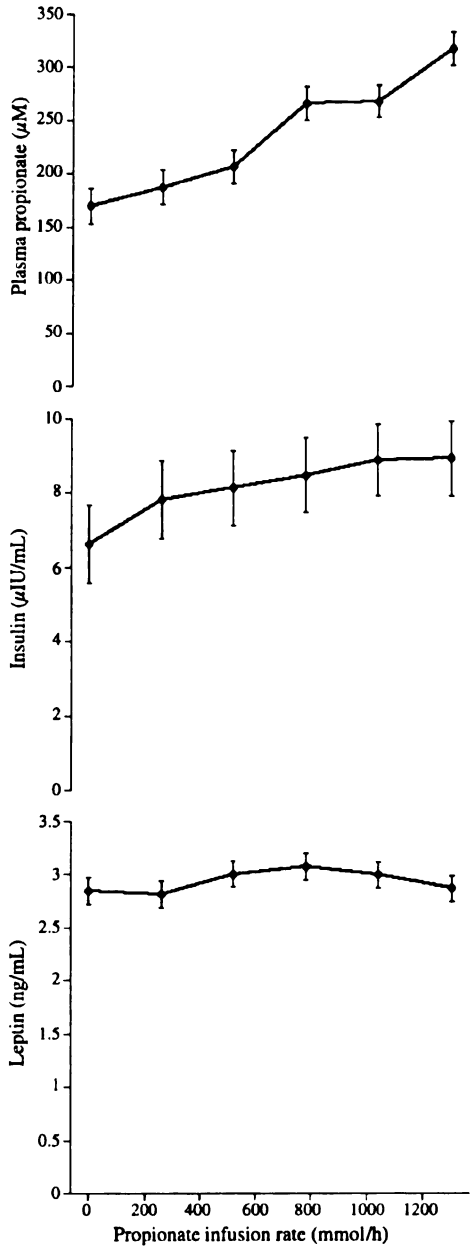

Fig. 3. Responses to continuous intraruminal Na propionate infusion. Sodium propionate was infused at rates of 0, 260, 520, 780, 1040, or 1300 mmol/h, with total SCFA infusion rate held constant at 1300 mmol/h by addition of Na acetate to the infusate. Coccygeal blood was sampled following 18 h of infusion. Increasing the rate of propionate infusion linearly increased plasma concentrations of propionate ($P < 0.001$) and insulin ($P < 0.05$) and quadratically altered leptin concentrations ($P < 0.05$).

during a 30 min intrajugular infusion and measured increased mRNA abundance for several genes in subcutaneous adipose tissue, including leptin. In mice, 500 μmol of propionate administered via gavage increased plasma propionate concentrations from 25 to 250 μM and resulted in an 80% increase in plasma leptin concentration [13]. Several groups have identified pathways mediating the effects of propionate in adipose tissue [11,13]. The G-protein coupled receptor GPR41 binds SCFA, particularly propionate, and is expressed primarily in adipose tissue in humans [11]. Recent sequencing of the bovine genome has identified a sequence (413563485, http://www.ncbi.nih.gov/Traces/) with a translated primary structure that shares 74% homology with a portion of human GPR41 protein (81% coverage). This finding is consistent with the possibility that propionate could regulate leptin gene expression via the bovine GPR41 ortholog.

The data obtained in these two experiments suggests that propionate plays a minor role, if any, in regulating plasma leptin concentrations in lactating dairy cows. In our first experiment, venous propionate concentrations peaked at more than 30 times baseline concentrations, although they decreased rapidly following this peak. Because leptin mRNA abundance was greatly increased just 2 h after initiation of propionate infusion in the sheep [12], it is likely that any stimulation of plasma leptin concentration by propionate would have been observed by 100 min post-infusion. However, it is possible that the very short-term effect on peripheral propionate concentrations prevented significant stimulation of leptin expression. Our second experiment addressed this possibility by administration of SCFA during 18 h intraruminal infusions. Although there was a significant quadratic response of plasma leptin to increasing rate of propionate infusion, plasma leptin concentration was increased by no more than 8% and did not produce a linear response.

It is possible that stimulation of leptin secretion requires peripheral propionate concentrations to be elevated to a greater extent than achieved with intraruminal infusions and for longer periods than in the intrajugular infusion experiment. If this were the case, however, propionate would not be expected to stimulate leptin secretion in any physiological situation. Hepatic uptake of propionate is extremely efficient in most species, with extraction efficiencies generally reported in the range of 90–100% in dairy cows [19]. As a result, despite the absorption of large amounts of propionate in dairy cows, venous propionate concentrations are relatively stable and are typically in the range of 70–150 μM. Propionate concentrations exceeding our treatment mean of 330 μM (1300 mmol/h Na propionate, experiment 2) would not occur under normal feeding conditions, and would likely indicate impaired liver function.

Another possibility is that peripheral concentrations of propionate in well-fed cows are adequate to constitutively stimulate GPR41 receptors. Baseline venous propionate concentrations of 160 μM in the cows in experiment 1 are approximately 50-fold higher than those in humans; healthy individuals in three studies had a mean propionate concentration of 3 μM [20–22]. Oral gavage of propionate stimulated leptin secretion in mice by increasing plasma propionate concentrations from 25 to 250 μM [13], suggesting that a near-maximal response might be achieved at 160 μM propionate. The reported EC_{50} for propionate stimulation of leptin secretion is 200 μM in primary cultures of murine adipocytes [13], however, differences in the primary structure of murine and bovine GPR41 may alter binding kinetics [11].

One weakness of in vivo experiments implicating propionate in regulation of leptin secretion [12] is that propionate is a potent insulin secretagogue in ruminants [23]. Insulin's effects on leptin secretion have been demonstrated in dairy cows [24], and in experiment 2, plasma leptin concentrations were significantly related to insulin, but not propionate concentrations. Insulin may alter transcription of the leptin gene by stimulating nutrient uptake and metabolism in adipocytes [25], as evidenced by the stimulatory effect of malonyl-CoA on leptin production in murine adipocytes [26]. However, increased leptin secretion following propionate infusion in rodents is probably not mediated by insulin because propionate does not stimulate insulin release in non-ruminants [27].

Several studies have considered the effects of fasting and refeeding on plasma leptin concentrations in cattle [28,29]. While propionate infusion might be expected to have a greater impact on leptin secretion in fasted animals, confounding effects on insulin, growth hormone, epinephrine, and other hormones would be an even greater problem in fasted animals. Furthermore, evidence that propionate can stimulate leptin secretion in rats fed ad libitum [13] led us to hypothesize that a similar response might occur in lactating cows. There was no evidence that leptin response to propionate infusion was greater for early lactation cows in experiment 2 (interaction $P = 0.91$), despite the fact that early lactation cows were in negative energy balance. Therefore, we expect that the direct effect of propionate on leptin secretion (independent of insulin, etc.) would be minor even in fasted animals.

Xiong et al. [13] suggested that hypophagia induced by infusion or dietary supplementation of propionate might be mediated by increased leptin production. Oba and Allen [16] reported that the infusions described here in experiment 2 linearly decreased metabolizable energy intake during the final 12 h of infusion (including energy provided by infused SCFA) as propionate infusion rate increased. The lack of a corresponding increase in leptin concentration refutes the hypothesis that leptin mediates the hypophagic effects of propionate in lactating dairy cattle. Potential alternative mechanisms for this response have been discussed elsewhere [30].

In conclusion, our findings do not support the hypothesis that propionate is an important regulator of plasma leptin concentration in lactating dairy cattle. Although propionate may have some influence on leptin production through its role as an insulin secretagogue, it is unlikely that hypophagic effects of propionate are mediated primarily through leptin.

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CHAPTER 4

Bradford, B. J. and M. S. Allen. 2005. Phlorizin administration increases hepatic gluconeogenic enzyme mRNA abundance but not feed intake in late-lactation dairy cows. J Nutr. 135(9):2206-2211.

Phlorizin Administration Increases Hepatic Gluconeogenic Enzyme mRNA Abundance but Not Feed Intake in Late-Lactation Dairy Cows[1–3]

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ABSTRACT Gluconeogenic capacity may be an important factor regulating dry matter intake (DMI) in lactating dairy cows. To determine whether increased glucose demand affects feed intake and hepatic gene expression, lactating Holstein cows were treated with phlorizin or vehicle (propylene glycol) for 7 d. Multiparous cows (n = 12; 269 ± 65 d in milk, mean ± SD) were randomly assigned to treatment sequence in a crossover design and were adapted to a common diet for 7 d before the beginning of the experiment. Phlorizin injected s.c. at 4 g/d caused glucose excretion in urine at the rate of 474 g/d. Although phlorizin decreased lactose synthesis and milk production (both $P < 0.01$), DMI and 3.5% fat-corrected milk production were not altered by treatment. A net deficit of 383 g glucose/d in milk and urine for phlorizin (relative to control) was likely replaced partially through increased gluconeogenesis. The molar insulin:glucagon ratio was decreased 17% by phlorizin ($P < 0.001$) and hepatic phosphoenolpyruvate carboxykinase, glucose-6-phosphatase, and pyruvate carboxylase mRNA abundance increased (all $P < 0.05$). Late-lactation dairy cows adapted quickly to an increase in peripheral glucose demand; adaptation mechanisms likely included enhanced gluconeogenic capacity, whereas DMI was not altered. J. Nutr. 135: 2206–2211, 2005.

KEY WORDS: • *dairy cows* • *phlorizin* • *glucose demand* • *gluconeogenesis* • *pyruvate carboxylase*

Dry matter intake (DMI)[5] is regulated by a wide range of signals related to the intake of specific nutrients, gut distention, adiposity, cephalic stimulation, and other factors (1). Forbes (2) suggested that these signals are integrated, driving feeding behaviors that minimize discomfort to the animal. Although this signal integration likely occurs in the hypothalamus, there is a large body of evidence indicating that some signals that regulate DMI originate in the liver and are delivered via the hepatic vagus (3,4). These signals are related to the energy status of the liver; increased hepatic ATP concentrations coincide with the ends of meals in rats (5).

Propionate is an important fuel for ruminants because it is the primary substrate for gluconeogenesis in lactating cows fed highly fermentable diets. Its importance in intake regulation was demonstrated by Oba and Allen (6), who showed that intraruminal infusions of propionate linearly decreased metabolizable energy intake (including both feed and infusions) compared with isomolar infusions of acetate. The liver was likely responsible for these effects because hepatic vagotomy eliminated hypophagic responses to portal infusions of propionate (7). These treatments have physiological importance for the regulation of meal size because propionate flux to the liver increases rapidly during meals (8).

If meal size and intermeal interval are dependent on the energy status of the liver, the intake effects of propionate should depend on the extent to which it stimulates hepatic oxidation. Therefore, increasing the flux of propionate to glucose might decrease its hypophagic effects while simultaneously providing more glucose for the mammary gland and other peripheral tissues. This mechanism, mediated by changes in insulin and glucagon secretion, may provide the link between increases in peripheral energy demand and increased DMI. Although this relation has been established in a variety of circumstances (9–11), well-controlled mechanistic studies in ruminants have not been conducted.

To test the effects of increased glucose demand on metabolic regulation and DMI, we used phlorizin to cause irreversible glucose loss in urine (12). We hypothesized that increased peripheral glucose demand would cause upregulation of gluconeogenic pathways, and that increased gluconeogenic capacity would increase DMI by increasing meal size. Therefore, the objective of this study was to measure responses in hepatic mRNA abundance, milk yield, DMI, feeding behavior, and plasma hormones and metabolites during phlorizin treatment. Although several studies have measured the effect of phlorizin

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[3] Supplemental Table 1 and Supplemental Figure 1 are available as Online Supporting Material with the online posting of this paper at www.nutrition.org.

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[5] Abbreviations used: BHBA, β-hydroxybutyrate; DMI, dry matter intake; G6PC, glucose-6-phosphatase catalytic subunit; PC, pyruvate carboxylase; PCK1, cytosolic phosphoenolpyruvate carboxykinase; PDK4, pyruvate dehydrogenase kinase 4; qRT-PCR, quantitative RT-PCR; SLC37A4, glucose-6-phosphatase translocase.

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on lactating cows (13,14), only one has considered its effect on DMI, and that study used only 4 cows with treatment periods of 2 d (14). To our knowledge, there have been no reports evaluating the effects of phlorizin on expression of metabolic genes in bovine liver.

MATERIALS AND METHODS

Experimental procedures were approved by the All-University Committee on Animal Use and Care at Michigan State University.

Design and treatments. Multiparous Holstein cows (n = 12; 269 ± 65 d in milk; 30.7 ± 5.0 kg/d milk yield; 2.6 ± 0.7 lactations; mean ± SD) were selected from the Michigan State University Dairy Cattle Teaching and Research Center and were randomly assigned to treatment sequence in a crossover design. Phlorizin, an inhibitor of renal glucose reabsorption (12), was administered via s.c. injection at the rate of 4 g/d, with propylene glycol as vehicle and control. Treatment periods were 7 d, and injections were given every 6 h during these periods. The cows were adapted to a single diet for a 7-d period before the first treatment period, and a 7-d rest period was included between the 2 treatment periods. The experimental diet (419 g dry matter/kg diet) contained dry, finely ground corn grain, corn silage, alfalfa silage, a premix of protein supplements, and a premix of minerals and vitamins (**Table 1**). The diet was formulated for 280 g/kg neutral detergent fiber and 160 g/kg crude protein.

Data and sample collection. Throughout the experiment, cows were housed in tie-stalls and fed once daily (1130 h) at 115% of expected daily intake. Cows were not allowed access to feed from 1000 to 1130 h, during which time orts and the amount of feed offered were weighed for each cow daily. During the final 4 d of each treatment period, feeding behavior was monitored by a computerized data acquisition system (15) throughout the day. Data on chewing activities, feed disappearance, and water consumption were recorded for each cow every 5 s, and mean daily values for number of meal bouts, interval between meals, meal size, and time spent eating were calculated. One cow was removed from feeding behavior analysis because equipment malfunction prevented collection of satisfactory chewing behavior data. On each of these 4 d, samples of all dietary ingredients (0.5 kg) and orts (12.5%) were collected, and on d 7 of treatment, fecal samples were collected 3 times at 8-h intervals. Cows were milked twice daily in the milking parlor during rest periods and in the tie stalls during treatment periods. Milk yield was recorded and samples were taken at each milking during the 4 collection days.

TABLE 1

Ingredients and nutrient composition of experimental diet

Component	Content
	g/kg dry matter
Diet ingredient	
Dry ground corn	382
Corn silage	264
Alfalfa silage	238
Protein mix[1]	94
Mineral and vitamin mix[2]	22
Nutrient composition	
Organic matter	939
Starch	346
Neutral detergent fiber	278
Indigestible neutral detergent fiber[3]	113
Crude protein	138

[1] Contents (g/kg dry matter): soybean meal, 750; blood meal, 200; SoyPlus (West Central Soy), 50.

[2] Contents (per kg dry matter): Na, 81 g; Cl, 41 g; Ca, 31 g; P, 26 g; Mg, 23 g; Fe, 3.2 g; Mn, 2.4 g; Zn, 2.3 g; K, 2 g; S, 2 g; Cu, 574 mg; I, 34 mg; Se, 16 mg; Co, 10 mg; vitamin A, 14 mg; vitamin D, 330 μg; vitamin E, 113 mg.

[3] Measured after 240 h of in vitro ruminal fermentation.

Urine was collected for a 24-h period starting on d 4 (1100 h) of each experimental period. Urinary catheters (Bardex Lubricath Foley 24FR, Bard Medical) were inserted and urine was collected into a container with 0.23 mol HCl to prevent glycolysis. Volumes were measured at 12-h intervals and samples were taken and frozen until analysis. According to the data of Amaral-Phillips et al. (14), phlorizin injected every 6 h causes glucose excretion at a constant rate, indicating that a single 24-h collection provided a valid estimate of daily glucose excretion. During the same 24-h period, blood samples were collected hourly from indwelling jugular catheters. Collected blood was immediately emptied into 2 tubes, one containing potassium EDTA and the other containing potassium oxalate with sodium fluoride as a glycolytic inhibitor (Vacutainer, Becton Dickinson). Both were centrifuged at 2000 × *g* for 15 min immediately after sample collection, and plasma was harvested and frozen at −20°C until analysis. One plasma sample from each tube containing K$_3$EDTA was preserved with benzamidine (0.05 mol/L final concentration), a proteolytic inhibitor that prevents glucagon degradation (16,17).

Liver biopsies were collected from phlorizin-treated cows immediately before the first injection on d 1 of each treatment period (control sample) and shortly after the final injection at the end of d 7 (treated sample). After local anesthetization with 2% lidocaine hydrochloride, biopsy instruments (14-gauge Vet-Core biopsy needles, Global Veterinary Products) were inserted between the 11th and 12th ribs on a line between the olecranon and the tuber coxae on the right side; 10 samples of ~20 mg were collected and immediately (<5 s) frozen in liquid nitrogen; the 200 mg sample was stored at −80°C until further processing.

Sample analysis. Diet ingredients, orts, and fecal samples were dried in a 55°C forced-air oven for 72 h and analyzed for dry matter concentration. All samples were ground with a Wiley mill (1-mm screen; Authur H. Thomas). Samples were analyzed for ash, neutral detergent fiber, indigestible neutral detergent fiber, crude protein, and starch. Ash concentration was determined after 5 h of oxidation at 500°C in a muffle furnace. Neutral detergent fiber was analyzed according to Van Soest et al. [(18), method A]. Indigestible neutral detergent fiber was measured as neutral detergent fiber residue after 240 h of in vitro fermentation (19). Ruminal fluid for the in vitro incubations was collected from a nonpregnant dry cow fed alfalfa hay only. Crude protein was analyzed according to Hach et al. (20). Starch was measured by an enzymatic method (21) after samples were gelatinized with sodium hydroxide; glucose concentration was measured using the glucose oxidase method. Concentrations of all nutrients were expressed as percentages of dry matter determined from drying at 105°C in a forced-air oven. Indigestible neutral detergent fiber was used as an internal marker to calculate total-tract digestibility of other nutrients.

Milk samples were analyzed for fat, true protein, and lactose with infrared spectroscopy by the Michigan Dairy Herd Improvement Association. Urinary nitrogen was quantified in duplicate by Dumas combustion using a commercial analyzer (LECO FP-2000). Urine and plasma samples were analyzed in duplicate for glucose content by the glucose oxidase method. Plasma samples were analyzed in duplicate using commercial kits to determine concentrations of FFA [NEFA C-kit; Wako Chemicals, as modified (22)], β-hydroxybutyrate (BHBA; procedure #2440, Stanbio Laboratory), insulin (Coat-A-Count, Diagnostic Products), and glucagon (Glucagon kit #GL-32K, Linco Research). Plasma L-lactate content was quantified with a clinical analyzer (YSI 1500, YSI Life Sciences).

Total RNA was isolated from liver tissue using a commercial kit (RiboPure, Ambion) and samples were treated with DNase to remove any DNA contamination. The quality of all RNA isolates was verified by analysis with an Agilent 2100 Bioanalyzer (Agilent Technologies). mRNA abundance for phosphoenolpyruvate carboxykinase (cytosolic form: PCK1, EC 4.1.1.32), glucose-6-phosphatase (EC 3.1.3.9, catalytic subunit: G6PC, regulatory subunit: SLC37A4), pyruvate carboxylase (PC, EC 6.4.1.1), and pyruvate dehydrogenase kinase 4 (PDK4) was analyzed by quantitative real-time RT-PCR (qRT-PCR) using a commercial kit (Superscript III Platinum Two-Step qRT-PCR Kit, Invitrogen). Reverse transcription was conducted using oligo-dT primers with 1 μg total RNA added as a template. After RNase H

treatment, the cDNA product was quantified by spectrophotometer. Real-time PCR was carried out in duplicate using 1 μg cDNA and was monitored using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Labeled LUX primers (FAM label) and complementary unlabeled primers (Invitrogen) were designed with online LUX Designer software (23) and included at 200 nmol/L in the PCR mix (Supplemental Table 1). Melting point analysis confirmed that only the transcripts of interest were amplified during PCR. Copy numbers for each gene were measured with 2 separate qRT-PCR analyses using standard curves to allow for absolute quantification (24). Clones used for standards were as follows. PCK1, bPEPCK-C2000 [(25), a gift from Dr. Shawn Donkin, Purdue University, West Lafayette, IN]; G6PC, GenBank accession #CB465348 (a gift from Dr. Tim Smith, USDA Meat Animal Research Center, Clay Center, NE); SLC37A4, accession #BF075927; PC, accession #BF604240; PDK4, accession #BE589194 (all from the Center for Animal Functional Genomics, Michigan State University, East Lansing, MI). Clones were cultured, plasmids were harvested (Wizard Plus Minipreps, Promega), and copy numbers were quantified by spectrophotometer for serial dilution. mRNA abundance was normalized using the geometric mean of copy numbers of cyclophilin, β-actin, and phosphoglycerate kinase 1 in each sample (26). Control genes were selected from genes used for normalization in the literature, based on consistent expression patterns in a preliminary study (unpublished data). Quantification of copy numbers for control genes was carried out in the manner described above, with the following clones used for standards: cyclophilin, accession #BG690429; β-actin, accession #BG689033; phosphoglycerate kinase 1, accession #BG688981 (all from the Center for Animal Functional Genomics, Michigan State University, East Lansing, MI).

Statistical analysis. Data were analyzed by the fit model procedure of JMP (version 5.0, SAS Institute) using the REML method according to the following model:

$$Y_{ijk} = \mu + P_i + T_j + C_k + PT_{ij} + e_{ijk}$$

where Y_{ijk} is a dependent variable, μ is the overall mean, P_i is the fixed effect of period (i = 1 to 2), T_j is the fixed effect of treatment (j = 1 to 2), C_k is the random effect of cow (k = 1 to 12), PT_{ij} is the interaction of period and treatment, and e_{ijk} is the residual error. Plasma analyses were conducted using the above model, but also included fixed effects of sample time. Sample time was a significant factor for all plasma variables except glucagon. mRNA abundance was analyzed with a model that included fixed effects of treatment and qRT-PCR run and random effect of cow. Values for PCK1, G6PC, PC, and PDK4 mRNA abundance were log-transformed for analysis, and values reported here are back-transformed. Although statistical analysis of mRNA abundance was carried out using values for copy numbers/μg cDNA, abundance is reported relative to control values for ease of interpretation. For all main effects, significance was declared at $P < 0.05$, and tendencies were declared at $P < 0.10$. There was a tendency for an interaction between period and treatment for plasma glucagon concentration ($P = 0.14$), but not for other variables of interest (all $P > 0.15$).

RESULTS

Glucose loss, milk production, and intake. Phlorizin treatment caused urinary excretion of 474 g glucose/d (**Table 2**), equivalent to 40% of daily lactose secretion in this group of cows. Milk yield was depressed by treatment ($P = 0.001$) primarily because of an 8% decrease in milk lactose production with phlorizin ($P < 0.01$). Milk fat and 3.5% fat-corrected milk yield were not altered by treatment, although milk protein production tended to decrease with phlorizin ($P = 0.08$). Although the response in milk lactose yield was significant, the decrease of 90 g/d accounted for a relatively small proportion of the 474 g/d of glucose lost in urine from phlorizin treatment. Contrary to our hypothesis, DMI was not altered by treatment (Table 2), nor was digestible DMI. Phlorizin did not alter feeding behavior as quantified by meal size and the number of meals/d (data not shown).

TABLE 2

Effects of phlorizin treatment on glucose loss, feed intake, and milk production in lactating Holstein cows[1]

Item	Control	Phlorizin	SEM	$P<$
Urinary glucose excretion, *g/d*	1	474	25	0.001
Dry matter intake, *kg/d*	20.7	20.2	0.8	0.37
Digestible dry matter intake, *kg/d*	13.6	13.2	0.5	0.34
Yield, *kg/d*				
Milk	24.9	22.8	1.4	0.001
3.5% fat-corrected milk	27.0	26.2	1.5	0.18
Solids-corrected milk	24.7	23.7	2.0	0.05
Milk fat	1.01	1.01	0.06	0.99
Milk protein	0.82	0.75	0.06	0.08
Milk lactose	1.18	1.09	0.07	0.01

[1] Values are least-squares means ± SEM, n = 12.

Plasma metabolites and hormones. Plasma glucose concentration was decreased by phlorizin ($P < 0.001$) and insulin concentration tended to decrease ($P = 0.09$), whereas lactate concentration was greater during phlorizin treatment ($P < 0.001$, **Table 3**). Concentrations of FFA and BHBA were also increased by phlorizin ($P < 0.001$), consistent with a decrease in fat deposition and increased hepatic oxidation of fatty acids. Similarly, the increase in plasma glucagon concentration ($P = 0.02$) and the decreased insulin:glucagon ratio ($P < 0.001$) during phlorizin treatment suggest that gluconeogenesis was enhanced by phlorizin. Phlorizin also tended to increase urinary N excretion from 135.7 to 151.6 g/d ($P < 0.10$).

Hepatic mRNA abundance. Expression patterns for genes involved in regulation of hepatic gluconeogenesis were altered by phlorizin (**Fig. 1**). Phlorizin increased PC mRNA abundance by 66% ($P < 0.001$) and abundance of PCK1 mRNA by 29% ($P < 0.05$); in addition, G6PC mRNA abundance was increased by 42% during phlorizin treatment ($P < 0.001$). SLC37A4 or PDK4 mRNA abundance was not affected.

DISCUSSION

The evidence available in this study indicates that gluconeogenic flux was likely greater in phlorizin-treated cows. The enzymes encoded by PCK1 and G6PC catalyze reactions that are considered to be potentially rate-limiting steps in gluconeogenesis (27,28); thus, the increased mRNA abundance of these genes likely led to enhanced gluconeogenic capacity. Given the effects on hepatic transcript abundance, hormone profile, and the relatively small decrease in milk lactose production, phlorizin treatment likely increased hepatic glucose export. However, the net increase in glucose production cannot be calculated because whole-body glucose oxidation was not measured. Phlorizin treatment decreased the proportion of plasma glucose oxidized to plasma CO_2 by 34% in steers (29), and some decrease in glucose oxidation probably resulted from phlorizin treatment in this study as well.

Regulation of transcription and/or mRNA stability is an important component of PC regulation, because Greenfield et al. (30) demonstrated that mRNA abundance was highly related to PC activity in bovine liver biopsies. The expression of PC is regulated by both insulin and glucagon (31), and short-term infusions of glucagon increased PC transcript abundance (32) and enzyme activity (33) in ruminants. Therefore, in this study, increased glucose demand probably increased

TABLE 3

Effects of phlorizin on blood plasma metabolites and hormones in lactating Holstein cows[1]

Item	Control	Phlorizin	SEM	$P <$
Glucose, *mmol/L*	3.62	3.54	0.07	0.001
Lactate, *mmol/L*	0.37	0.44	0.02	0.001
FFA, *μmol/L*	70.0	100.1	11.8	0.001
BHBA, *μmol/L*	624	807	38	0.001
Insulin, *pmol/L*	94.7	87.5	9.3	0.09
Glucagon, *pmol/L*	36.9	37.9	2.3	0.02
Insulin:glucagon ratio, *mol/mol*	2.48	2.06	0.38	0.001

[1] Values are least-squares means ± SEM, $n = 12$.

transcription of PC by causing a decrease in plasma glucose concentration and a corresponding decrease in the insulin: glucagon ratio (Table 3). The significant increase in PC mRNA abundance in response to increased glucose demand by phlorizin also agrees with recent reports in transition dairy cows with increased glucose demand (30,34) and suggests that the liver responds to increased glucose demand in part by limiting oxidation of intracellular pyruvate.

Pyruvate carboxylase is generally recognized as an important enzyme for the production of glucose from lactate and glucogenic amino acids (30,35). However, its importance in regulating the flux of propionate to glucose is not generally discussed, with notable exceptions (36,37). For net oxidation of propionate to occur, it must be converted to pyruvate (through oxaloacetate and phosphoenolpyruvate), oxidized by pyruvate dehydrogenase, and directed toward the tricarboxylic acid cycle as acetyl-CoA (see Supplemental Fig. 1). In one of the few studies in which this pathway was studied in lactating cows, Black et al. (38) calculated that ~13% of propionate was converted to acetyl-CoA. Although this seems to be a relatively insignificant proportion of the propionate entering the liver, it represents the only net loss of glucogenic carbon from propionate in the bovine liver; all other propionate that is not utilized in gluconeogenesis is converted to lactate or glucogenic amino acids. Enhancing PC activity, therefore, should not only increase gluconeogenesis from lactate and amino acids, but also increase the proportion of propionate that can be utilized for glucose production. This may be especially true because of the transient nature of propionate absorption (8); during meals, propionate is rapidly taken up by the liver, and propionate influx may exceed the liver's capacity for gluconeogenesis. If this build-up causes an increase in pyruvate concentration in hepatocytes, the relative activities of PC and pyruvate dehydrogenase are important in determining the proportion of glucogenic substrates that are preserved.

Past research using phlorizin indirectly supports these assertions. Veenhuizen et al. (29) used phlorizin to increase irreversible loss of glucose by 36% and measured the end-products of propionate metabolism. Phlorizin significantly increased the proportion of propionate that was utilized for gluconeogenesis, both with and without supplemental dietary propionate. Similarly, the relative proportion of propionate used for glucose production vs. CO_2 production was increased in hepatocytes harvested from phlorizin-treated wethers (39). Although no enzyme activity or gene expression data are available from these studies, it is possible that enhanced PC activity was responsible for increasing the efficiency of gluconeogenesis from propionate.

Although phlorizin treatment increased the demand for glucose specifically, adaptations to the treatment included altered metabolism of fatty acids as well as glucose and its precursors. Phlorizin increased plasma FFA concentration, which likely increased hepatic FFA uptake because the liver takes up FFA in proportion to its concentration in plasma (40). The increased uptake of FFA likely resulted in greater hepatic fatty acid oxidation because plasma BHBA concentration was increased by phlorizin. Although lipolysis contributes only small amounts of carbon for gluconeogenesis (glycerol), hepatic fatty acid oxidation plays an important role in supporting gluconeogenesis (41). Acetyl-CoA and NADH produced by oxidation of fatty acids provide energy and reducing equivalents to drive gluconeogenesis and allosterically inhibit pyruvate dehydrogenase activity, which increases the proportion of pyruvate retained for glucose production. Therefore, pyruvate dehydrogenase activity was likely decreased by phlorizin, despite the fact that mRNA abundance of the regulatory protein PDK4 (42) was not altered by treatment. Work by Chow and Jesse (43) indicated that gluconeogenesis from propionate was decreased when fatty acid oxidation, and therefore acetyl-CoA and NADH accumulation, was limited by tetradecylglycidic acid. The authors were surprised by this result because acetyl-CoA does not activate any of the enzymes in the direct gluconeogenic pathway from propionate through phosphoenolpyruvate. However, carbon cycling among oxaloacetate, phosphoenolpyruvate, and pyruvate (see Supplemental Fig. 1) could explain this surprising result because of the allosteric effects of acetyl-CoA and NADH on PC and pyruvate dehydrogenase (41).

Plasma lactate concentrations were increased by phlorizin in this study; however, this does not indicate whether glucose production from lactate was altered in phlorizin-treated cows. Baird et al. (44) found that lactate turnover through the Cori cycle was decreased in lactating cows that were food deprived for 4 d, even though plasma lactate concentrations nearly doubled. Although net hepatic uptake of lactate increases with increasing demand for glucose production in ruminants (45), the majority of this lactate is apparently not used for glucose production because it accounted for only 7% of total glucose production in early-lactation cows (46). Fatty acid oxidation inhibits the conversion of lactate to pyruvate by decreasing the

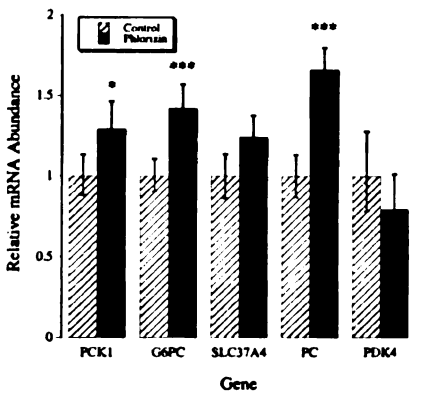

FIGURE 1 Effects of phlorizin administration in lactating Holstein cows on hepatic mRNA abundance relative to control for genes involved in gluconeogenesis. Values are means ± SEM, $n = 12$. *Different from control, $P < 0.05$; ***different from control, $P < 0.001$.

NAD:NADH ratio, and nonessential amino acids are probably more important sources of gluconeogenic substrate in cases of increased glucose demand (39). In this study, the tendency for an increase in urinary N excretion during phlorizin treatment suggests that increased PC activity may have increased glucose production from nonessential amino acids.

Despite the indirect evidence that gluconeogenic capacity was increased (and the possibility that propionate oxidation was decreased) by phlorizin treatment, voluntary DMI did not increase. Although this result is contrary to our hypothesis, the treatment effect on hepatic fatty acid oxidation makes it impossible to reject the hypothesis. A decrease in propionate oxidation was expected to increase DMI only if it delayed prandial increases in hepatic energy status. In this study, however, enhanced hepatic fatty acid oxidation could have replaced an energy deficit created by a decrease in propionate oxidation.

The decision to use late-lactation cows in this study was based on the assumption that DMI is limited primarily by metabolic factors rather than distention of the reticulorumen when cows are producing at less than the peak of lactation (47). In cows whose intake is limited primarily by rumen distention, the energy status of the liver likely has less effect on DMI. However, considering the results of the present study, other animal or experimental models may be more appropriate for testing the effects of increased glucose demand on DMI. Early-lactation cows generally mobilize large amounts of fat from adipose tissue, making it possible that a decrease in the insulin:glucagon ratio would have little additional effect on lipolysis. Maintaining a constant rate of lipolysis across treatments would likely prevent hepatic fatty acid oxidation from confounding the effects of glucose demand on DMI. An alternative model would be to use late-lactation cows and to administer tetradecylglycidic acid, an inhibitor of carnitine palmitoyltransferase, during both phlorizin and control injections. The drawback to this model is that tetradecylglycidic acid would limit fatty acid oxidation in muscle and adipose tissue in addition to the liver. Nevertheless, the strength of the evidence relating meal patterns to the energy status of the liver in rodents (48) encourages continued investigation of this potential mechanism of DMI regulation in cattle.

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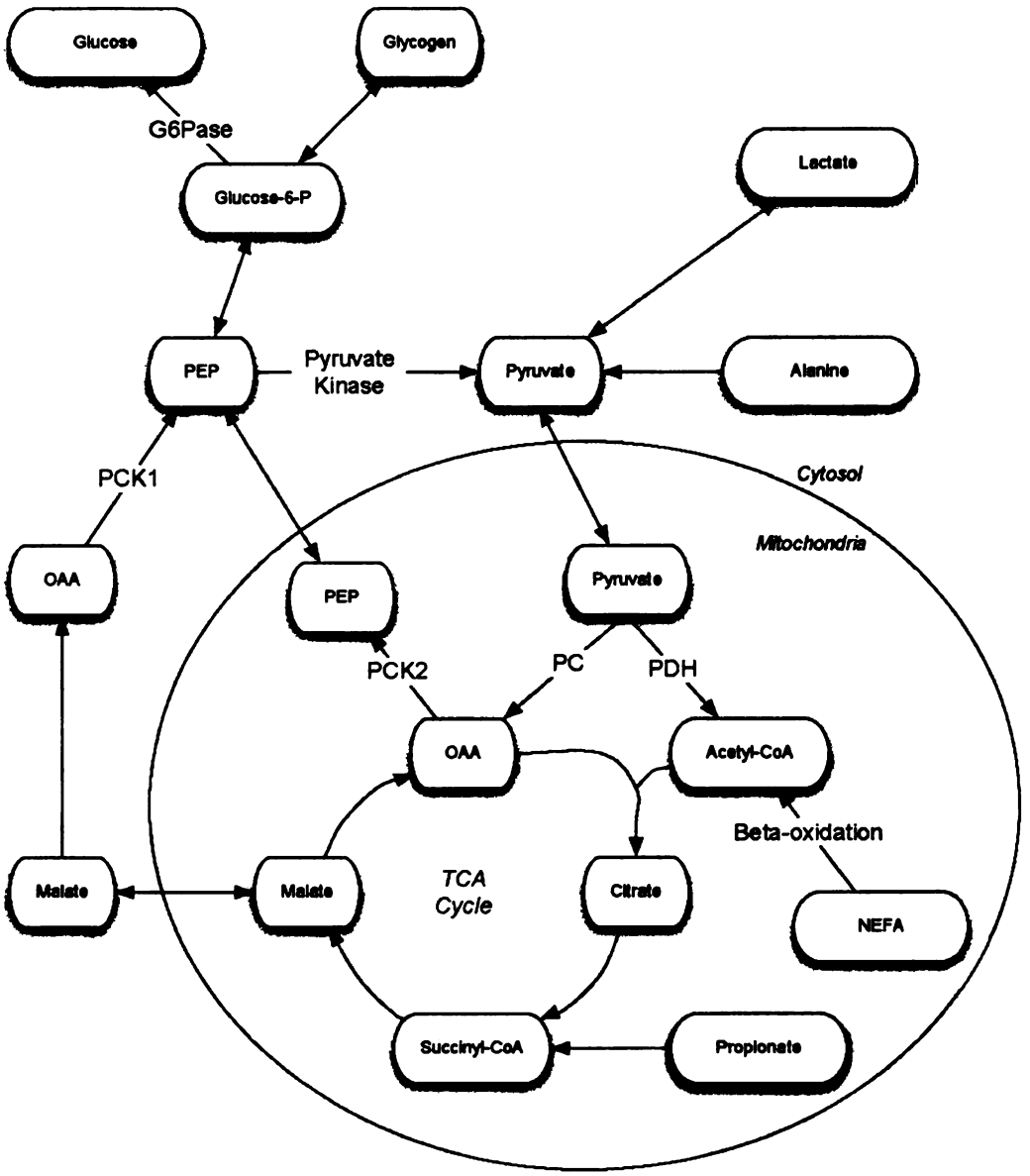
SUPPLEMENTAL TABLE 1

Genes, primers, and homology to reference sequences for sequences analyzed by qRT-PCR

Gene of interest[1]	Forward primer (5' – 3') Reverse primer (5' – 3')	Reference Sequence	Sequence Homology	Alignment Region
PCK1	GTCCTGGCCCTGAAGCAGA TCCTGCTCCTGGTGCGTTG*	AY145503.1	100%	1972 - 2060
G6PC	GTCCTCTTCCCCATTTGGTTC* TCCTCTGGGTAGCTGTGATTGG	NM000151.1 (*H. sapiens*)	92%	212 - 282
SLC37A4	CACCTCCCATGCCATTG* TTTGGCAATGGTGCTGAAGG	NM001467.3 (*H. sapiens*)	96%	1762 – 1839
PC	CGTCTTTGCCCACTTCAAGG GGCGCGTATTGAGGCTG*	NM177946	100%	3113 - 3177
PDK4	CAGGCCAACCAATTCATATTG* GGCCCTCATTGCATTCTTGA	NM002612.2 (*H. sapiens*)	89%	904 – 985
PPIH	TGGCAAATTCAAGCCCTG* AACTTCCTGGCCGCCAAT	NM028677.2 (*H. sapiens*)	90%	78 – 142
ACTB	TGGGCCAGAAGGACTCG* GGGTACTTGAGGGTCAGGATGC	AY141970.1	100%	179 – 248
PGK1	CAGTGGAGCCAAGTCAGTTG* GCAACTGGCTGCAAGGAGTA	NM000291.2 (*H. sapiens*)	90%	228 – 314

[1]PCK1, cytosolic phosphoenolpyruvate carboxykinase; G6PC, catalytic glucose-6-phosphatase; SLC37A4, regulatory subunit glucose-6-phosphatase; PC, pyruvate carboxylase; PDK4, pyruvate dehydrogenase kinase 4; PPIH, cyclophilin; ACTB, β-actin; PGK1, phosphoglycerate kinase 1.

*FAM-labeled LUX primers.



SUPPLEMENTAL FIGURE 1 Overview of hepatic metabolic pathways related to gluconeogenesis. Pathways and enzymes discussed in the text are labeled (*G6Pase*, glucose-6-phosphatase; *PCK1*, *PCK2*, cytosolic and mitochondrial phosphoenolpyruvate carboxykinase; *PC*, pyruvate carboxylase; *PDH*, pyruvate dehydrogenase).

CHAPTER 5

PHLORIZIN INDUCES LIPOLYSIS AND ALTERS MEAL PATTERNS IN BOTH EARLY AND LATE LACTATION DAIRY COWS

ABSTRACT

Increased glucose demand likely alters hepatic propionate metabolism, but it also stimulated lipolysis in past studies in ruminants. To assess whether increased glucose demand selectively increases dry matter intake (DMI) for cows in negative energy balance, phlorizin was administered to blocks of early and late lactation cows. Six Holstein cows in early lactation (19 ± 6 DIM, 50.0 ± 1.8 kg/d milk, mean ± SD) and six Holstein cows in late lactation (228 ± 18 DIM, 30.6 ± 1.9 kg/d milk) were randomly assigned to treatment sequence in a crossover design. Periods were 14 d with 7 d adaptation periods and 7 d of treatment. Phlorizin (4 g/d) and propylene glycol (carrier and control) were administered subcutaneously every 6 h throughout the treatment periods. Feeding behavior and DMI data were collected for the final 4 d of each treatment period, and blood samples and total urine output were collected on d 4 of each treatment period. Phlorizin caused urinary loss of glucose at 333 g/d in early lactation and 532 g/d in late lactation cows. Phlorizin increased plasma non-esterified fatty acid concentration similarly in early and late lactation cows, but did not significantly alter plasma insulin concentrations. Phlorizin treatment tended to decrease meal size, but also decreased intermeal interval, resulting in no effect on DMI. We

conclude that phlorizin's effects on lipolysis, feeding behavior, and DMI are not dependent on relative energy balance.

INTRODUCTION

Physiological regulation of feed intake must respond to a variety of environmental and endogenous cues to allow growth or maintain body weight, support milk production, and supply nutrients for fetal growth. The mechanisms that direct changes in feeding behavior often interact, making it difficult to identify the signals that are most responsible for increased dry matter intake (DMI) at parturition or following the initiation of bST treatment, for example. Pharmacological treatments that have more specific impacts on physiological processes can help identify specific mechanisms that may contribute to the regulation of feed intake.

Phlorizin inhibits renal reabsorption of glucose (Ehrenkranz et al., 2005), resulting in urinary excretion of glucose and a significant increase in whole-body glucose demand. Increasing glucose demand in lactating cows led to an increase in transcript abundance for potentially rate-limiting gluconeogenic enzymes (Bradford and Allen, 2005). We hypothesized that greater gluconeogenic capacity would increase utilization of propionate for glucose production and decrease its oxidation, altering hepatic energy status. Decreased hepatic ATP production may result in delayed satiety during meals, because preventing ATP production by trapping inorganic phosphate increased feed intake in rats (Rawson et al., 1994), as did inhibition of fatty acid oxidation (Horn et al., 2004). Therefore, we predicted that phlorizin would increase meal size and DMI in lactating cows by limiting propionate oxidation (Allen et al., 2005).

However, in our previous work, we showed that late-lactation cows were able to adapt to phlorizin administration for 7 d without increasing DMI (Bradford and Allen, 2005). Phlorizin's effects on glucose metabolism were confounded by a concomitant increase in fatty acid (FA) delivery to the liver, which likely supplied substrate to replace propionate removed from oxidative pathways. We speculated that early-lactation cows, already in negative energy balance, would be less flexible in their use of nutrients to drive gluconeogenesis, and that phlorizin treatment would result in increased DMI in this model. This experiment was designed to test our revised hypothesis.

MATERIALS AND METHODS

Experimental procedures were approved by the All-University Committee on Animal Use and Care at Michigan State University.

Design and treatments

Multiparous Holstein cows were selected from the Michigan State University Dairy Cattle Teaching and Research Center and assigned to blocks of early lactation (n = 6; 19 ± 6 DIM; 2.3 ± 0.5 lactations; 657 ± 68 kg BW; mean ± SD) and late lactation (n = 6; 228 ± 18 DIM; 2.3 ± 0.8 lactations; 689 ± 77 kg BW) cows. Stalls were assigned to block and treatment sequence to assure balance within blocks, and cows were randomly assigned to stalls within block. Phlorizin (Sigma Chemical Co., St. Louis, MO) was administered via subcutaneous injection at the rate of 4 g/day, with propylene glycol as vehicle and control.

Treatment periods were 7 d, and injections were given every 6 h during these periods. Animals were adapted to a single diet for a 7-d period prior to the first treatment period, and a 7-d rest period was included between the two treatment periods. One of the 28 injections during period 2 was missed, however, no data was collected for 36 h after the missed injection, and glucose excretion stabilizes relatively quickly following injection of phlorizin (Amaral-Phillips et al., 1993). Because there were no significant period x treatment interactions for DMI, feeding behavior, or plasma analyte concentrations in subsequent statistical analyses (all $P > 0.15$), we conclude that the missed injection had little or no effect on measured responses to treatment.

Data and sample collection

Throughout the experiment, cows were housed in tie-stalls and fed a single experimental diet (**Table 5.1**) as a TMR once daily (1130 h) at 115% of expected daily intake. Cows were not allowed access to feed from 1000 to 1130 h, during which orts and the amount of feed offered were weighed for each cow daily. During the final 4 d of each treatment period, feeding behavior was monitored by a computerized data acquisition system (Dado and Allen, 1993) throughout the day. Data on chewing activities, feed disappearance and water consumption were recorded for each cow every 5 s, and mean daily values for number of meal bouts, interval between meals, and meal size were calculated. On each of these 4 d, samples of all dietary ingredients (0.5 kg) and orts (12.5%) were collected and frozen for later analysis. Starting on d 4 (1000 h) of each experimental

period, urine was collected for a 24-h period and blood was sampled hourly from an indwelling jugular catheter as previously described (Bradford and Allen, 2005). Cows were milked twice daily in the milking parlor during rest periods and in the tie stalls during treatment periods. Milk yield was recorded and samples were taken at each milking during the 4 collection days.

Table 5.1 Ingredients and nutrient composition of experimental diet[1].

Item	
Diet ingredients	
Corn silage	31.8
High moisture corn grain	29.1
Alfalfa haylage	11.5
Soybean meal	11.8
Modified expeller soybean meal[2]	9.0
Mineral and vitamin mix[3]	6.9
Nutrient composition	
DM, % as-fed	43.4
Organic matter	94.0
Starch	28.2
NDF	27.1
CP	17.8

[1]Values other than DM are expressed as % of dietary DM.

[2]SoyPlus (West Central Soy, Ralston, IA).

[3]Mineral and vitamin mix contained 74.7% dry ground corn, 10.9% limestone, 5.5% salt, 5.3% dicalcium phosphate, 1.8% magnesium oxide, 1.4% trace mineral premix, and 0.4% vitamin ADE premix.

Sample analysis

Diet ingredients, orts, and fecal samples were dried in a 55°C forced-air oven for 72 h and analyzed for dry matter concentration. Feed samples were ground with a Wiley mill (1-mm screen; Arthur H. Thomas, Philadelphia, PA) and analyzed for ash, neutral detergent fiber, crude protein, and starch concentrations as

previously described (Bradford and Allen, 2005). Concentrations of all nutrients were expressed as percentages of dry matter determined from drying at 105°C in a forced-air oven.

Milk samples were analyzed for fat, true protein, and lactose with infrared spectroscopy (AOAC, 1990) by Michigan DHIA (East Lansing, MI). In addition, milk samples from d 7 of each treatment period were analyzed for fatty acid profile by gas chromatography as previously described (Bradford and Allen, 2004). Plasma samples were composited into a single sample for each cow period for all analyses except non-esterified fatty acid (NEFA) and growth hormone (GH) concentration. Urine and plasma samples were analyzed in duplicate for glucose concentration by the glucose oxidase method (Raabo and Terkildsen, 1960). Plasma samples were analyzed in duplicate using commercial kits to determine concentrations of NEFA [NEFA C-kit; Wako Chemicals USA, Richmond, VA, as modified (McCutcheon and Bauman, 1986)], β-hydroxybutyrate (procedure #2440, Stanbio Laboratory, Boerne, TX), insulin (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA), and glucagon (Glucagon kit #GL-32K, Linco Research Inc., St. Charles, MO). Plasma GH concentrations were quantified with a double-antibody radioimmunoassay (Gaynor et al., 1995).

Statistical analysis

One cow in the late lactation group decreased DMI by approximately 50% at the beginning of each data collection period, likely because of sensitivity to the chew halter apparatus. Because the cow was an outlier for DMI, and plasma analyses were indicative of an unusual catabolic state, this cow was removed from all analyses.

Data were analyzed according to the following mixed-effects model:

$$Y_{ijk} = \mu + P_i + S_j + T_k + C_l(S_j) + ST_{jk} + PT_{ik} + e_{ijkl}$$

in which Y_{ijkl} is a dependent variable, μ is the overall mean, P_i is the fixed effect of period ($i = 1$ to 2), S_j is the fixed effect of block ($j = 1$ to 2) T_k is the fixed effect of treatment ($k = 1$ to 2), C_l is the random effect of cow nested within stage ($l = 1$ to 6), ST_{jk} is the interaction of block and treatment, PT_{ik} is the interaction of period and treatment, and e_{ijkl} is the residual error. Analysis of plasma NEFA data also included fixed effects of sample time, time by treatment interaction, and time by stage interaction. Urinary glucose and plasma BHBA values were not normally distributed and were log-transformed for analysis; reported means are back-transformed. For all main effects, significance was declared at $P < 0.05$, and tendencies were declared at $P < 0.10$. For interactions, significance was declared at $P < 0.10$, and tendencies were declared at $P < 0.15$.

RESULTS AND DISCUSSION

As expected, urinary glucose excretion increased dramatically with phlorizin administration, from 0.4 g/d to 333 g/d in early lactation and 532 g/d in late lactation ($P < 0.001$, **Table 5.2**). The significant treatment by stage of lactation interaction ($P < 0.01$) is likely related to the significantly higher plasma glucose concentrations in late lactation. Phlorizin-treated cows in late lactation had a mean glucose concentration of 68.2 mg/dL compared to 59.9 mg/dL for treated early-lactation cows. Because phlorizin inhibits renal glucose reabsorption, greater delivery of glucose to the kidneys in late-lactation cows would be expected to result in greater glucose excretion.

As reported previously (Amaral-Phillips, et al., 1993; Bradford and Allen, 2005; Overton et al., 1998), phlorizin treatment increased plasma NEFA concentration ($P < 0.03$), which is indicative of the rate of lipolysis in ruminants (Dunshea et al., 1989). Although Amaral-Phillips and coworkers (1993) reported increased lipolysis in response to phlorizin treatment in cows at 6 weeks postpartum, the mean NEFA concentration for the control treatment (181 μEq/L) suggested that the cows were not in an severe catabolic state. Nevertheless, our results in cows with higher plasma NEFA concentrations (278 μEq/L) confirm these findings, and further demonstrate that early and late lactation cows respond to phlorizin with a comparable increase in lipolysis. We and others have suggested that phlorizin-induced lipolysis is mediated by decreased plasma insulin concentrations (Bradford and Allen, 2005; Vranic et al., 1984), however, in this experiment, we

Table 5.2 Effects of phlorizin and stage of lactation on glucose excretion, blood plasma metabolites and hormones, and feeding behavior[1].

	Early Lactation		Late Lactation		*P*		
Item	Control	Phlorizin	Control	Phlorizin	Stage[2]	Trt[2]	Int[2]
Urine glucose (g/d)	0.40 ± 0.03	323 ± 19	0.45 ± 0.03	549 ± 36	< 0.01	< 0.001	0.02
Plasma glucose (mg/dL)	61.2 ± 2.3	59.9 ± 2.3	72.8 ± 2.6	68.2 ± 2.6	< 0.01	0.25	0.51
Plasma NEFA (μEq/mL)	278 ± 27	322 ± 27	43 ± 30	96 ± 30	< 0.001	0.04	0.84
Plasma BHBA (mg/dL)	11.8 ± 1.2	15.4 ± 1.2	12.4 ± 1.1	9.4 ± 0.8	0.04	0.97	0.03
Plasma insulin (μIU/mL)	2.87 ± 0.61	2.83 ± 0.61	7.81 ± 0.68	7.23 ± 0.68	< 0.001	0.64	0.69
Plasma glucagon (pg/mL)	121 ± 12	131 ± 11	130 ± 11	137 ± 11	0.63	0.21	0.80
Insulin:glucagon (mol:mol)	0.35 ± 0.15	0.31 ± 0.15	1.30 ± 0.16	1.13 ± 0.16	< 0.001	0.52	0.69
Plasma GH (ng/mL)	6.96 ± 0.53	6.76 ± 0.53	2.72 ± 0.58	3.09 ± 0.58	< 0.001	0.78	0.32
Dry matter intake (kg/d)	22.2 ± 0.7	23.6 ± 0.7	21.4 ± 0.7	21.8 ± 0.7	0.08	0.15	0.37

[1]Values are least square means ± SEM, n = 6 for early lactation and n = 5 for late lactation.

[2]Stage = main effect of block (stage of lactation); Trt = main effect of treatment; Int = interaction of block and treatment.

found that phlorizin did not significantly alter plasma insulin concentration or insulin:glucagon ratio (Table 5.2).

We then assessed whether GH was responsible for this lipolytic response, because plasma GH concentrations increase in response to hypoglycemia (De Feo et al., 1989) and GH acts to suppress lipogenesis in ruminant adipose tissue (Liesman et al., 1995). Again, we found no effect of phlorizin on plasma concentration of GH (Table 5.2). Finally, we considered the work of Brockman (1984), who demonstrated that hypoglycemia directly stimulated lipolysis in an elegant study of alloxan diabetic, adrenal-denervated sheep. However, phlorizin treatment did not cause hypoglycemia in this study; in fact, phlorizin did not significantly alter plasma glucose concentration (Table 5.2). While we are unable to address the possibility that phlorizin treatment caused a stress response in these animals, Brockman's observation that phlorizin causes lipolysis in animals without active adrenal glands (1984) demonstrates that a stress response is not a required component of phlorizin-induced lipolysis. Therefore, the cause of phlorizin-induced lipolysis in lactating dairy cows remains unclear. It is possible that small, statistically undetectable changes in plasma insulin and glucose concentration were adequate to increase plasma NEFA concentration to the extent that was observed in this experiment. Also, despite the validation of plasma NEFA concentration as an index of lipolysis (Dunshea, et al., 1989), it is important to note that we did not directly measure lipolytic rate in this experiment.

We found a significant stage of lactation by treatment interaction for plasma BHBA concentration ($P < 0.03$), but the reason for this response is unclear.

Phlorizin did not alter yield of milk, milk lactose, or milk fat (**Table 5.3**). However, there was a tendency for an interaction between stage of lactation and treatment for milk protein yield ($P < 0.15$). We also used milk FA profile to help assess metabolic changes in response to phlorizin injection. Consistent with observed effects on plasma NEFA concentration, long-chain milk FA yield tended to increase with phlorizin treatment ($P = 0.07$, Table 5.3). Long-chain FA in milk are derived from circulating FA rather than *de novo* synthesis in the mammary gland (Barber et al., 1997), so a tendency for increased secretion of long-chain FA suggests that more circulating FA were available for uptake by the mammary gland. We also found that early lactation cows had a significantly greater proportion of unsaturated FA in milk fat than late lactation cows (39.6 vs. 31.3 g/100 g FA, $P < 0.001$). Greater *delta*-9 desaturase activity in early lactation cows (*delta*-9 desaturase index: 0.40 vs. 0.30, $P < 0.01$) accounted for the majority of the difference in milk FA saturation, in agreement with the findings of Kay et al. (2005).

Contrary to our primary hypothesis, there was no interaction of treatment and stage of lactation for DMI in this study ($P = 0.37$). This is perhaps not surprising given that indicators of lipolysis (plasma NEFA concentration and long-chain FA secretion) responded to treatment in a similar manner in both early and late

Table 5.3 Effects of phlorizin and stage of lactation on milk and milk component yield[1].

	Early Lactation		*Late Lactation*		*P*		
Item	Control	Phlorizin	Control	Phlorizin	Stage[2]	Trt[2]	Int[2]
Milk yield (kg/d)	50.0 ± 2.1	49.9 ± 2.1	31.7 ± 2.3	29.5 ± 2.3	< 0.001	0.50	0.55
Milk lactose yield (kg/d)	2.60 ± 0.12	2.64 ± 0.12	1.50 ± 0.14	1.48 ± 0.14	< 0.001	0.96	0.79
Milk protein yield (kg/d)	1.36 ± 0.06	1.40 ± 0.06	1.00 ± 0.07	0.96 ± 0.07	< 0.01	0.93	< 0.15
Milk fat yield (kg/d)	2.04 ± 0.09	2.16 ± 0.09	1.17 ± 0.10	1.26 ± 0.10	< 0.001	0.13	0.81
Short-chain FA yield[3] (g/d)	121 ± 10	122 ± 10	63 ± 11	54 ± 11	< 0.001	0.54	0.47
Medium-chain FA yield[3] (g/d)	324 ± 15	346 ± 15	264 ± 17	245 ± 17	< 0.01	0.90	0.09
C16 FA yield (g/d)	622 ± 30	650 ± 30	382 ± 33	412 ± 33	< 0.001	0.12	0.96
Long-chain FA yield[3] (g/d)	940 ± 55	1004 ± 55	431 ± 60	527 ± 60	< 0.001	0.07	0.69

[1]Values are least square means ± SEM, n = 6 for early lactation and n = 5 for late lactation.

[2]Stage = main effect of block (stage of lactation); Trt = main effect of treatment; Int = interaction of block and treatment.

[3]Fatty acids defined as follows: short-chain FA, C4 – C8; medium-chain FA, C10 – C15; long-chain FA, >C16.

lactation cows. However, the results of this study do contradict our original hypothesis that phlorizin would increase DMI by increasing meal size. Phlorizin treatment tended to decrease mean meal size ($P = 0.07$, **Figure 5.1**), which resulted in a compensatory decrease in intermeal interval ($P = 0.02$) and no effect on DMI ($P = 0.15$, Table 5.2).

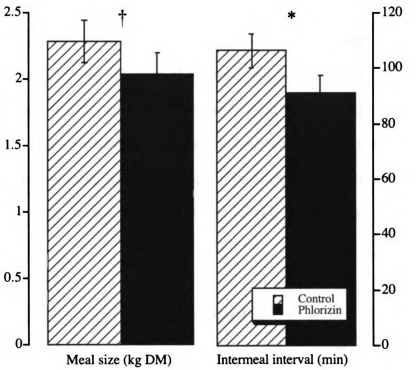


Figure 5.1 Effects of phlorizin administration on feeding behavior. Values are least square means ± SEM, n = 11. † $P < 0.10$; * $P < 0.05$.

Although we have previously shown that phlorizin administration results in increased gluconeogenic capacity, we also observed an increase in NEFA available for hepatic oxidation (Bradford and Allen, 2005). In this study we have

again shown that phlorizin increased circulating NEFA, and the total amount of substrate available for oxidation in the liver may have increased with phlorizin treatment. Furthermore, we have previously suggested that propionate and acetyl-coA may interact to shorten meal size in cows mobilizing body fat (Oba and Allen, 2003a). Cows in negative energy balance often combine high rates of acetyl-CoA production (from FA oxidation) with a relative shortage of glucogenic substrate. This situation results in a deficit of TCA cycle intermediates and high NADH:NAD+, preventing oxidation of acetyl-CoA (Lopes-Cardozo et al., 1975). Propionate can stimulate acetyl-CoA oxidation both by providing TCA intermediates and by utilizing NADH during its conversion to glucose. Such an interaction would lead to rapid ATP production during meals, and could have played a role in the tendency for a decrease in meal size for the phlorizin treatment.

CONCLUSIONS

In agreement with past results, phlorizin not only increased glucose demand but also stimulated lipolysis in both early and late lactation cows. Phlorizin did not significantly alter plasma concentrations of glucose, insulin, glucagon, or GH. Finally, phlorizin tended to decrease meal size but also decreased intermeal interval, resulting in no change in DMI in either early or late lactation cows. Phlorizin provides a model for increased glucose demand, but confounding effects on lipid metabolism complicate interpretation of its effects on hepatic energy metabolism in lactating cows.

CHAPTER 6

PHLORIZIN ADMINISTRATION DID NOT ATTENUATE HYPOPHAGIA INDUCED BY INTRARUMINAL PROPIONATE INFUSION

ABSTRACT

Infusion data from ruminants has shown that propionate stimulates satiety and decreases meal size, possibly because of increased propionate oxidation in the liver. In this experiment, phlorizin was used to increase glucose demand, which was expected to decrease propionate oxidation and attenuate the decrease in dry matter intake (DMI) caused by propionate infusion. Twelve multiparous, ruminally-cannulated Holstein cows (49 ± 33 DIM, 40 ± 7 kg/d milk; mean ± SD) were randomly assigned to square and treatment sequence in a replicated 4x4 Latin square experiment with a 2x2 factorial arrangement of treatments. Treatments were subcutaneous injection of phlorizin or propylene glycol in combination with intraruminal infusion of either Na acetate or Na propionate. Following a 7 d adaptation period, phlorizin (4 g/d) and control injections were administered every 6 h for 7 d. During the final 2 d of injections, Na acetate or Na propionate solutions (1 mol/L, pH 6.0) were infused continuously at the rate of 0.80 L/h. Feeding behavior data were collected during the final 2 d of treatment. Phlorizin caused urinary excretion of 400 g glucose/d across infusion treatments. Phlorizin tended to increase plasma non-esterified fatty acid and beta-hydroxybutyrate concentrations to a greater extent with Na acetate compared to Na propionate infusion. Phlorizin decreased and Na propionate increased

plasma insulin and glucose concentrations. Infusion of Na propionate decreased DMI (18.4 vs. 21.1 kg/d) through an increase in intermeal interval (89.2 vs. 77.3 min), resulting in fewer meals/d (11.6 vs. 13.7). Phlorizin did not alter DMI or measures of feeding behavior, nor were there interactions with infusion type. Increasing glucose demand does not limit the extent to which propionate decreases DMI in lactating dairy cows.

INTRODUCTION

Propionate, a product of ruminal carbohydrate metabolism, depresses feed intake of ruminants. Although the mechanism for this hypophagic response is unknown, a strong body of evidence indicates that the liver is involved. Propionate infusion depresses feed intake to a greater extent when infused into the portal circulation compared to peripheral infusion (Anil and Forbes, 1980; Baile, 1971), and blocking autonomic nervous system pathways between the brain and liver eliminated hypophagic responses to propionate infusion (Anil and Forbes, 1980; Anil and Forbes, 1988). Forbes has pointed out that propionate and many other substrates oxidized by the liver have demonstrable effects on food intake, while the products of these oxidation reactions do not (Forbes, 1988). Increased feed intake was induced in nonruminants by blocking hepatic oxidation of fatty acids (Horn et al., 2004; Langhans and Scharrer, 1987) and by preventing ATP production through phosphate trapping (Rawson et al., 1994), providing more direct evidence for the involvement of hepatic oxidation in feed intake regulation.

Evidence regarding the feed intake effects of hepatic oxidation by ruminant liver is currently lacking. Although at least some propionate is oxidized by ruminant liver (Black et al., 1966), the majority of propionate entering the liver is utilized for gluconeogenesis. Propionate infusion alters feed intake primarily through decreased meal size (Oba and Allen, 2003a), and we hypothesized that the increased flux of propionate into the liver during meals (Benson et al., 2002)

results in excess glucogenic substrate availability and greater oxidation of propionate, stimulating satiety. To test this hypothesis, we used phlorizin to cause urinary loss of glucose; we previously provided evidence of an adaptive increase in gluconeogenic capacity in response to phlorizin administration in lactating cows (Bradford and Allen, 2005). We expected phlorizin to direct more propionate toward glucose production and to limit the hypophagic response to propionate infusion.

MATERIALS AND METHODS

Experimental procedures were approved by the All-University Committee on Animal Use and Care at Michigan State University.

Design and treatments

Twelve multiparous lactating Holstein cows (49 ± 33 days in milk, 40 ± 7 kg/d milk; mean ± SD) with ruminal cannulas were selected from the Michigan State University Dairy Cattle Teaching and Research Center and randomly assigned to square and treatment sequence in a replicated 4x4 Latin square design balanced for carry-over effects. A 2x2 factorial arrangement of treatments was used to assess effects of phlorizin in combination with infusion of Na acetate or Na propionate. During treatment periods, phlorizin (Sigma Chemical, St. Louis, MO) was administered via s.c. injection every 6 h at the rate of 4 g/d, with propylene glycol as vehicle and control. Treatment periods were 7 d, and during the final 2 d of treatment, solutions of Na acetate or Na propionate (1.0 mol/L, pH 6.0) were

continuously infused into the rumen at the rate of 0.80 ± 0.07 L/h. Solutions were infused using 4-channel peristaltic pumps (#78016–30, Cole-Parmer Instrument, Vernon Hills, IL) and Tygon tubing (7.5 m x1.6 mm i.d.; Fisher Scientific Co., Pittsburgh, PA). The cows were adapted to a single diet for a 7-d period before the first treatment period, and 7 d of rest were included between treatment periods. Throughout the experiment, cows were housed indoors in tie stalls and fed a total mixed ration once daily (1130 h) at 110% of expected intake. The diet (455 g dry matter / kg diet, **Table 6.1**) was formulated to meet nutrient requirements (NRC, 2001) and to promote endogenous propionate production. One cow was removed from treatment in period 1 for clinical ketosis and a second cow was removed from the experiment in period 4 for mastitis, leaving 11 observations for each of the acetate treatments.

Data and sample collection

Access to feed was prevented for 90 min each day (1000 to 1130 h) while orts and the amount of feed offered were weighed for each cow. During the final 2 d of each treatment period, feeding behavior was monitored by a computerized data acquisition system (Dado and Allen, 1993) throughout the day. Data on feed disappearance and water consumption were recorded for each cow every 5 s, and mean daily values for number of meal bouts, interval between meals, and meal size were calculated. On each data collection day, samples of all dietary ingredients (0.5 kg) and orts (12.5%) were collected. Cows were milked in the tie stalls during data collection and in the milking parlor during the rest of the

experiment. Milk yield was recorded and samples were taken at each milking during collection days.

Table 6.1 Ingredients and nutrient composition of the experimental diet.

Component	Content
	g/kg dry matter
Diet ingredient	
Corn silage	297
High moisture corn	291
Alfalfa silage	165
Modified expeller soybean meal[1]	81
Soybean meal	76
Mineral and vitamin mix[2]	89
Nutrient composition	
Organic matter	922
Starch	316
Neutral detergent fiber	275
Crude protein	153

[1] SoyPlus (donation from West Central Soy, Ralston, IA).

[2] Contained (/kg dry matter): Ca, 44 g; Na, 38 g; Cl, 36 g; Mg, 14 g; P, 13 g; K, 2.8 g; Fe, 1.4 g; S, 1.4 g; Mn, 467 mg; Zn, 453 mg; Cu, 113 mg; I, 6.7 mg; Se, 3.4 mg; Co, 1.7 mg; β-carotene, 24 mg; cholecalciferol, 369 μg; 2-ambo-α-tocopherol, 148 mg.

Urine was collected for a 24-h period on d 7 (1100 h) of each treatment period. Urinary catheters (Bardex Lubricath Foley 24FR, Bard Medical) were inserted and urine was collected into a container with 0.23 mol HCl to prevent glycolysis. Volumes were measured at 6-h intervals and samples were taken and frozen until analysis. During the same 24-h period, blood samples were collected at 3-h intervals from indwelling jugular catheters. Collected blood was processed and stored as described (Bradford and Allen, 2005). At the end of each data collection period, ruminal contents were sampled from 5 sites throughout rumen and squeezed through a nylon screen (1 mm pore size) to collect the liquid phase. Ruminal fluid pH was measured using a portable pH meter (model 230A,

ATI Orion, Boston, MA), and samples were frozen at -20° C until analysis.

Sample analysis

Diet ingredients, orts, and fecal samples were dried in a 55°C forced-air oven for 72 h and analyzed for dry matter concentration. Ingredient samples were ground with a Wiley mill (1-mm screen; Authur H. Thomas) and analyzed for ash, neutral detergent fiber, crude protein, and starch content as previously described (Bradford and Allen, 2005). Concentrations of all nutrients are expressed as percentages of dry matter determined from drying at 105°C in a forced-air oven. Milk samples were analyzed for fat, true protein, and lactose with infrared spectroscopy by the Michigan Dairy Herd Improvement Association (Lansing, MI). In addition, milk samples from d 7 of each treatment period were analyzed for fatty acid profile by gas chromatography as previously described (Bradford and Allen, 2004). Urine and plasma samples were analyzed in duplicate for glucose content by the glucose oxidase method (Raabo and Terkildsen, 1960). Plasma samples were analyzed using commercial kits to determine concentrations of FFA, β-hydroxybutyrate (BHBA), insulin, and glucagon as previously described (Bradford and Allen, 2005). Concentrations of short-chain fatty acids (SCFAs) in ruminal fluid were determined as previously described (Oba and Allen, 2003b).

Statistical Analysis

Data were analyzed by the fit model procedure of JMP (version 5.0, SAS Institute, Cary, NC) using the REML method according to the following model:

$$Y_{ijkl} = \mu + P_i + S_j + C_k(S_j) + T_l + PT_{il} + V + VT_l + e_{ijkl}$$

where Y_{ijkl} is a dependent variable, μ is the overall mean, P_i is the fixed effect of period ($i = 1$ to 4), S_j is the fixed effect of square ($j = 1$ to 3), C_k is the random effect of cow within square ($k = 1$ to 4), T_l is the fixed effect of treatment ($l = 1$ to 4), PT_{il} is the interaction of period and treatment, V is the effect of infusion volume, VT_l is the interaction of infusion volume and treatment, and e_{ijk} is the residual error. Infusion volume was included in the model to account for potential bias caused by variation in infusion rate across cows and periods. Period by treatment interactions were not significant for any variable except milk lactose yield; modeling potential carryover effects for this variable did not resolve the issue, so treatment effects could not be assessed. Plasma data were analyzed by a model including the above terms as well as the effects of sample time and time by treatment interaction; repeated measures over time were modeled with a heterogeneous autoregressive [ARH(1)] covariance structure using the repeated statement of SAS (version 9.0, SAS Institute, Cary, NC), and denominator degrees of freedom were estimated using the Kenward Rogers method. For all main effects, significance was declared at $P < 0.05$, and tendencies were declared at $P < 0.10$. Interactions were declared significant at $P < 0.10$ and tendencies for interactions were declared at $P < 0.15$.

RESULTS AND DISCUSSION

Phlorizin administration caused urinary loss of glucose at the rate of 400 g/d across infusion treatments (**Table 6.2**), consistent with previous results in lactating cows (Bradford and Allen, 2005). Also as expected, infusion of acetate or propionate increased ruminal concentrations of the respective SCFAs ($P < 0.001$). More surprisingly, phlorizin treatment tended to decrease ruminal propionate concentration ($P < 0.10$), and propionate infusion decreased ruminal butyrate concentration relative to acetate ($P < 0.01$). Plasma glucose concentration was altered both by substrate availability and by peripheral demand; glucose concentrations increased in response to propionate infusion ($P < 0.001$) and decreased with phlorizin treatment ($P < 0.01$). Changes in plasma insulin and glucagon concentrations were expected responses to treatment effects on plasma glucose concentration (Table 6.2).

In past experiments, we have consistently found that both increased dietary starch fermentability and propionate infusion depress feed intake primarily through decreased meal size (Oba and Allen, 2003a; Oba and Allen, 2003b; Oba and Allen, 2003d). However, in this experiment, propionate infusion increased intermeal interval ($P < 0.04$, **Table 6.3**) with no effect on meal size. Management, ration formulation, and infusion protocols were similar in this experiment compared to our past work, making it difficult to determine why the feeding behavior response was different in this case.

TABLE 6.2 Effects of phlorizin and SCFA infusion on glucose excretion, ruminal SCFAs, and blood plasma metabolites and hormones[1].

	Na Acetate		Na Propionate			*P* <		
Item	Control	Phlorizin	Control	Phlorizin	SEM	Prop[2]	Phlor[2]	Int[2]
Urinary glucose excretion, *g/d*	2	398	1	403	40	0.92	0.001	0.91
Ruminal acetate, *mmol/L*	79.0	77.8	51.6	52.1	3.5	0.001	0.90	0.76
Ruminal propionate, *mmol/L*	19.4	17.7	35.5	31.7	1.8	0.001	0.10	0.50
Ruminal butyrate, *mmol/L*	10.8	10.4	8.0	8.1	0.77	0.01	0.85	0.76
Plasma glucose, *mmol/L*	3.09	2.88	3.32	3.23	0.09	0.001	0.01	0.17
Plasma glycerol, *mg/dL*	2.59	2.94	2.43	2.68	0.17	0.10	0.02	0.70
Plasma FFA, *µmol/L*	91	132	82	79	30	0.04	0.22	0.14
Plasma BHBA, *µmol/L*	850	1250	410	460	130	0.001	0.04	0.11
Plasma triglycerides, *mg/dL*	3.65	3.84	3.82	3.54	0.25	0.75	0.85	0.25
Plasma insulin, *pmol/L*	60	47	79	68	10	0.001	0.01	0.77
Plasma glucagon, *pmol/L*	58.4	61.3	49.9	54.2	3.7	0.001	0.05	0.69

[1]Values are least square means ± SEM, n = 11 for acetate treatments and n = 12 for propionate treatments

[2]Prop = effect of propionate infusion; Phlor = effect of phlorizin administration; Int = interaction of treatments.

TABLE 6.3 Effects of phlorizin and SCFA infusion on feeding behavior and yield of milk components[1].

	Na Acetate		Na Propionate			*P* <		
Item	Control	Phlorizin	Control	Phlorizin	SEM	Prop[2]	Phlor[2]	Int[2]
Dry matter intake, *kg/d*	21.5	20.8	18.7	18.1	1.4	0.001	0.39	0.91
Meal size, *kg dry matter*	1.53	1.53	1.61	1.58	0.09	0.34	0.87	0.86
Number of meals, */d*	14.2	13.4	11.6	11.8	0.7	0.001	0.61	0.32
Intermeal interval, *min*	76.6	77.9	92.1	86.2	6.6	0.04	0.67	0.50
Milk lactose yield[3], *kg/d*	2.26	2.22	2.27	2.26	0.15			
Milk protein yield, *kg/d*	1.15	1.09	1.07	1.06	0.06	0.04	0.20	0.30
Milk fat yield, *kg/d*	1.68	1.80	1.54	1.63	0.09	0.01	0.04	0.74
Milk long-chain fatty acid yield[4], *g/d*	680	718	590	669	56	0.20	0.29	0.71
Milk odd-chain fatty acid yield, *g/d*	37.4	38.7	55.0	50.6	3.4	0.001	0.61	0.38

[1]Values are least square means ± SEM, n = 11 for acetate treatments and n = 12 for propionate treatments.

[2]Prop = effect of propionate infusion; Phlor = effect of phlorizin administration; Int = interaction of treatments.

[3]Treatment effects could not assessed because of a significant period by treatment interaction.

[4]Long-chain fatty acids are defined as fatty acids with more than 16 carbons.

In previous experiments, phlorizin induced a catabolic state resulting in increased availability of FFA at the liver (Bradford and Allen, 2005). Phlorizin caused increases of similar magnitude in plasma FFA and BHBA concentrations with acetate infusion in this experiment (Table 6.2). However, propionate infusion significantly decreased FFA and BHBA concentrations; furthermore, tendencies for treatment interactions for both FFA and BHBA suggest that marginal responses to phlorizin were decreased by propionate infusion (Table 6.2). Plasma FFA analyses provide a reasonable assessment of lipolytic activity in ruminants (Dunshea et al., 1989). On the other hand, plasma glycerol is also typically indicative of lipolysis (Brockman, 1984), and phlorizin increased plasma glycerol concentrations across infusion treatments ($P < 0.02$, Table 6.2). Analysis of milk components offers the advantage of providing a composite view of nutrient availability over the course of the day. *De novo* lipogenesis in the ruminant mammary gland produces fatty acids with chain lengths up to 16 carbons, but long-chain fatty acids (LCFA) exceeding 16 carbons are plasma-derived (Barber et al., 1997). Because phlorizin treatment had no influence on dietary lipid intake, any treatment effects on LCFA can be attributed to differences in lipolytic activity. Although there was no significant effect of phlorizin on LCFA yield ($P < 0.29$, Table 6.3), numerical increases in LCFA yield accounted for 32% and 88%, respectively, of the significant increase in milk fat yield for phlorizin treatment with acetate and propionate infusions. No other class of fatty acids could account for the increase in milk fat yield with phlorizin.

Taking into account treatment effects on plasma NEFA, plasma glycerol, and milk LCFA yield, it seems likely that lipolysis, and delivery of fatty acids to the liver, was increased by phlorizin across infusion treatments.

As expected, propionate infusion decreased DMI relative to acetate infusion (18.7 vs. 21.5 kg/d, P <0.001, Table 6.3); according to our working hypothesis, this was because propionate infusion stimulated hepatic oxidation of propionate (Allen et al., 2005). Phlorizin is expected to increase utilization of propionate for gluconeogenesis (Veenhuizen et al., 1988), and we predicted that this would limit the hypophagic effects of propionate infusion. However, we found no overall effect of phlorizin on DMI (P < 0.39, Table 6.3), nor was an interaction with infusion treatment evident (P < 0.91). These data do not support our hypothesis; however, treatment effects on fatty acid availability for hepatic oxidation confound the targeted effects on propionate and glucose metabolism.

A number of alternative hypotheses have been suggested to explain the hypophagic effects of propionate in ruminants. Propionate is an insulin secretagogue, and both propionate infusions and rations with large amounts of highly digestible starch typically increase plasma insulin concentrations (Harmon, 1992). Insulin is a potent satiety factor when administered via intracerebroventricular infusion in sheep (Foster et al., 1991), and it can access the central nervous system through the blood-brain barrier (Plum et al., 2005). However, propionate infusions have decreased DMI without increasing plasma

insulin concentrations (Farningham and Whyte, 1993), and in one study, propionate infusion failed to decrease DMI despite nearly tripling jugular insulin concentrations (Leuvenink et al., 1997). Within propionate infusions in this experiment, phlorizin treatment tended to decrease insulin concentration (68 vs. 79 pmol/L, $P < 0.06$) while numerically *decreasing* DMI; at least in this case, insulin did not mediate propionate's hypophagic effects.

Nutrient receptors in the veins draining the splanchnic bed have been proposed for propionate in ruminants (Baile, 1971; Leuvenink, et al., 1997) and glucose in nonruminants (Novin et al., 1973). Glucose is hypophagic in a variety of nonruminants (Forbes, 1995), but intestinal and intravenous glucose infusions have not decreased energy intake of ruminants (Allen, 2000). The absence of effects of portal glucose infusion on feed intake by sheep (Baile and Forbes, 1974) casts doubt on the hypothesis that sensory neurons in the portal vein mediate the hypophagic effects of glucose, given that mechanisms regulating feed intake are well conserved across divergent species (Chiang and MacDougald, 2003). There is broad consensus now that portal glucose infusions modulate feed intake of nonruminants through oxidation of glucose (Mobbs et al., 2005), but there is disagreement as to whether this occurs in sensory neurons or hepatocytes. Because ruminant neural tissue metabolizes glucose (Lindsay and Setchell, 1976), it seems unlikely that hypophagia is induced by glucose oxidation in sensory receptors. Rather, differences in hypophagic effects of glucose infusion observed between ruminants and nonruminants are likely

because of differences in hepatic oxidation of glucose; liver hexokinase activity is low in ruminants compared with nonruminants (Ballard, 1965), and in mature ruminants, hepatic removal of glucose appears to be negligible (Stangassinger and Giesecke, 1986). Likewise, the responses attributed to the proposed propionate receptor can be explained equally well by the hepatic oxidation hypothesis, without the need to propose dramatic evolutionary divergence in mechanisms regulating feed intake.

Evidence regarding the mechanism for propionate's hypophagic effects in ruminants remains elusive. These data and other recent findings suggest that short-term (7 d) increases in glucose demand do not alter feed intake response to propionate infusion or highly fermentable diets (Bradford and Allen, 2005). Adaptive physiological responses such as increased lipolysis and protein breakdown may provide oxidative fuel and glucogenic substrate for enhanced gluconeogenesis during phlorizin administration, preventing the increase in feed intake that was expected in response to urinary loss of ~5% of net energy intake.

CHAPTER 7

RATE OF PROPIONATE INFUSION WITHIN MEALS DID NOT INFLUENCE FEEDING BEHAVIOR

ABSTRACT

Propionate has been shown to depress feed intake of ruminants, but it is unknown whether the rate of propionate infusion influences this response. To test this possibility, rate of propionate infused within meals was altered while total amount of propionate infused was held constant. Eight multiparous Holstein cows (51 ± 19 DIM, 44.0 ± 4.8 kg/d milk; mean ± SD) were randomly assigned to treatment sequence in a crossover experiment with a 10-d diet adaptation period and 3 d between treatments. Treatments were intraruminal infusion of 1.26 mol Na propionate (2.33 ± 0.06 L, 0.54 M, pH 6.0) over the course of either 5 min (FAST) or 15 min (SLOW) at each spontaneous meal. The experimental diet included high moisture corn and was formulated for 27% NDF, 36% starch, and 17.5% CP. Feeding behavior was monitored by a computerized data acquisition system that triggered infusion pumps at the initiation of meals, and consecutive infusions began at least 15 min apart under both treatment protocols. Feeding behavior data were analyzed to quantify meal size, meal length, number of meals, and intermeal interval. Propionate infusions depressed feed intake by 20% and 23%, respectively, for SLOW and FAST. However, rate of propionate infusion did not significantly alter dry matter intake, meals/d, meal size, meal length, or intermeal interval. We found no evidence that rate of infusion, within

the range of typical meal lengths, determines the extent of hypophagia from propionate.

INTRODUCTION

Propionate plays an important role as a regulator of feed intake in ruminants fed high starch diets. Continuous infusion of propionate depressed feed intake of lactating cows in a dose-dependent manner, primarily by decreasing meal size (Oba and Allen, 2003a; Oba and Allen, 2003d). We have also shown that intraruminal infusion of Na propionate at spontaneous meals decreases feed intake relative to isomolar infusions of Na acetate and NaCl (Choi and Allen, 1999). These observations, in combination with data suggesting that propionate delivery to the liver can increase significantly within the course of a meal (Benson et al., 2002), lead us to hypothesize that propionate depresses feed intake by decreasing the time required to stimulate satiety within a meal (Allen et al., 2005). However, if hepatic oxidation of propionate is required for its satiating effects, then the fate of propionate within the liver will determine the extent to which feed intake is depressed. If propionate flux into the liver outpaces capacity for glucoeneogenesis, excess propionate will likely be oxidized. Therefore, rate of propionate absorption may be as important as total propionate uptake as a limitation to feed intake. To test this hypothesis, we assessed the effects of propionate infusion rate, independent of infusion amount, on feeding behavior and dry matter intake (DMI) of lactating dairy cows.

MATERIALS AND METHODS

Eight multiparous Holstein cows (51 ± 19 DIM, 44.0 ± 4.8 kg/d milk; mean ± SD) with ruminal cannulas were selected from the Michigan State University Dairy

Cattle Teaching and Research Center and randomly assigned to treatment sequence in a crossover experiment with a 10-d diet adaptation period and 3 d between treatments. Treatments were intraruminal infusion of 1.26 mol Na propionate (2.33 ± 0.06 L, 0.54 M, pH 6.0) over the course of either 5 min (FAST) or 15 min (SLOW) at each meal for 24 h. The experimental diet is shown in **Table 7.1**; the diet was formulated to promote endogenous propionate production so that propionate would provide the primary limitation to feed intake during the experiment. Throughout the experiment, cows were housed indoors in tie stalls and fed a total mixed ration once daily (1130 h) at 110% of expected intake. Cows were not allowed access to feed between 1000 h and 1130 h each day while orts and the amount of feed offered were weighed for each cow. Diet ingredients and orts samples were dried in a 55°C forced-air oven for 72 h and analyzed for dry matter concentration. Ingredient samples were ground with a Wiley mill (1-mm screen; Authur H. Thomas) and analyzed for ash, neutral detergent fiber, crude protein, and starch content as previously described (Bradford and Allen, 2004). At the end of each infusion period, ruminal contents were sampled from 5 sites throughout rumen and squeezed through a nylon screen (1 mm pore size) to collect the liquid phase. Ruminal fluid pH was measured using a portable pH meter (model 230A, ATI Orion, Boston, MA), and samples were frozen at -20° C until analysis. Concentrations of volatile fatty acids in ruminal fluid were determined as previously described (Oba and Allen, 2003b).

Table 7.1 Ingredients and nutrient composition of experimental diet.[1]

Item	
Diet ingredients	
Corn silage	31.0
High moisture corn grain	28.4
Alfalfa haylage	14.8
Modified expeller soybean meal[2]	8.9
Mineral and vitamin mix[3]	8.6
Soybean meal	8.4
Nutrient composition	
DM	46.8
Organic matter	92.8
Starch	30.1
NDF	27.3
CP	16.1

[1]Values other than DM are expressed as % of dietary DM.

[2]SoyPlus (donation from West Central Soy, Ralston, IA).

[3]Mineral and vitamin mix contained 67.9% dry ground corn, 13.1% limestone, 8.5% magnesium sulfate, 6.4% salt, 2.9% dicalcium phosphate, 0.9% trace mineral premix, and 0.3% vitamin ADE premix.

On treatment days, feeding behavior was monitored by a computerized data acquisition system (Dado and Allen, 1993) that triggered peristaltic infusion pumps at the initiation of each new meal. Weight of the feed manger was monitored every 5 s, and when the running SD reached a threshold, a computerized "eating flag" was triggered. Because of differences in behavior, the SD threshold was set for each cow individually based on 3 d of observation prior to the initial treatment (range = 1.25 to 2.75). Infusions were initiated only when the eating flag was triggered 5 times in a 100 s period. Mid-meal infusions were prevented by requiring that the eating flag was triggered less than 13 times in the preceding 5 min. Consecutive infusions began at least 15 min apart under both

treatment protocols to prevent potential treatment bias. Feeding behavior data were analyzed to quantify meal size, meal length, number of meals, and intermeal interval, and statistical analyses were carried out using a mixed model with fixed effects of period, treatment, and period by treatment interaction, and the random effect of cow.

RESULTS AND DISCUSSION

Triggering infusions based on real-time monitoring of feeding behavior resulted in reasonably good relationships between infusion events and meals identified by *post-hoc* analysis. There were a mean of 2.9 false positives (infusions without meals) out of a total of 15.6 infusions per cow/d, and 0.1 false negatives (meals without infusions) per cow/d. A numerically greater volume of Na propionate was infused for the SLOW treatment compared to FAST (38.6 vs. 34.1 L/d, $P = 0.21$), due in part to a numerically greater number of false positive infusions (3.63 vs. 2.25 per d, $P = 0.19$).

Compared to the days prior to infusion, propionate infusion at meals depressed DMI by 20% and 23%, respectively, for SLOW and FAST. However, rate of infusion had no detectable influence on DMI or on measures of feeding behavior (all $P > 0.10$, **Table 7.2**). In addition, to assess whether the numerically greater amount of propionate infused for SLOW biased these results, energy intake from both infusate and feed consumed was calculated; treatment had no effect on calculated ME intake (data not shown). Propionate infusion rate did not alter

ruminal pH (P = 0.38, mean = 6.38) or ruminal propionate concentration (P = 0.74) at the end of the infusion periods.

Table 7.2 Effects of propionate infusion rate on feeding behavior of lactating cows[1].

Item	FAST	SLOW	SEM	P
Dry matter intake (kg/d)	18.6	19.1	1.3	0.61
Meal size (kg DM)	1.48	1.49	0.17	0.93
Meals/d	12.88	12.75	0.70	0.87
Meal length (min)	22.6	24.3	1.1	0.20
Intermeal interval (min)	82.8	82.6	5.7	0.98

[1]Values are least square means, n = 8.

Data from 2 preliminary studies were included in an expanded dataset to examine whether DMI responses to infusion rate are dose-dependent. Protocols from these 2 studies were identical to those described above, except that solutions of Na propionate were of different concentrations (0.27 M and 1.0 M, respectively). The preliminary experiments were randomized complete block designs, so DMI depression is expressed as a percent of DMI on the preceding 2 d to account for individual variation in baseline DMI. Intraruminal infusion of propionate at the beginning of spontaneous meals decreased DMI in a dose-dependent manner (**Figure 7.1A**), similar to what has been observed previously with continuous infusion in cows (Oba and Allen, 2003d) and meal-initiated infusions in goats (Baile and Mayer, 1969). Depression of DMI was not influenced by FAST or SLOW across experiments. However, amount of propionate infused per meal better predicted intake responses than did total amount of propionate infused (**Figure 7.1B**).

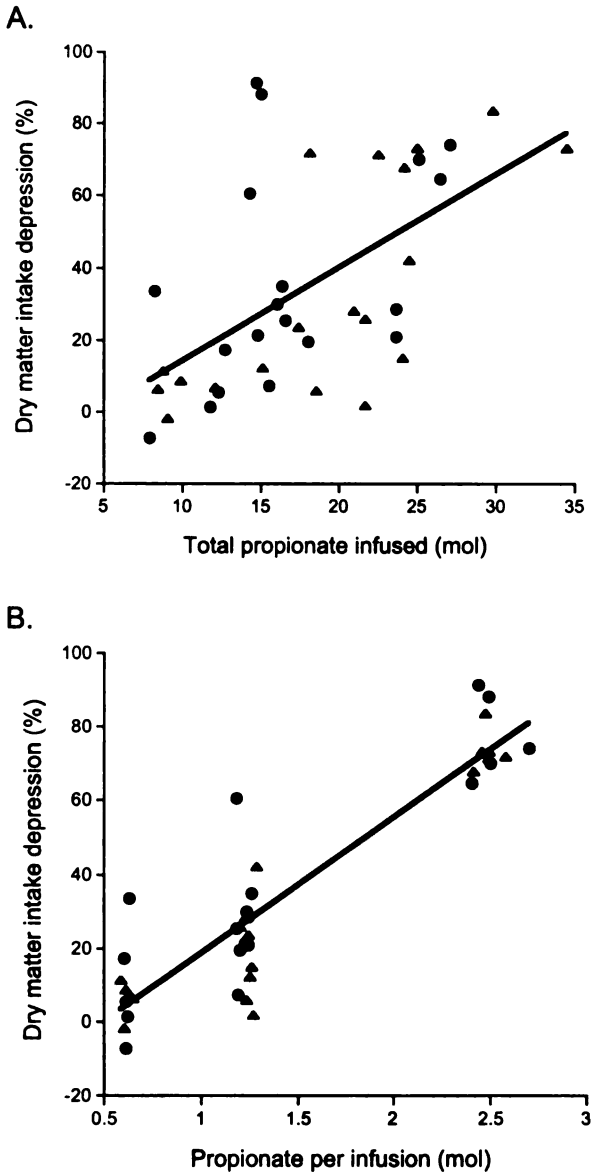


Figure 7.1 Propionate infusion at spontaneous meals depresses feed intake in a dose-dependent manner. FAST: ●, SLOW: ▲. (A) Total amount of propionate infused over 24 h, but not infusion rate, determined degree of dry matter intake depression by infused propionate. $R^2 = 0.33$, $P < 0.001$, n = 38. (B) Amount of propionate infused at each meal was a better predictor of dry matter intake depression than total amount infused. $R^2 = 0.83$, $P < 0.001$, n = 38.

The lack of effect of infusion rate on feeding behavior and dry matter intake may be because our infusion rates were not sufficiently different in this experiment. Meal lengths across treatments averaged 23.1 ± 14.2 min (SD), and 73% of meals were longer than 15 min (**Figure 7.2**), suggesting that the SLOW treatment could have been infused over a longer period. Another possibility is that rate of propionate activation by propionyl-CoA synthetase may be limiting, resulting in little change in hepatic uptake of propionate across treatments. Propionyl-CoA synthetase has a reported Km for propionate of 1.3 mM (Ricks and Cook, 1981), and enzyme activity increases as the concentration of propionate reaching the liver increases. However, portal propionate concentrations in cows nearing peak lactation *averaged* 1.86 mM over the course of the day (calculated from Reynolds et al., 2003). Here we observed similar DMI with higher starch and NDF concentrations in the diet (compared to that of Reynolds et al., 2003), which was expected to promote greater endogenous propionate production. Combining propionate infusion with rapid propionate production during meals in this experiment may have increased propionate concentrations at the liver to the extent that hepatic propionyl-CoA synthetase activity was maximized. Although hepatic extraction of propionate is typically quite efficient, we have reported that peripheral propionate concentrations increased linearly with rate of intraruminal propionate infusion (Bradford et al., 2006b). Therefore, it is plausible that liver capacity for activation of propionate was saturated during meals in this experiment. Given these potential limitations,

the possibility remains that the post-absorptive fate of propionate influences DMI in lactating dairy cows.

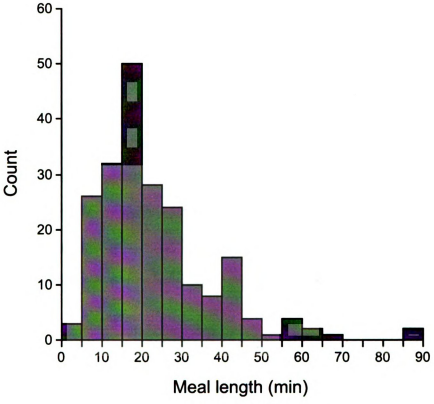


Figure 7.2 Distribution of meal lengths on infusion days. The histogram represents 210 meal bouts from 2 d (n = 8 cows).

CHAPTER 8

CONCLUSIONS

The aim of these investigations was to provide insight into the mechanism by which propionate affects feeding behavior of lactating dairy cows. We initially used dietary treatments to alter propionate flux to the liver and measured a large number of potentially related variables in 32 cows. Few relationships between feed intake response and metabolic parameters, production variables, or hepatic gene expression patterns were discovered. However, significant relationships between feed intake response and 2 measures of insulin dynamics do suggest that metabolic state influences individual response to propionate.

In our second study, we tested the hypothesis that propionate depresses feed intake by stimulating leptin secretion. Plasma leptin responses to both short-term jugular infusions and longer-term ruminal infusions of propionate were minimal and did not explain feed intake responses to ruminal infusion. We concluded that propionate's effects on feed intake are not mediated by leptin.

In a series of 3 experiments, we investigated the effect of increased glucose demand on feed intake response to highly fermentable diets and propionate infusion. In each of the experiments, phlorizin not only increased glucose demand but also stimulated lipolysis in cows in both positive and negative energy balance. This secondary effect of phlorizin most likely increased hepatic uptake

of fatty acids, which serve as important fuels for the ruminant liver. Because of this confounding effect on fatty acid metabolism, the lack of an effect of phlorizin on feed intake is not inconsistent with the hepatic oxidation hypothesis.

Finally, we investigated whether rate of propionate infusion administered at voluntary meals would influence feed intake. We expected that a more rapid infusion rate would depress feed intake to a greater extent because more propionate would be forced into oxidative pathways, as opposed to gluconeogenesis. However, we found no effect of infusion rate on feeding behavior. Despite the fact that our hypothesis was not supported, a number of factors could explain the lack of treatment effect, including the possibility that our treatments did not change flux of propionate into the liver. Therefore, in this experiment, the hepatic oxidation hypothesis could not be critically evaluated.

The complexities of the hepatic oxidation hypothesis make it extremely difficult to design experiments that could reasonably lead to its rejection. According to the hypothesis, satiety is driven by the accumulation of hepatic ATP; this single concentration is dependent on the balance between both catabolic and anabolic processes in the liver, and is undoubtedly variable over time. While it is attractive to attempt to measure changes in hepatic energy charge associated with treatments, we have not had success in measuring significant changes over the course of a meal (unpublished data, Longuski, Bradford, and Allen). Furthermore, *in vivo* assessment of fluxes through oxidative and anabolic

processes in ruminants is technically challenging, and because multiple fuels must be considered (eg. fatty acids and propionate), extremely expensive. Despite the difficulty of "ruling in or ruling out" the hepatic oxidation hypothesis, the evidence in its favor is compelling enough to warrant further investigation into its potential role in regulation of feed intake of ruminants.

One difficulty in studying feed intake regulation in general is that multiple mechanisms are always at play in determining feed intake and feeding behavior, and it can be difficult to ensure that the mechanism in question is the dominant one during the experiment. A potential solution to this problem is to focus on intermediates in the proposed pathway. Although this approach is somewhat less satisfying in that feeding behavior is the variable of greatest interest, biochemical measurements are less likely to be masked by confounding factors than are measures of animal behavior.

If the hepatic oxidation hypothesis is valid, then some means of communication between hepatocytes and vagal afferents is required. This may be a fruitful point in the pathway for assessing the effects of propionate infusions on potential mediators. One clear candidate for investigation is AMPK; phosphorylation state of AMPK can be maintained by careful preservation of liver samples, and specific antibodies for phospho-AMPK are commercially available for use in immunoblotting procedures. Infusion of Na propionate vs. Na acetate over the course of several hours should decrease the relative phosphorylation state of

hepatic AMPK if it is involved in the signaling pathway mediating propionate's effects. The other proposed mediator at that step, Na^+/K^+ ATPase, is more difficult to investigate, because ATP is thought to alter its activity primarily at the substrate level. However, phosphorylation of various ion channels could be quantified in the same manner as for AMPK. Because of the large number of ion channels that could be involved, a proteomics approach may be ideal for this investigation. Plasma membranes could be isolated from liver samples, and mass spectroscopy could be used to identify peptide fragments derived from ion channels with and without phosphate groups attached. In this way, the relative phosphorylation state of multiple ion channels could be measured simultaneously.

In addition to a greater focus on pathway intermediates, different approaches to applied studies may also yield more conclusive data. The results of the phlorizin experiments described in chapters 4 –6 suggest that our focus at the outset was too narrowly focused on metabolism of propionate. One of the strengths of the hepatic oxidation hypothesis is its ability to integrate metabolism of carbohydrate, fats, and proteins into a single mechanism, and future research needs to more explicitly consider potential interactions of nutrient classes. For example, increasing fatty acid oxidation (e.g. in early lactation or with phlorizin treatment) increases the amount of acetyl-CoA generated through β-oxidation. When glucose demand is high relative to availability of gluconeogenic substrates, oxidation of acetyl-CoA can be limited by accumulation of reducing equivalents

(high NADH:NAD^+) and by availability of TCA cycle intermediates. When propionate enters the hepatocyte, it can be quickly converted to oxaloacetate, providing substrate for the citrate synthase reaction, and during conversion to glucose, propionate utilizes reducing equivalents. Therefore, propionate can stimulate ATP production by removing the primary constraints to oxidation of acetyl-CoA, even if it is not oxidized itself.

In support of this view, Oba and Allen (2003a) showed in a dose-response experiment that low rates of propionate infusion decreased feed intake in early lactation cows, but not in mid lactation cows. Infusion of propionate at lower doses increased plasma glucose concentration in both stages of lactation, suggesting that this rate of infusion did not overwhelm gluconeogenic capacity and likely did not greatly increase propionate oxidation. However, propionate likely stimulated oxidation of acetyl-CoA, increasing ATP production in early lactation cows despite the use of propionate for glucose production. This hypothesis is supported by the observed decrease in plasma BHBA concentration in early lactation cows at lower infusion rates while NEFA concentration remained elevated (Oba and Allen, 2003a). Differences in the concentration of acetyl-CoA in the liver might help explain differences in feeding behavior responses between early and mid-lactation cows as well as differences among individual cows. The experiment described in chapter 1 is an ideal design to assess whether hepatic concentration of acetyl-CoA explains individual differences in response to highly fermentable diets; unfortunately, samples were

not stored for preservation of acetyl-CoA. Future work regarding the hepatic oxidation hypothesis for ruminants will undoubtedly need to consider such potential interactions.

Despite our inability to add support for the hepatic oxidation hypothesis in these experiments, we have made progress on the mechanism for propionate regulation of feed intake. In the work described in chapter 2, we presented strong evidence that leptin secretion is not an important component of feed intake regulation by propionate in lactating cows fed *ad libitum*. The experiment reported in chapter 6 added to the evidence that insulin is not a required component of the mechanism for propionate's effects on feed intake. Finally, the series of phlorizin studies provide strong evidence that increased glucose demand and energy requirements do not result in compensatory increases in feed intake within 7 d. These results narrow the scope of possible mechanisms by which propionate may act to alter feeding behavior. Hopefully, future research will be able to more clearly define the mechanism for propionate regulation of feed intake, allowing for better predictions of individual responses to dietary formulations and improving the management of lactating dairy cows.

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