AN ABSTRACT OF THE THESIS OF

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Title:α-Tocopherol is a Potential Diagnostic Indicator of Metabolic Diseases in Early Lactation Dairy Cows

Abstract approved: _____

Gerd Bobe

Milk fever (MF), retained placenta (RP), and left displaced abomasum (LDA) are three common and costly metabolic diseases in cows during the first days of lactation. Some studies suggest that circulating concentrations of α-tocopherol (ATOC) are decreased by these three diseases. It is, however, unknown if and how long lower circulating ATOC precede and/or remain after recovery from these three diseases. The hypothesis or the thesis is that lower serum ATOC concentrations precede and persist in cows after MF, RP and LDA. The objective of the thesis is to examine the association between MF, RP, and LDA and serum concentrations of ATOC, metabolites, refore, the hypothesis of this project was to compare with healthy cows, lower serum ATOC concentration precede and persist in cows after MF, RP and LDA. Using a nested case-control study design, the relationship between the incidence of those three diseases and serum concentrations of ATOC, metabolites, acute phase proteins, and minerals, measured at day -21, -14, -7, -3, -1, 0, 1, 3, 7, 14, 21, 28, 35, 42, and 49 postpartum, was evaluated in multiparous Holstein cows.

In chapter 2, serum concentrations of ATOC, metabolites, acute phase proteins, and minerals of 7 multiparous Holstein cows diagnosed with LDA between day 6 and 32 postpartum were compared with 10 healthy Holstein cows. Besides indicators of

negative energy balance and inflammation, lower serum ATOC concentrations preceded LDA and persisted after LDA correction. At the last blood sampling before LDA diagnosis, cows had 45% lower serum ATOC concentrations (5.0 ± 0.9 vs. 9.1 ± 0.9 µM; $P = 0.004$) and 39% lower ATOC to cholesterol molar ratios (1.90 ± 0.19 vs. 3.09 ± 0.26; $P = 0.003$) compared with healthy cows. Serum ATOC concentrations remained lower (<10 vs. ~15 µM) in cows that had LDA up to day 49 postpartum (all $P < 0.03$).

In chapter 3, serum concentrations of ATOC, metabolites, acute phase proteins, and minerals of 32 multiparous Holstein cows with retained fetal membranes for more than 24 h were compared with those of 32 diseased cows and those of 32 visually healthy cows. Besides indicators of negative energy balance and inflammation, cows that developed RP had prepartum 30% lower prepartal serum ATOC concentrations (8.7 ± 0.6 vs. 12.5 ± 0.6 µM; $P< 0.001$) and 23% lower ATOC to cholesterol molar ratios (3.12 vs. 4.03 µM/mM; $P< 0.001$) compared with visually healthy cows. These group differences were already significant three weeks before calving for ATOC concentrations (8.3 ± 0.7 vs. 11.9± 0.7 µM; $P< 0.001$) and ATOC to cholesterol molar ratios (2.68 vs. 3.66 µM/mM; $P = 0.001$). Up to day 28 postpartum, serum ATOC concentrations remained lower in RP than in visually healthy cows (<10 vs. ~13 µM; all $P < 0.001$). Serum ATOC concentrations and ATOC to cholesterol molar ratios did not differ between diseased cows with RP than with other diseases.

In chapter 4, serum concentrations of ATOC, metabolites, acute phase proteins, and minerals of 9 multiparous Holstein cows with serum calcium concentrations below 6 mg/dl and being treated for milk fever within the first 48 hpostpartum were compared with those of 10 healthy cows and with those of 31 diseased cows with serum calcium

concentrations above 6 mg/dL in the first 48 hpostpartum Besides indicators of negative energy balance and inflammation, cows that later developed MF had 37% lower prepartal serum ATOC concentrations (9.0 ± 0.9 vs. 14.2 ± 0.8 μM; $P< 0.001$) and 35% lower ATOC to cholesterol molar ratios (3.08 vs. 4.78 μM/mM; $P< 0.001$) compared with healthy cows. These group differences were already significant three weeks before calving for ATOC concentrations (8.3 ± 0.9 vs. 13.8 ± 0.8 μM; $P< 0.001$) and ATOC to cholesterol molar ratios (2.86 vs. 4.18 μM/mM; $P = 0.003$). Up to day 28 postpartum, serum ATOC concentrations remained lower in MF than in healthy cows (<9 vs. ~13 μM; all $P < 0.002$). Serum ATOC concentrations and ATOC to cholesterol molar ratios did not differ between diseased cows with MF than with other diseases.

In summary, depleted serum ATOC concentrations preceded the three investigated metabolic diseases (MF, RP, and LDA). Thus, lower serum ATOC concentrations maybe a potential diagnostic indicator for metabolic diseases in multiparous dairy cows during early lactation. Serum ATOC concentrations remained lower than in healthy cows for several wk after disease treatment. The focus of future studies will be if and how vitamin E alimentation may prevent or improve response to conventional treatments of metabolic diseases in multiparous cows.

α-Tocopherol is a Potential Diagnostic Indicator of Metabolic Diseases in Early Lactation Dairy Cows

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Yang Qu, Author

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CONTRIBUTION OF AUTHORS

Chapter 2

Dr. Bobe – obtained funding, designed project, performed statistical analysis, and provided oversight during all phases of the study

Kelli Lytle – did part of the vitamin E analysis

Dr. Traber – provided technical assistance and oversight for vitamin E analysis and manuscript writing assistance

Chapter 3 & 4

Dr. Bobe – obtained funding, designed project, performed statistical analysis, and provided oversight during all phases of the study

Nicole Fadden – did part of the chemical analysis

Dr. Traber – provided technical assistance and oversight for vitamin E analysis and manuscript writing assistance

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ABBREVIATION LIST

α-TTP: α-tocopherol transfer protein
α-CEHC: 2,5,7,8-tetramethyl-2-(2'-carboxyethyl)-6-hydroxychroman
γ-CEHC: 2,7,8-trimethyl-2-(2'-carboxyethyl)-6-hydroxychroman
AA: Ascorbic acid
AlP: Alkaline phosphatase
ACP: Acidic phosphatase
APX: Ascorbate peroxidase
AST: Aspartate transaminase
ATOC: α-tocopherol
BCS: Body condition score
BLT-1: Block lipid transport
BHBA: beta-hydroxybutyric acid
CM: Chylomicrons
CMR: Chylomicron remnants
Ca: Calcium
Cl: Chloride
CK: Creatine kinase
DMI: Dry matter intake
DHA: Reduced dehydroascorbate
DHAR: Dehydroascorbate reductase
dL: deciliter
(E •): Vitamin E radical
Eq: Equivalent
GGT: Gamma-glutamyl transpeptidase
GPX: Glutathione peroxidase
GR: Glutathione reductase
GSH: Glutathione
GSSG: Glutathione disulfide
HDL: High density lipoprotein
H_2O_2: Hydrogen peroxide
IDL: Intermediate density lipoprotein
IM: Intramuscular injection
IV: Intravenous infusion
IU: International units
K: Potassium
Kg: Kilogram
L: Liter
LCAT: Lecithin:cholesterol acyltransferase
LDA: Left displaced abomasum
LDL: Low density lipoprotein
LH: Lipid hydroperoxide
(LO_2 •)˙: Peroxyl radical

M: Mol
µM: Micromol
MF: Milk fever
Mg: Magnesium
µEq: Microequivalent
mg: Milligram
µg: Microgram
mL: Milliliter
µL: Microliter
NADPH: Reduced nicotinamide adenine dinucleotide phosphate
NEFA: Non-esterified fatty acids
NPC1L1: Niemann-pick C1-like 1
(• OH): Hydroxyl radical
P: Phosphorus
PLTP: Phospholipid transfer protein
PUFA: Poly-unsaturated fatty acids
RP: Retained placenta
ROS: Reactive oxygen species
SAA: Serum amyloid A
SCC: Somatic cell count
SRB-1: Scavenger receptor B-1
SC: Subcutaneous injection
TMR: Total mixed ration
TP: Total protein
VLDL: Very low density lipoprotein
Zn: Zinc

CHAPTER 1 LITERATURE REVIEW

Vitamin E Status in Dairy Cows during the Transition Period

The transition period (also called the periparturient period), defined as between -4 to 4 weeks after calving, is critical to the health of dairy cows (Sharma et al., 2011). In the last 4 weeks prepartum, feed intake decreases as a consequence of the rapidly growing fetus (Campbell and Miller, 1998). Cholesterol concentrations in blood are decreased (Drackley, 1999), indicating decreased lipid and vitamin E absorption and transport. In contrast to the decreased nutrient intake and absorption, there is a growing nutrient demand for a) the exponentially growing fetus and b) the developing mammary tissue (Sordillo and Aitken, 2009). As a result, many multiparous cows experience in the last weeks before calving a negative balance of nutrients, including vitamin E, as indicated by decreased vitamin E concentrations in blood and mobilization of non-esterified fatty acids (NEFA) (Drackley, 1999).

After calving the negative nutrient balance gets worse, as nutrient intake is depressed because of a) feed changes, b) moving the cows from the dry cow group to the lactation cow group, and c) the calving-associated cortisol and cytokine release (Contreras and Sordillo, 2011). Furthermore, absorption and transport of lipids, including vitamin E, is depressed as indicated by low lipoprotein concentrations in the first weeks after calving (Herdt and Smith, 1996). Concomitant with the decreased vitamin E input and transport, there are increased vitamin E requirements. Calves are born vitamin E deplete (Walsh et al., 1993). Colostrum provides the only source of vitamin E to the offspring; thus, vitamin E and other vitamins as well as minerals are utilized in large amounts for colostrum synthesis. In addition, nutrients, including vitamin E, are utilized for a)

mammary tissue synthesis and maintenance, b) milk synthesis (Weiss et al., 1990), c) tissue repair from calving (Politis et al., 2012), and d) pathogen defense, as the uterus, ovarian tract, and mammary gland are most vulnerable to pathogen invasion after calving (Jukola et al., 1996). Vitamin E requirements are exponentially increased to remove reactive oxygen species (ROS), specifically oxygen singlets from lipids, and break the lipid peroxidation cycle generated by a) fatty acid oxidation, b) colostrum and milk synthesis, c) pathogen defense, and d) tissue repair and synthesis (Drackley, 1999; LeBlanc et al., 2004; Politis et al., 2012).

Vitamin E concentrations in blood and probable in tissue rapidly decrease during the first week after calving (LeBlanc et al., 2004) and cows are in a depleted vitamin E status. The inadequate vitamin E status may explain in part the increased riskof dairy cows for metabolic and infectious diseasesshortly after calving (Mudron et al., 1997).

Chemical Structure of Vitamin E

Vitamin E is a group of compounds named chromanols which play a crucial role in breaking lipid peroxidation and protecting cell membrane function (Niki and Traber, 2012). Animals depend on chromanols from feed stuff as they cannot synthesize vitamin E, as plants do.

Tocochromanols consist of a consist of a chromanol ring system, called ‘head’, and a hydrophobic prenyl side chain, called ‘tail’. Tocochromanols are divided into two groups, tocopherols and tocotrienols, which differ in the saturation of their 12-carbon lipid tail (saturated in tocopherols versus three double bonds in tocotrienols) (**Figure 1**). Tocopherol and tocotrienols exist each in four different forms, α-, β-, γ-, and δ, which

differ in the number of number and position of methyl groups on the chromanol head group. Plants synthesize *RRR*-tocopherols based on three centers of asymmetry in their tail region (C-2 of the heterocyclic ring and C-4' and C-8' in the carbon tail). Industrially produced vitamin E has eight stereoisomers (*RRR, RSR, RSS, RRS, SRR, SSR, SRS,* and *SSS*) and is called *all-rac*-tocopherol; it (adapted from Traber, 2014). The most common of vitamin E sold to farmers in vitamin mixes is *all-rac*-α-tocopheryl acetate, which has a 50% lower activity than *RRR*-α-tocopherol because only α-tocopherol with an R formation in the C-2 position of the heterocyclic ring (*2R*-α-tocopherol) is not quickly excreted.

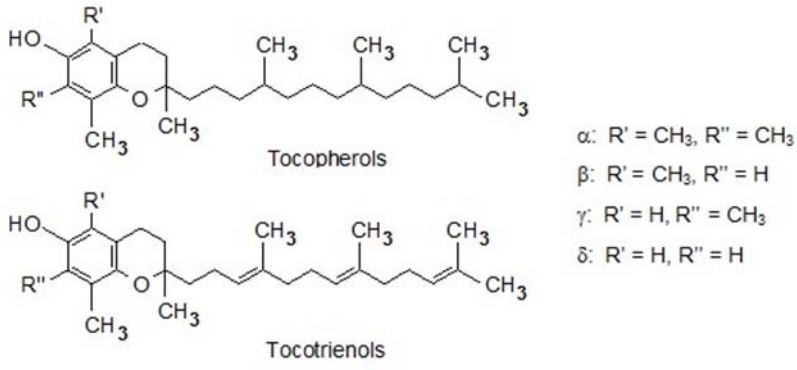

Figure 1. Chemical structure of chromanols (adapted from Lampi, 2011)

Dietary Sources of Vitamin E

The primary dietary vitamin E forms are α- and γ-tocopherol (**Table 1**). α-Tocopherol is the most biologically active form of vitamin E in the body, while other forms of tocopherol and tocotrienol are rapidly metabolized and excreted (Traber and Atkinson, 2007). Primary dietary sources of α-tocopherol are oils and forages; thus, the vitamin E content is often converted into 2*R*-α-tocopherol equivalents. Primary dietary sources of vitamin E are oils, forages, and grains. In the absence of fresh forages, cows can become rapidly vitamin E deplete (McDowell et al., 1996).

Table 1: Vitamin E content of oils, cereal grains and seeds (per kg edible portion) (adapted fromSheppard et al., 1993)

Product	**Tocopherol (mg)**				**Tocotrienol (mg)**	
	α-	**β-**	**γ-**	**δ-**	**α-**	**β-**
Oils						
Canola	210	1	42	0.4	0.4	-
Coconut	5	-	-	6	5	1
Corn	112	50	602	18	-	-
Cottonseed	389	-	387	-	-	-
Olive	119	-	7	-	-	-
Palm	256	-	316	70	146	32
Peanut	130	-	214	21	-	-
Safflower	342	-	71	-	-	-
Sesame	136	-	290	-	-	-
Soyabean	75	15	797	266	2	1
Sunflower	487	-	51	8	-	-
Wheatgerm	1330	710	260	71	26	181
Grains						
Barley	2	0.4	0.3	0.1	11	3
Corn	6	-	45	-	3	-
Oats	5	1	-	-	11	2
Rye	16	4	-	-	15	8
Wheat	10	7	-	-	4	28
Seeds						
Sesame	-	-	227	-	-	-
Sunflower	495	27	-	-	-	-

The vitamin E content in cow feed is highly variable (**Table 2**) and differs depending on plant species, stage of maturity, environmental conditions, time of cutting, time from cutting to dehydration, processing, and storing conditions (Jukola et al., 1996; Tramontano et al., 1993; McDowell et al., 1996). Rapid losses of vitamin E in the diet may occur under oxidative conditions, such as high temperature, moisture, oxygen, iron or other oxidizing salts, rancid fat, and pelleting (McDowell et al., 1996). Vitamin E concentrations in stored forages are lowest in Spring and early Summer shortly before the harvest for the new season, when fresh cows are at greatest risk for metabolic and infectious diseases (Mohebbi-Fani et al., 2012).

Table 2: Vitamin E content of feed in mg/lbs (adapted from Cort et al., 1983; Colak et al., 2006; Muller et al., 2007; Mogensen et al., 2012)

Product	**Tocopherol (mg/lbs)**				**Tocotrienol (mg/lbs)**	
	α-	**β-**	**γ-**	**δ-**	**α-**	**γ -**
Alfalfa dehydrated	16.4-29	-	1.7-15.3	0.0-6.0	-	0-4.2
Alfalfa meal	12.5-38	0.4	1.6-3.3	-	-	-
Animal fat	1.2-9.3	0.1	0.1	3.0-8.3	trace	0-0.7
Barley	3.2-4.4	0.3-0.7	0.8-1.7	Trace	7.9-12.9	1.5
Corn	0.9-6.8	0.2-0.3	9.1-25.0	-	1.8-4.5	2.7-11
Corn gluten meal	2.3-6.6	-	5.4-17.8	0-0.4	5.3-25.5	10.7
Cottonseed meal	0.5-8.3	-	2.2-8.0	-	-	0.4-1.1
Fish meal	0.2-3.7	-	-	-	-	-
Meal and bone meal	Trace	-	-	-	-	-
Oats	2.0-3.6	0.3-0.5	trace	-	2.8-10.0	-
Soybean meal	0.4-1.3	-	1.4-15.2	0.9-2.4	-	Trace
Wheat	2.3-5.5	1.1-2.6	-	-	0.3-1.4	-
Forages						
Alfalfa, fresh	116	-	5	-	-	-
Alfalfa, hay	31	-	2	-	-	-
Fresh grass	53-74	-	0-4	-	-	-
Grass silage	34	-	5.3	-	-	-
Grass hay	24-30	-	1.6-3.5	-	-	-
Fresh grass silage	31.6	-	-	-	-	-
Stored grass silage	29.7	-	-	-	-	-
Fresh corn silage	28.5	-	-	-	-	-
Stored corn silage	12.7	-	-	-	-	-
Fresh grain silage	51.0	-	-	-	-	-
Stored grain silage	28.1	-	-	-	-	-

Vitamin E Requirements in Dairy Cows

The Institute of Medicine (IOM) defined the vitamin E requirement in milligrams of 2*R*-α-tocopherol, with 1 mg *all-rac*-α-tocopherol equal to 0.5 mg of *RRR*-α-tocopherol (adapted from Traber, 2014). One international unit (IU) of *all-rac*-α-tocopherol is equal to 0.5 mg of 2*R*-α-tocopherol, and 1 IU of *RRR*-α-tocopherol or its esters being equal to 0.67 mg 2*R*-α-tocopherol (DRI, 2000). Based on feeding experiments in dairy cows, 1 mg *all-rac*-α-tocopherol acetate is equivalent to 0.5 mg of *RRR*-α-tocopherol acetate (Weiss et al., 2009). Similar results have been reported by Meglia et al. (2006). α-Tocopherol esters usually have a lower availability than natural ATOC, which can be explained by the fact that ATOC esters have to be first hydrolyzed before they can be absorbed (Hidiroglou et al., 1994).

Current NRC recommendations for ATOC in dairy cows are 2.6 IU/kg of body weight, which includes vitamin E from feed stuffs and from supplements (NRC, 2001). One marker to assess vitamin E adequacy is to measure serum/plasma concentrations of ATOC. Vitamin E concentrations below 0.5 μg ATOC/mL are considered deficient (McDowell et al., 1996). Deficiency symptoms, such as lesions of white muscle disease, can be observed at concentrations below 1.5 μg ATOC/mL (McDowell et al., 1996). Concentrations of 1.5 or 2 to 3 μg ATOC/mL (equivalent to 7 μM in serum/plasma) are considered marginal, whereas higher ATOC of at least 3 or 4 μg ATOC/mL are required for adequacy (McDowell et al., 1996; NRC, 2001).

To reach adequate blood values, current NRC recommendations for supplemental α-tocopherol in dairy cows are 1.6 IU/kg BW (approximately 80 IU/kg DMI) during the dry period and 0.8 IU/kg BW (approximately 20 IU/kg DMI) during lactation (NRC, 2001).

The latter recommendations may be insufficient during times of low feed intake or increased vitamin E excretion, such as in the first weeks postpartum. In addition, when vitamin E deficient forages and concentrations are fed (as in Oregon), when forages of poor quality are fed, when harvesting, drying or storage conditions decreased vitamin E, when other feed components require higher vitamin E doses (e.g., PUFA, high nitrates in water), when cows have greater requirements for production, stress, feed efficiency, or disease, or the total mixed ration (TMR) is low in vitamin E, greater amounts of vitamin E supplements are needed (McDowell et al., 1996). In contrast, if fresh forages are fed or cows are on pasture, vitamin E supplementation needs are lower. Feeding extra vitamin E may also increase the proportion of PUFA's in milk of pasture-fed cattle and reduce oxidized flavors. Thus, some experts recommend injectable vitamin E for cows 2 weeks before calving (McDowell et al., 1996; Weiss et al., 1998; Baldi, 2005; Politis, 2012).

Absorption and Transport of Vitamin E

Absorption ofVitamin E

There is limite information about the mechanism of vitamin E absorption in dairy cattle (Baldi et al., 1997; Bontempo et al., 2000). In humans, the absorption efficiency for vitamin E is low (15-45%) and decreases further in the absence of adequate pancreatic function, bile secretion, triacyglycerols (TAG) and cholesterol (Traber, 1999). In lactating cows, the bioavilability for oil-based *all-rac* ATOC is estimated to be 47% (Bontempi et al., 1997). **Figure 2** displays the steps of vitamin E absorption, starting with hydrolysis of the ester bond of lipids, including vitamin E esters, in the rumen and by pancreatic esterase in the duodenum (Hidiroglou and Ivan, 1992; Frank, 2005). As ruminants lack a duodenal oil phase, bile acids, adapted to the lower pH in ruminants by a greater taurine content, act as detergent to emulsify vitamin E from the insoluble particulate phase. Next, vitamin E is then incorporated into mixed micelles containing lipids and bile acids (adapted fromTraber, 2014), which are then transported to the brush border membrane of the enterocytes for uptake.

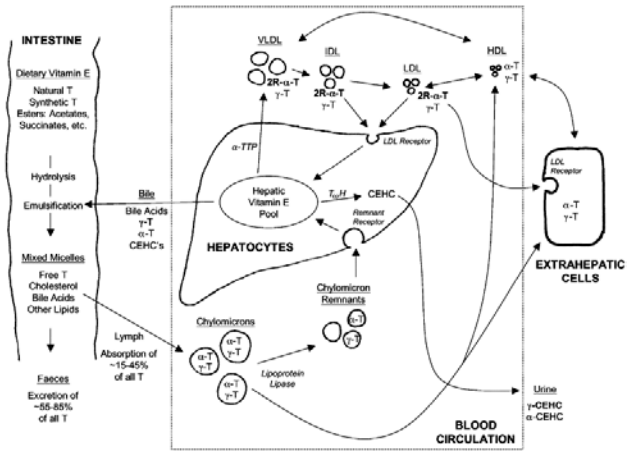

Figure 2. Absorption and transport of tocopherols (adapted from Frank, 2005)

Tocopherol is absorbed in the proximal small intestine (adapted fromTraber, 2014). The mechanism of tocopherol absorption is not completely understood. No intestinal tocopherol transfer proteins have been discovered. Thus, it is assumed that tocopherol is absorbed by passive diffusion. In *in vitro* intestinal model systems (Takada and Suzuki, 2010), the Niemann-Pick C1-Like 1 (NPC1L1) protein facilitates uptake of tocopherol (Takada and Suzuki, 2010) and cholesterol (Narushima et al., 2008) by enterocytes. In addition, the scavenger receptor B-1 (SRB-1) facilitates tocopherol and cholesterol efflux by enterocytes (Reboul et al., 2006, 2012).

Vitamin E in Circulation

After being absorbed by enterocytes, tocopherols are either bound to SRB-1 and packaged into HDL complexes that are secreted into the blood (Anwar et al., 2007) or, in the presence of sufficient TAG and apolipoprotein B-100, tocopherols are packaged into chylomicrons and secreted into the lymph system (Hidiroglou et al., 1994Frank, 2005). In circulation, vitamin E is transported with the lipoprotein fraction; no specific tocopherol transport protein has been identified. This explains why vitamin E concentrations are adjusted for serum lipids, specifically cholesterol concentrations. Lactating dairy cows have lower plasma/serum concentrations of ATOC than humans (10-15 μM versus 20-40 μM). Most of the circulating lipids, including vitamin E, are in the HDL fraction (70-80%) in ruminants. To our knowledge, the half-life of vitamin E in circulation in lactating dairy cows has not been determined. The time until maximum vitamin E concentrations are obtained in blood is 57.5 h in dairy cows (Baldi et al., 1997). In humans, the half-life of *RRR*-α-tocopherol in circulation is between 48 h (Traber et al.,

1994) and 60 h (Bruno et al., 2005). In contrast, *SRR*-α-tocopherol (Traber et al., 1994) and γ-tocopherol (Leonard et al., 2005) have a much shorter half life of 15 h.

In the endothelial capillaries, lipoprotein lipase hydrolyzes lipid esters. Vitamin E is either a) taken up by target tissues through LDL receptors, b) taken up by tissue membranes, c) remains in chylomicron remnants, which are taken up by the liver, or d) is bound to HDL, which readily transfer vitamin E to other lipoproteins utilizing phospholipid transfer protein (PLTP). In lactating dairy cows, chylomicrons have a very short half life of 5 to 11 min, as chylomicrons are quickly transported to the mammary gland for lipid release.

Tissue Distribution ofVitamin E

Our knowledge about transport of vitamin E within tissues is limited. Vitamin E remains in the lipid soluble fraction of the tissue. In liver, to move ATOC through the hydrophilic cytosol, α-tocopherol transfer protein (α-TTP), a 30 to 35 kDa, highly conserved, cytosolic, hydrophobic ligand binding protein is required, which moves ATOC from liposomes to microsomes. The α-TTP is primarily expressed in the liver but also at lower levels in brain, lung, kidney, and spleen. Absence of α-TTP in humans results in vitamin E deficiency and tissue damage (Niki and Traber, 2012). Binding to α-TTP is highly specific to *2R*-α-tocopherol, as α-TTP requires for binding three methyl groups in the chromanol ring, a phytyl tail , and the R- configuration at C-2 where the side chain is being attached to the chromanol ring (Traber and Atkinson, 2007). Other vitamin E forms bind at much lower rates than ATOC to α-TTP, which explains the much lower half-life and biological activity of other vitamin E forms (**Table 1**).

Liver, specifically hepatic parenchymal cells, are the primary storage tissues for vitamin E, while adrenal glands, spleen, kidney, and heart muscle also store vitamin E in rats (Uchida et al., 2012). In contrast to humans, vitamin E storage in bovine adipose tissue is low (**Table 3**). In general, body tissues have limited storage capacity of ATOC. The ATOC exchange between tissues is relatively rapid; specifically between liver, erythrocytes, spleen, and serum/plasma (Hidiroglou et al., 1994). Thus, serum/plasma concentrations provide an acceptable indicator of whole-body ATOC status. Heart, muscle, and spinal cord have a slower ATOC exchange, while brain ATOC exchange is slowest (Hidiroglou and Ivan, 1992).

Table 3: Tissue α-tocopherol concentration (*μg/g fresh tissue*) in Holstein steers after a single gastric dose of 50 IU α-tocopherol/ kg of body weight (Eicher et al., 1997)

Tissues	***RRR*-α-Tocopherol**	***all- rac*-α-Tocopherol acetate**
Initial plasma (*μg/mL*)	3.4	3.3
Final plasma (*μg/mL*)	6.1	4.4
Spleen	17.5	27.0
Liver	30.8	24.0
Adipose	2.5	2.0
Muscle	7.4	7.5
Gut	6.7	4.6
Kidney	24.0	12.3
Heart	14.0	13.2

Transport of α-Tocopherols from Liver and other Tissues

Tocopherols stored in the liver are subsequently transferred to very-low density lipoproteins (VLDLs) to circulate in blood for biological processes. The cytosolic 30-kDa α-tocopherol transfer protein (α-TTP) allows the incorporation of ATOC into nascent VLDLs, which are released from the liver. α-Tocopherol transfer protein belongs to the CRAL-TRIO family of lipid binding proteins (Panagabko et al., 2003), which specifically binds to 2*R*-α-tocopherol. Deletion of the α-TTP gene has been shown to

contribute to vitamin E deficiency in mice (Terasawa et al., 2000; Yokota et al., 2001), indicating that α-TTP is essential for vitamin E transport and tissue distribution. Once ATOC is secreted from the liver, ATOC is either exchanged to other lipoporteins or taken up by tissues via the LDL receptor.

Another important protein involved in ATOC transport from the liver is ATP-binding cassette transporter A1 (ABCA1), an ATP-binding cassette (ABC) transport protein, which facilitates ATOC secretion mediated by α-TTP, when Apo-A1 is the acceptor protein. Besides ATOC, ABCA1 also facilitates transport of cholesterol and phospholipids out of cells into HDL (Reboul et al., 2009).

Metabolism and Excretion ofα-Tocopherols

Metabolism of Vitamin E

Unlike other fat-soluble vitamins, vitamin E does not bioaccumulate to toxic levels in the liver. As a consequence, efficient metabolism and excretion are crucial to maintain vitamin E concentration (Traber, 2007). So far, the only known site of vitamin E metabolism is the liver. The first step of vitamin E metabolism is ω-hydroxylation of the lipid tail and followed by β-oxidation. Hepatic CYP4F2 and CYP3A are involved in the tail shortening reaction, which occurs in the mitochondria (adapted from Traber, 2014). High ATOC concentrations promote the tail-shortening reaction of α-tocopherol as well as γ-tocopherol. The primary endproducts of the tail shortening reaction are α-CEHC (2,5,7,8-tetramethyl-2-(2'-carboxyethyl)-6-hydroxychroman) for α-tocopherol and γ-CEHC (2,7,8-trimethyl-2-(2'-carboxyethyl)-6-hydroxychroman) for γ-tocopherol, which are both found in plasma and urine (Birringer et al., 2002; Lodge et al., 2001).

Excretion of Vitamin E

To increase water solubility, CEHCs are sulfated or, to a smaller extent, glucuronidated (Pope et al., 2002; Swanson et al., 1999) before being excreted into urine or bile (Brigelius-Flohe and Traber, 1999). In the biliary excretion, multidrug resistance gene product (MDR) 1 and 2 are involved (adapted from Traber, 2014). In addition, because of the low absorption of vitamin E from intestines, non-metabolized vitamin E will be excreted in small amounts in feces (Hidiroglou et al., 1990; Hidiroglou and Ivan, 1992; Frank, 2005).

Functions of Vitamin E

Vitamin E as Antioxidant

The most important function of vitamin E is to stop lipid peroxidation. Electron transport in mitochondria or endoplasmic reticulum of eukaryotic cells can result in formation of lipid damaging reactive oxygen species (ROS), which are capable of reacting with other ROS or non-reactive species. The iron-mediated reduction of H_2O_2 by O_2 produces the hydroxyl radical (• OH) (Miller et al., 1993). The hydroxyl radical will react with PUFAs producing lipid radicals. The unstable lipid radical can react with oxygen which is called propagation, producing a lipid peroxyl radical. Lipid peroxyl radicals are also unstable and can convert normal lipids to radicals (**Figure 3**).

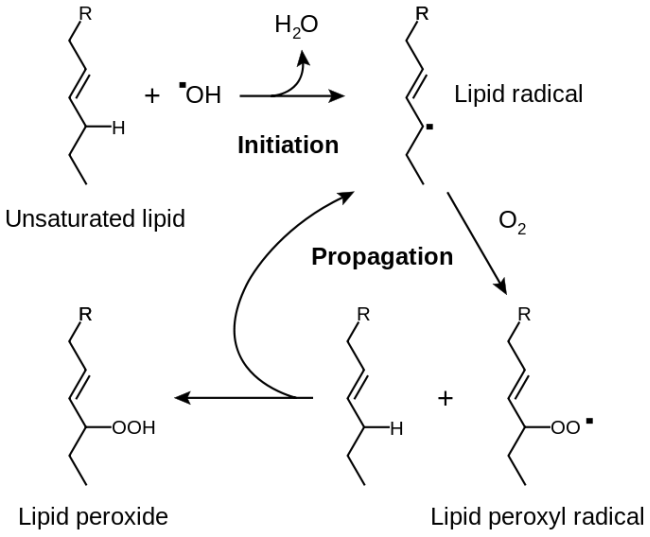

Figure3. Peroxidation of polyunsaturated fatty acids (adapted from Wikimedia.org)

Vitamin E stops the lipid peroxidation chain reaction (Niki and Traber, 2012) by the following mechanism: vitamin E donates the hydrogen from the hydroxyl group of the phenolic ring to the lipid peroxyl radical (LO_2 •) producing a vitamin E radical (E •)

(**Figure4**). In the absence of sufficient ascorbic acid (AA) and glutathione peroxidase (GPX), the vitamin E radical reacts with lipids (LH) or lipid hydroperoxides which propagate the lipid peroxide chain reaction. Alternatively, vitamin E radicals may also react with lipid peroxyl radicals to produce adducts or react with other vitamin E radicals to produce non-reactive dimers. The whole process is called tocopherol-mediated lipid peroxidation which has not been documented to occur *in vivo*.

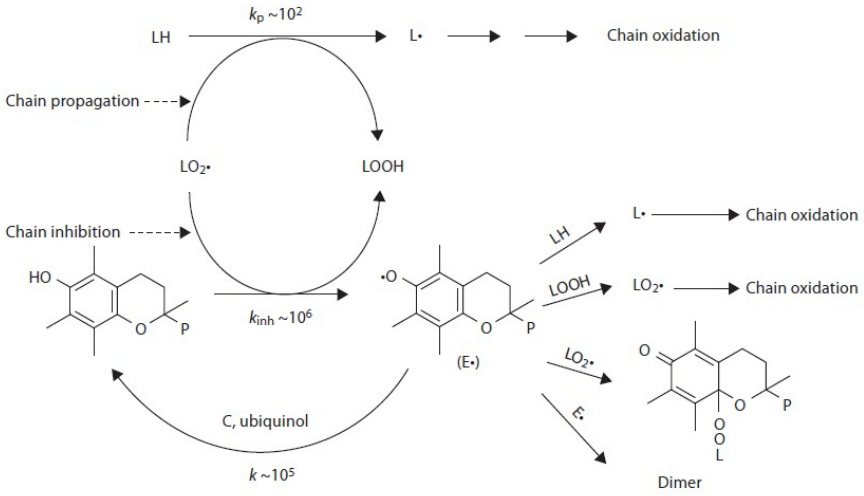

Figure 4. α-Tocopherol acts as a lipid radical scavenging antioxidant (adapted from Niki and Traber, 2012)

In the presence of sufficient AA and GPX, the vitamin E radical gets detoxified by the following mechanism (**Figure 5**): vitamin E donate a hydrogen atom to lipid peroxy radicals and form a tocopheroxyl radical. The tocopheroxyl radical is reduced to tocopherol by AA, is oxidized to dehydroascorbate (DHA). Next, DHA is reduced to AA by dehydroascorbate reductase (DHAR), while converting reduced glutathione (GSH) to glutathione disulfide (GSSG). Ascorbic acid reduces in the presence of ascorbate peroxidase (APX) H_2O_2 to water. Alternatively, H_2O_2 is converted by glutathione peroxidase (GPX) to water, while GSH is converted to GSSG. Finally, glutathione

reductase (GR) reduces oxidized GSSG to reduced GSH in the presence of NADPH.

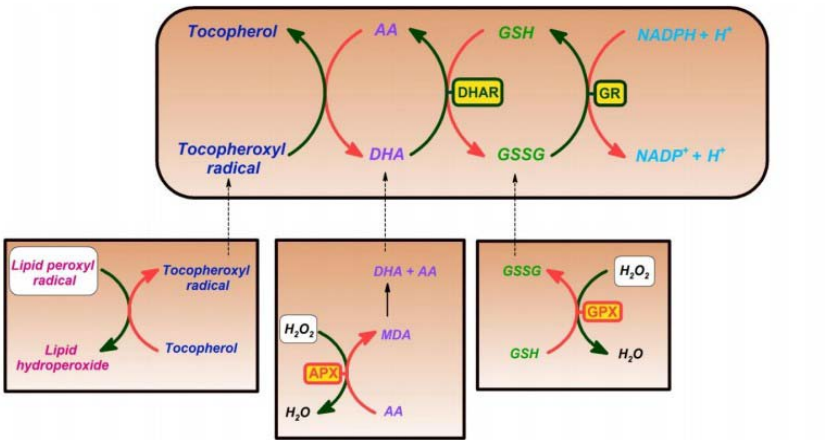

Figure 5. Relationship among tocopherol, ascorbates and glutatahione peroxidase (adapted from Szarka et al., 2012)

Therefore, adequate levels of vitamin E, AA, and GPX are essential to break the lipid peroxidation chain reaction. Why is this important? Poly-unsaturated fatty acids (PUFAs) are important cell membrane components. Metabolites of PUFAs are hormones (for example prostaglandins) and signal molecules that are important to maintain cellular processes. Peroxidation of PUFAs alters these processes and can result in lipoprotein damage (oxidized lipoproteins) as well as reproductive failure.

Vitamin E as Immune-Enhancer

Adequate vitamin E status plays an important role in immune function (Chew, 1995). Cells involved in the immune system are rich in PUFAs and utilize oxygen radicals to combat pathogens (Sanchez Perez et al., 2006). Cows with depleted vitamin E status have a depressed neutrophil function(Willshire and Payne, 2011). There are several potential mechanisms by which depleted vitamin E may indirectly impair immune function: a) increased lipid peroxidation decreases function and survival of immune cells,

b) increased lipid peroxidation induces proinflammatory gene expression, c) increased lipid peroxidation modulates signal transduction, d) low vitamin E modulates cell membrane structure and, thereby, alters cell signaling and cell-to-cell communication (Chew, 1995). One of the pathways most affected by vitamin E is age-dependent changes in CD4+T cells and age-related increased production of prostaglandin E_2, which both can be reversed by vitamin E (Meydani and Wu, 2008; Molano and Meydani, 2012). Currently, it is not clear how vitamin E can reverse the age-related defect in T-cell receptor transduction. However, these results indicate that the health of multiparous cows in particular may be at risk under vitamin E deficiency.

Metabolic Disease in Dairy Cows

Left Displaced Abomasum

Left displaced abomasum (LDA) affects approximately 3.5% of U.S. dairy cows (USDA, 2009). The cost per case, including surgery, milk loss, and mortality, is estimated between U.S. $ 250 and 400 (Bartlett et al., 2006). The cost estimate does not include the costs associated with decreasedbody weight, delayed reproductive performance, and increased culling rates (Østergaard and Gröhn, 1999; Raizman and Santos, 2002). As shown in **Figure 6**, the abomasum, which is the equivalent to our stomach, is shifted below the rumen to the left side. As a result, no freshly predigested food can enter the abomasum. The animal will stop eating and the stool, if any, is hard and has a lipid coating (Mueller, 2011).

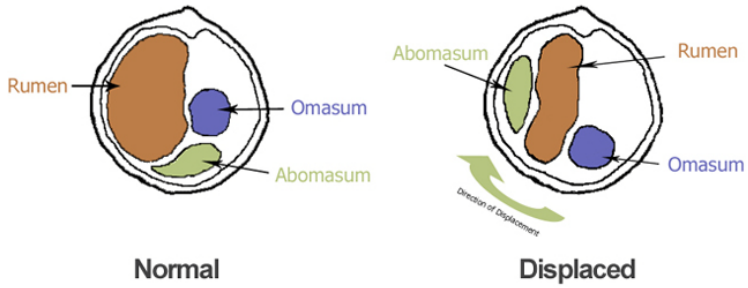

Figure 6. Left Displaced Abomasum (adapted from My dairy vet.com)

Many factors play a role in the etiology of LDA, including genetics, age (>3 yr), early lactation, late winter and early spring, obesity, endotoxemia, rapid weight loss, sudden diet changes, high grain and corn silage, and low NDF rations, low feed intake, rumen fill, and rumen motility, impaired liver function, pregnancy with multiples, and co-morbidities (as reviewed by Geishauser, 1995; Shaver, 1997; Doll et al., 2009). There are two major types of LDA: primary LDA, which occurs throughout lactation in response to feed management errors, and secondary LDA, the more common form, which

occurs in multiparous cows during the first mo of lactation as part of the peripartal disease complex.

The most commondiagnostic indicator of LDA is a “ping” during simultaneous auscultation and percussion of the abdomen in the area marked by a line from the tuber coxae to the point of the elbow, and from the elbow toward the stifle. The ping characteristic of an LDA is detected in an area between ribs 9 and 13 in the middle to upper third of the left abdomen; thought it may be slightly ventral, caudal, or both. Pings associated with rumen gas caps are usually more dorsal, less resonant, and extend more caudally through the left paralumbar fossa (Merck Vet Manual, 2002-2012). The LDA is usually corrected by the “roll and toggle” procedure (Bartlett et al., 2006). To prevent infections, the animal receives s.c. or i.m. antibiotics. In addition, solutions containing dextrose or calcium borogluconate are given orally to improve the energy status of the cow. Some veterinarians also recommend antioxidant alimentation, including vitamin E.

Cows with LDA have 40% lower serum/plasma α- tocopherol concentrations compared to healthy controls (Mudron et al., 1997; Hasanpour et al., 2011; **Table 4**). Prepartal α-tocopherol alimentation decreased numerically LDA incidence in one study (Erskine et al., 1997) but not in another (LeBlanc et al., 2002).

Retained Placenta

Retained placenta (**RP**) is defined as failure to expel fetal membranes within either 12 or 24 h after parturition. Normally, expulsion occurs within 3 to 8 h after calf delivery. Retained placenta affects approximately 7.8% of U.S. dairy cows (USDA 2009). Depending on the study, the incidence rates can vary between 1.3% and 39.2% (Kelton et

al., 1998). The average cost, including treatment costs, milk loss, and increased days open, is estimated to be $285 per case (Kelton et al., 1998). Not included in the cost estimate are that RP cows have increased culling rates and incidences of other metabolic and infectious diseases (Peters and Laven, 1996). The primary risk factors associated with RP are premature births, multiple calves, dystocia (calving problems), abortion, still births, uterine infections, age of cow, milk fever, antioxidant (selenium, vitamin E, and β-carotene) deficiency, and season (reviewed in Laven and Peters, 1996; Drillich, 2011).

The most common diagnostic indicator for RP is degrading, discolored fetal membranes hanging out of the vulva. Manual removal of retained membranes is not recommended because it could result in internal bleeding at the site of tear. The most common treatment is antibiotic flushes with tetracycline to prevent uterine infections. If the uterine infections are not treated successfully, endometritis and infertility are common for cows with RP. In addition, antibiotics are given i.m. to prevent systemic infections. Usually, the retained fetal membrane will fall off after 2 to 14 d after calving.

Vitamin E is usually not given during RP treatment. A meta-analysis documented that vitamin E alimentation during the last month before calving decreases the risk in dairy cows to develop RP by 53% (Bourne et al., 2007). Using data from a large vitamin E supplementation trial in the Guelph area in Canada showed that cows that developed RP had in the last wk postpartum significantly lower serum α-tocopherol concentrations than cows that did not develop RP (LeBlanc et al., 2004) (**Table 4**).

Milk Fever

Milk fever (parturient paresis, **MF**) is an acute to peracute, afebrile, flaccid paralysis of mature dairy cows that occurs within 48 or 72 h of calving (Merck Vet Manual, 2002-2012). Insufficient concentrations of soluble calcium (**Ca**) and magnesium (**Mg**) impair motor, nerve, and immune function in the cows. There are three stages of milk fever: during stage 1, cows are hypersensitive and excitable. During stage 2, cows are unable to stand. During stage 3, cows lose consciousness progressively to the point of coma (Kelton et al., 1998). Another name for MF is clinical hypocalcemia, as low circulating concentrations of calcium is the most common cause of MF. Normal blood calcium concentrations for dairy cows are 8.5 to 10 mg Ca/dL (Goff, 2008). Subclinical hypocalcemia is usually defined as 5.5 to 8 mg Ca/dL, where the lower threshold varies among studies between 5.5 and 6 mg Ca/dL (Goff, 2008; DeGaris and Lean, 2008; Reinhard et al., 2011). Blood concentrations between 6 and 8 mg Ca/dL can occur also in response to infections and, thus, are not a good indicator of hypocalcemia and MF (Goff, 2008). Clinical hypocalcemia or milk fever can be assumed if blood concentrations are below 6 mg/dL within 48 h of calving and cows show lack of appetite.

Clinical MF affects approximately 4.9% of US dairy cows (USDA, 2009). The estimates range between 0.03% and 22.3% (Kelton et al., 1998). The average cost, including treatment costs, milk loss, and increased days open, is estimated to be $335 per case (Kelton et al., 1998). Not included in the cost estimate are that decreased muscle and immune function in MF cows increases incidence rates of other metabolic (RP, LDA, ketosis) and infectious (metritis and mastits) diseases as well as cow mortality from

downer cown syndrome (Curtis et al., 1983). The primary risk factors associated with MF are diet, parity (up to 10% in older cows), obesity, and season (Goff, 2008).

Efforts to decrease MF incidences have shifted from treatment to prevention. For prevention, the late dry cow diet has a low cation-anion difference and is low in calcium to stimulate calcium mobilization. The problem with a low cation-anion diet, however, is that such a diet decreases feed intake. Another method for MF prevention is providing prophylactically oral calcium treatments, usually calcium priopionate or calcium borogluconate, in the first days after calving to all cows with 3 or more parities. The additional benefit of oral prophylactic treatment is that it promotes feed intake, which is suppressed with subclinical hypocalcemia. Treatment of MF is directed toward restoring normal serum calcium levels as early as possible to avoid muscle and nerve damage and recumbency. If the cow can still stand, cows receive i.v. a calcium-magnesium-phosphorus-potassium-dextrose solution. If the cow cannot stand, cows receive i.v. a Ca salt (commonly Ca borogluconate or possibly containing Mg), administered between 8.5-11.5g Ca/ 500 mL (Goff, 2008). The most effective dose is around 2 g Ca/ 100 kg BW (Goff, 2008). A significant proportion of recumbent MF cows (4.5%) progress to alert downer cow syndrome, which have a mortality rate of 20 to 67% (Ménard and Thompson, 2007).

The role of vitamin E in the etiology of MF is not known. Prepartal ATOC alimentation did not affect incidence of MF (Erskine et al., 1997; LeBlanc et al., 2002). Furthermore, Goff et al. (1990) reported for Jersey cows and Sivertsen et al. (2005) for Norwegian dairy cows reported that cows with or without MF had similar plasma ATOC concentrations (**Table 4**).

Effects of Periparturient Diseases on Serumα-Tocopherol Concentrations

The effects of common periparturient diseases on plasma/serum ATOCconcentrations of dairy cows are shown in **Table 4**. Most diseases did not consistently affect plasma/serum ATOC concentrations of dairy cows or had only a small effect, as indicated by <20% lower ATOC concentrations in diseased cows. The exceptions were fat cow syndrome (Hidroglou and Hartin, 1982) and LDA (Mudron et al., 1997; Hasanpour et al., 2011), which were associated with over 40% decreased plasma or serumATOC concentrations. The ATOC concentrations were below 7.0 µM in cows with fat cow syndrome and left displaced abomasum, which indicate deficient absorption and/or transport of ATOC.

Table 4. Effect of diseases on plasma/serumα-tocopherol concentrations of dairy cows

Disease	**Effect**	**Reference**
Left displaced abomasums	- (-47%)[3]	Mudron et al. (1997)
	- (-42%)[3]	Hassanpour et al. (2011)
Retained placenta	0[2]	Brezezinska-Slebodzinska et al. (1994)
	-[3]	LeBlanc et al.(2004; small effect 1 wk prepartum)
Milk fever	0[2]	Goff and Stabel (1990); Sivertsen et al. (2005)
	-[3]	LeBlanc et al. (2004; small effect)
Ketosis	0[2]	Sivertsen et al. (2005)
Mild fatty liver	0[2]	Rosendo et al. (2010)
Fat cow syndrome	- (-50% at calving)[3]	Hidiroglou and Hartin (1982)
Laminitis	0[2]	Kilic et al. (2007)
Metritis	- (-31%)[3]	Kizil et al. (2010)
Clinical Mastitis	- (-19%)[3]	Atroshi et al. (1987; small effect)
	- (-13%)[3]	Erskine et al. (1987; small effect)
	0[2]	Weiss et al. (1990); Nizamlioglu et al. (1993); LeBlanc et al. (2004; also no predisease effect); Sivertsen et al. (2005); Rezamand et al. (2007; small postdisease effect)
Subclinical Mastitis/SCC	0[2]	Weiss et al. (1990); Jukola et al., (1996b)
SCC	-[3]	Nyman et al.(2008; lower around calving)

1: increase; 2: no changes; 3: decrease; in parenthesis relative changes of plasma/serumα-tocopherol concentrations

Potential Predictors and Indicators for Metabolic Diseases in Transition Dairy Cows

Predictors and Indicators for Left Displaced Abomasum

As shown in **Table 5**, the most consistent predictors of LDA are NEFA concentrations pre- and postpartum and AST activity and BHBA concentrations postpartum. Those biomarkers, however, lack specificity, because they are associated with many diseases in the early postpartal period. Elevated lipid mobilization during late gestation suggests that cows are already in late gestation in a negative energy balance and/or experience inflammation. The elevated BHBA concentrations around calving suggest that the liver function for gluconeogenesis is inadequate and increased AST activity suggest tissue damage prior to LDA. Low calcium concentrations or insulin or urea concentrations are not consistently associated with LDA (**Table 5**).

Table 5. Predictive biomarkers for left displaced abomasum in dairy cows

Blood Parameter	**Effect**	**Reference**
NEFA	+[1]	Cameron et al. (1998); LeBlanc et al. (2005); Chapinal et al. (2011) all pre-and postpartum
	+/0	Van Winden et al., (2003; postpartum)
	0[2]	Seifi et al. (2011; 1st wk postpartum)
BHBA	+[1]	Geishauser et al. (1997, 1998); Van Winden et al. (2003); Seifi et al. (2011) all postpartum
	+[1]	LeBlanc et al. (2005); Chapinal et al. (2011) all pre-and postpartum
	+[1]	Ospina et al.(2010; only postpartum)
AST	+[1]	Geishauser et al.(1997, 1998); Van Winden et al. (2003) all postpartum
Glucose	-[3]	Geishauser et al. (1998; 2nd wk postpartum)
	-/0	Van Winden et al. (2003; postpartum)
	0[2]	Seifi et al. (2011; 1st wk postpartum)
Insulin	-[3]	Van Winden et al. (2003; postpartum)
Urea	0[2]	Geishauser et al. (1998; postpartum)
Calcium	0[2]	LeBlanc et al. (2005; pre- and postpartum)
	-[3]	Curtis et al. (1983;); Massey et al. (1993; at calving)
	-[3]	Geishauser et al. (1998; 2nd wk postpartum)
	-[3]	Van Winden et al. (2003; postpartum)
	-[3]	Chapinal et al. (2011;); Seifi et al. (2011; 1st wk postpartum)

1: increase; 2: no changes; 3: decrease

As shown in **Table 6**, the most consistent indicators of LDA are elevated concentrations of NEFA, BHBA, bilirubin, enzymes (AST, CK, GGT, GLDH), and markers of inflammation (haptoglobin, serum amyloid A), indicating tissue damage. Concentrations of cholesterol and macrominerals are usually decreased. Glucose concentrations are increased, decreased, or not altered by LDA. The inconsistent effect on glucose concentrations can be explained by the fact that many cows have been treated prior with glucose therapy or have been transported to the clinic for surgery, which increased cortisol release. To our knowledge, the long-term effect of LDA on concentrations of blood parameters has not been examined.

Table 6.Indicators for left displaced abomasum in dairy cows

Blood Parameter	Effect	Reference
Haptoglobin	+[1]	Hirvonen and Pyorala (1998); Guzelbektes et al (2010); Stengärde et al. (2010)
Serum Amyloid A	+[1]	Guzelbektes et al. (2010)
AST, GGT	+[1]	Itoh et al. (1998); Zadnik et al. (2003; also GLDH)
	+[1]	Wittek et al. (2004; GLDH, CK instead of GGT)
	+[1]	Stengärde et al. (2010; GLDH instead of GGT)
	+/0	Guzelbektes et al. (2010; GGT only significant)
NEFA, BHBA	+[1]	Muylle et al. (1990; only in cows with FL)
	+[1]	Itoh et al. (1998); Zadnik et al. (2003); Guzelbektes et al. (2010); Stengärde et al. (2010);
	+[1]	Wittek et al. (2004; only BHBA)
Bilirubin	+[1]	Zadnik et al. (2003); Wittek et al. (2004); Guzelbektes et al. (2010); Stengärde et al. (2010)
Cholesterol	-[3]	Itoh et al. (1998); Wittek et al. (2004); Guzelbektes et al. (2010); Stengärde et al. (2010)
	0[2]	Zadnik et al. (2003)
Glucose	+[1]	Vanmeirhaeghe et al. (1988); Zadnik et al. (2003)
	+/0	Guzelbektes et al. (2010)
	0[2]	Muylle et al. (1990); Itoh et al. (1998); Stengärde et al. (2010)
Lactate	+[1]	Wittek et al. (2004)
Ca, Na, K, Cl	-[3]	Zadnik et al. (2003)
Ca, Mg, P, K	-[3]	Kalaitzakis et al.(2011)
P	-[3]	Grunberg et al.(2005)
Urea, Creatinine	0[2]	Guzelbektes et al. (2010)
Urea	+[1]	Wittek et al. (2004)
Protein	-[3]	Wittek et al. (2004)
Protein, Albumin	0[2]	Hirvonen and Pyorala (1998); Guzelbektes et al. (2010)

1: increase; 2: no changes; 3: decrease

Predictors and Indicators for Retained Placenta

Potential predictive biomarkers for RP are shown in **Table 7**. None of the blood parameters consistently differed between cows with or without RP. Even NEFA concentrations were only elevated in 6 of 10 studies, while serum cholesterol concentrations were decreased in 4 of 6 studies.

Table 7. Predictive biomarkers for retained placenta in dairy cows

Blood Parameter	**Effect**	**Reference**
NEFA	0[2]	Chassagne et al. (1998);Kaneene et al. (1997); Seifi et al. (2007); Quiroz-Rocha et al. (2009)
	+[1]	Zhang et al. (2012); LeBlanc et al. (2004); Koenyves et al. (2009); Ospina et al. (2010); Chapinal et al. (2011); Moyes et al. (2013)
BHBA	0[2]	Chassagne et al. (1998);Seifi et al. (2007); Quiroz-Rocha et al. (2009); Ospina et al. (2010); Chapinal et al. (2011); Moyes et al. (2013)
Cholesterol	-[3]	Kudlac et al. (1995); Kaneene et al. (1997); Zhang et al. (2002); Quiroz-Rocha et al. (2009)
	0[2]	Seifi et al. (2007); Konyves et al. (2009)
Triglyceride	-[3]	Seifi et al. (2007)
Glucose	+[1]	Chassagne et al. (1998)
	0[2]	Konyves et al. (2009); Quiroz-Rocha et al. (2009); Moyes et al. (2013)
	-[3]	Kudlac et al. (1995); Zhang et al. (2002)
Urea	0[2]	Chassagne et al. (1998); Quiroz-Rocha et al. (2009); Seifi et al. (2007)
	+[1]	Kudlac et al. (1995)
Albumin	+/0	Seifi et al. (2007)
	0[2]	Chassagne et al. (1998)
AST	+[1]	Kudlac et al. (1995)

	0[2]	Konyves et al., (2009)
GGT	0[2]	Kudlac et al. (1995); Chassagne et al. (1998)
Creatinine	+[1]	Kudlac et al. (1995; also LDH, bilirubin; acid phosphatase)
Total Protein	0[2]	Kudlac et al. (1995; also AlP); Zhang et al. (2002)
Mg	0[2]	Kudlac et al. (1995); Chassagne et al. (1998)
Calcium	-[3]	Chassagne and Chacornac (1994); Zhang et al. (2002)
	0[2]	Kudlac et al. (1995); Quiroz-Rocha et al. (2009)

1: increase; 2: no changes; 3: decrease

After calving, cows with RP have elevated concentrations of haptoglobin and decreased concentrations of calcium (**Table 8**). Other blood parameters are not consistently different in cows with or without RP. To our knowledge, the long-term effect of RP on concentrations of blood parameters has not been examined.

Table 8.Indicators for retained placenta in dairy cows

Blood Parameter	**Effect**	**Reference**
Haptoglobin	+[1]	Skinner et al. (1991); Crawford et al. (2005); Mordak (2009)
NEFA	0[2]	Kaneene et al. (1997); Melendez et al.(2004); Kaczmarowski and Malinowski.(2005); Ospina et al. (2010)
	+/0	Seifi et al. (2007)
	+[1]	Civelek et al.(2011)
BHBA	0[2]	Melendez et al. (2004); Kaczmarowski and Malinowski.(2005); Ospina et al. (2010)
	+[1]	Seifi et al. (2007); Civelek et al. (2011)
Cholesterol	0[2]	Kaneene et al. (1997); Kaczmarowski and Malinowski.(2005); Civelek et al. (2011)
	-/0	Seifi et al. (2007)
	-[3]	Semacan and Sevinc(2005)
HDL Chol.	-[3]	Semacan and Sevinc (2005); Civelek et al. (2011)

LDL/VLDL Chol	0[2]	Civelek et al. (2011)
LDL Chol	-[3]	Semacan and Sevinc (2005)
Triglyceride	0[2]	Seifi et al. (2007); Civelek et al. (2011)
	-[3]	Semacan and Sevinc (2005)
Bilirubin	0[2]	Semacan and Sevinc (2005)
AST, GGT	+[1]	Semacan and Sevinc (2005)
CPK	0[2]	Semacan and Sevinc (2005)
Glucose	0[2]	Melendez et al. (2004); Kaczmarowski et al. (2005); Civelek et al. (2011)
	-[3]	Semacan and Sevinc (2005)
Mg, P	0[2]	Melendez et al. (2004)
P	-[3]	Semacan and Sevinc(2005)
Ca	-[3]	Semacan and Sevinc(2005); Melendez et al. (2004); Seifi et al. (2007)
Urea,Creatinine	0[2]	Semacan and Sevinc(2005)
Urea	-[3]	Seifi et al. (2007)
Protein	0[2]	Semacan and Sevinc(2003; also globulin)
	-[3]	Civelek et al. (2011)
Albumin	0[2]	Civelek et al. (2011)
	-[3]	Semacan and Sevinc (2005); Seifi et al. (2007)

1: increase; 2: no changes; 3: decrease

Predictors and Indicators for Milk Fever

The information about the association between clinical MF and prepartal concentrations of blood parameters is limited: NEFA, glucose, and urine pH were increased and phosphorus and zinc were decreased in the last wk before calving (Seifi et al., 2003; Moyes et al., 2013) (**Table 10**). Cows with clinical MF had elevated concentrations of NEFA and decreased concentrations of calcium, phosphorus, cholesterol, and phospholipids within the first 3 days after calving (**Table 10**). The long-term effect of clinical MF on concentrations of blood parameters has not been examined.

Table 9. Predictive biomarkers of clinical milk fever in dairy cows

Blood Parameter	Effect	Reference
NEFA	+[1]	Moyes et al. (2013; wk -1 prepartum)
BHBA	0[2]	Moyes et al. (2013; wk -1 prepartum)
Glucose	+[1]	Moyes et al. (2013; wk -1 prepartum)
Calcium	0[2]	Goff et al. (1990; <5 mg/dL; recumbent) prepartum
	-[3]	Seifi et al. (2003; recumbent; d -2/-1 prepartum)
Phosphorus	-[3]	Seifi et al. (2003; recumbent; d -2/-1 prepartum)
Zinc	-[3]	Goff et al. (1990; <5 mg/dL; recumbent) prepartum
Urine PH	+[1]	Seifi et al. (2003; recumbent; d -2/-1 prepartum)

1: increase; 2: no changes; 3: decrease

Table 10. Indicators of clinical milk fever in dairy cows

Blood Parameter	Effect	Reference
NEFA	+[1]	Oikawa and Katoh (2002<6.6 mg/dLl; recumbent)
	+[1]	Melendez et al.(2009; recumbent within 72 h)
BHBA	0[2]	Oikawa and Katoh (2002; <6.6 mg/dL; recumbent)
Haptoglobin	0[2]	Skinner et al. (1991)
	+[1]	Crawford et al. (2005)
Cholesterol	-[3]	Oikawa and Katoh (2002; <6.6 mg/dL; recumbent)
Phospholipids	-[3]	Oikawa and Katoh (2002; <6.6 mg/dL; recumbent)
AST, CPK	0[2]	Oikawa and Katoh (2002; <6.6 mg/dL; recumbent)
Bilirubin	0[2]	Oikawa and Katoh (2002; <6.6 mg/dL; recumbent)
Calcium	-[3]	Oikawa and Katoh (2002; <6.6 mg/dL; recumbent); Starič and Zadnik(2010 ;<6.6 mg/dL recumbent); Liesegang et a. (1998; <6.6 mg/dL recumbent); Goff et al. (1990; <5 mg/dL; recumbent)
Phosphorus	-[3]	Liesegang et a. (1998; recumbent); Oikawa and Katoh. (2002; <6.6 mg/dL; recumbent); Starič and Zadnik (2010; recumbent)
AlP	0[2]	Starič and Zadnik (2010; recumbent)
Mg	0[2]	Liesegang et a. (1998; recumbent); Starič and Zadnik (2010; recumbent)

1: increase; 2: no changes; 3: decrease

Summary

Chapter 1 provided a short overview what is currently known about vitamin E and the relationship between vitamin E and three economically important metabolic diseases, LDA, RP, and MF, in early lactation dairy cows. Chapter 1 provided evidence that dairy cows under the current production systems have a low vitamin E status in the first weeks postpartum, which may negatively affect metabolic and immune function. The literature about the relationship between vitamin E and LDA, RP, and MF is limited and warrants further studies. In addition, currently used blood biomarkers, NEFA, BHBA, calcium, and haptoglobin, are limited in their success to predict which cow will or will not become sick. Last, to our knowledge, the long-term effect of LDA, RP, and MF on concentrations of blood parameters have not been examined.

The focus of the following chapter 2 to 4 is to evaluate the association between three metabolic diseases, LDA, RP, and MF, in early lactation dairy cows and their serum concentrations of vitamin E, metabolites, acute phase proteins, and minerals between 3 weeks before and 7 weeks after calving. The hypothesis is that low serum ATOC concentrations precede LDA, RP, and MF and, thus, could be used as a prognostic indicator for LDA, RP, and MF. Furthermore, the hypothesis is that low serum ATOC concentrations persist for weeks after clinical disease signs have subsided and the cow appear healthy.

CHAPTER 2 DEPLETED SERUM VITAMIN E CONCENTRATIONS PRECEDE LEFT DISPLACED ABOMASUM IN EARLY LACTATION DAIRY COWS

INTERPRETIVE SUMMARY: **Depleted serum vitamin E concentrations precede left displaced abomasum in early lactation dairy cows.** By *Qu et al.* To determine the association between vitamin E status and left displaced abomasum, serum vitamin E concentrations were measured between -3 and 7 weeks postpartum in multiparous cows that developed left displaced abomasum in early lactation and compared to those in healthy cows. Lower vitamin E concentrations preceded and persisted after left displaced abomasum, indicating lower serum α-tocopherol concentrations as a potential early indicator for developing left displaced abomasum.

DEPLETED SERUM VITAMIN E CONCENTRATIONS PRECEDE LEFT DISPLACED ABOMASUM IN EARLY LACTATION DAIRY COWS

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ABSTRACT

Cows with left displaced abomasum (LDA), a costly disease occurring primarily in multiparous dairy cows during early lactation, have been reported to have 40% lower circulating concentrations of vitamin E. It is unknown, however, whether the lower circulating α-tocopherol concentrations precede LDA or remain after LDA. Using a nested case-control design, blood samples taken at day -21, -14, -7, -3, -1, 0, 1, 3, 7, 14, 21, 28, 35, 42, and 49 postpartum from 7 multiparous Holstein cows diagnosed with LDA between day 6 and 32 postpartum and 10 healthy Holstein cows from the same herd were analyzed for serum concentrations of α-tocopherol and indicators of energy and nutrient status and inflammation. In addition to indicators of negative energy balance and inflammation, lower serum α-tocopherol concentrations preceded LDA and persisted after LDA correction. At the last blood sampling before LDA diagnosis, cows had serum α-tocopherol concentrations 45% lower (5.0 ± 0.9 vs. 9.1 ± 0.9 μM; $P = 0.004$) and α-tocopherol to cholesterol molar ratios 39% lower (1.90 ± 0.19 vs. 3.09 ± 0.26; $P = 0.003$) than those of healthy cows. Serum α-tocopherol concentrations remained lower (<10 vs. ~15 μM) up to day 49 postpartum in cows that had LDA (all $P < 0.03$). These findings indicate that lower serum α-tocopherol concentrations are a potential early indicator for the development of LDA in multiparous cows.

Key Words: dairy cow; early lactation; left displaced abomasum; vitamin E.

INTRODUCTION

Left displaced abomasum (**LDA**) is an economically important disease that affects approximately 3.5% of US dairy cows (USDA, 2009). The cost per case, including surgery, milk loss, and mortality, is estimated between U.S. $ 250 and 400 (Bartlett et al., 1995) and does not include costs associated with decreased BW, delayed reproductive performance, and increased culling rates (Østergaard and Gröhn, 1999; Raizman and Santos, 2002). Many factors play a role in the etiology of LDA, including genetics, age (>3 yr), early lactation, late winter and early spring, obesity, endotoxemia, rapid weight loss, sudden diet changes, high grain and corn silage, and low NDF rations, low feed intake, rumen fill, and rumen motility, impaired liver function, pregnancy with multiples, and co-morbidities (as reviewed by Geishauser, 1995; Shaver, 1997; Doll et al., 2009). There are two major types of LDA: primary LDA, which occurs throughout lactation in response to feed management errors, and secondary LDA, which occurs in multiparous cows during the first mo of lactation as part of the peripartal disease complex. This study focuses on the latter type.

α-Tocopherol, the most abundant and biologically active form of vitamin E, is a lipid soluble antioxidant that prevents PUFA oxidation (as reviewed by Baldi, 2005; Traber and Atkinson, 2007; Politis, 2012). Current NRC recommendations for supplemental α-tocopherol in dairy cows are 1.6 IU/kg BW (approximately 80 IU/kg DMI) during the dry period and 0.8 IU/kg BW (approximately 20 IU/kg DMI) during lactation (NRC, 2001). Dietary vitamin E requirements are elevated in early lactation because lipid peroxidation is increased (Castillo et al., 2006; Sordillo and Aitken, 2009) and significant amounts of vitamin E are secreted in the colostrum (Weiss et al., 2009). Depressed feed intake,

inflammation, and low lipid absorption and transport may decrease dietary vitamin E utilization (Baldi, 2005).

It has been previously reported that cows with LDA have 40% lower circulating α-tocopherol concentrations than control cows (Mudron et al., 1997; Hasanpour et al., 2011). We hypothesized that depleted α-tocopherol concentrations precede LDA and remain after LDA correction. Thus, the objective of this study was to determine serum α-tocopherol concentrations of multiparous dairy cows with secondary LDA during the first mo of lactation and without disease between -3 to 7 wk postpartum.

MATERIALS AND METHODS

Animals and Study Design

All procedures involving animals were approved by the Oregon State University Institutional Animal Care and Use committee. The research was conducted on a 1,000-head commercial dairy farm in Oregon's Central Willamette Valley during Spring and Summer 2010. The cohort consisted of 161 multiparous Holstein cows (parity 2 to 7). Seven cows (2 or 3 parities) that were diagnosed with LDA (identified at d 6, 7, 13, 13, 17, 22, and 32, respectively) and 10 control cows (not treated for diseases during the sampling period) that were similar in parity, calving month, and age, were selected for this nested case-control study.

During the last 4 wk before expected calving, cows were housed in a straw-bedded free stall barn and were fed once in the morning (7:30) a TMR based on corn, corn silage, and alfalfa and triticale hay, which met NRC guidelines (NRC, 2001) and contained supplemental vitamin E at 167 IU/kg DM (**Table 1**). After calving, healthy cows stayed the first 2 d in the hospital pen, and then for 4 wk in the early lactation pen, and then based on body size in 3 mid-lactation pens. Cows diagnosed with LDA were moved back to the hospital pen for treatment. Cows from the hospital, early lactation, and the mid-lactation pen were fed at 7:00, 9:00, 10:00, respectively, and 13:30 for all cows, a TMR based on corn, corn silage, and alfalfa hay, which met NRC guidelines (NRC, 2001) and contained supplemental vitamin E at 24.5 IU/kg DM (**Table 1**).

Starting 28 d before predicted calving date, BCS of cows were scored weekly until 4 wk postpartum and then at wk 7 and 14 postpartum (Edmonson et al., 1989). During the study period, cows were monitored daily for flakes in the milk, gait, appetite, general

appearance, alertness, vaginal discharge, and retained placenta. Uterine discharge was checked twice a week, and urinary ketones and body temperature were checked if cow appeared not healthy. Medical treatments were administered based on the standard operating procedures of the dairy farm, which was for LDA: after LDA diagnosis by the herd manager, cows were moved to an isolation pen and the herd manager performed the "roll and toggle" procedure (Bartlett et al., 1995). After LDA correction, cows were moved to the hospital pen, received i.v. 0.5 L of dextrose (50% dextrose; Aspen Veterinary Resources® Ltd, Liberty, MO) and 2 capsules of the fiber, electrolyte, and vitamin A supplement Pecti-cap (Bio-Vet, Inc., Blue Mounds, WI), and were then injected i.m. with 20 mL of vitamin B complex (Aspen Veterinary Resources® Ltd). To prevent infections, cows were injected i.m. for up to 7 d with penicillin (40 mL/d, Penicillin G Procaine; Aspen Veterinary Resources® Ltd), followed by treatment for up to 7 d with sulfadimethoxine (30 g/d, Sulfasol soluble powder; Med-Pharmex®, Pomona, CA).

Blood Collection and Analysis

Blood samples were taken at d -21 (-24 to -18), -14 (-17 to -11), -7 (-10 to -5), -3 (-4 or -3), -1 (-2 or -1), 0, 1, 3, 7, 14, 21, 28, 35, 42, and 49 postpartum within 10 min after morning feeding. Blood (5 to 8 mL) was obtained from the coccygeal vein or artery in 10 mL serum vacutainer tubes (BD Vacutainer® Plus Plastic Serum Tubes, BD Diagnostics, Franklin Lakes, NJ), placed on ice, and transported to the laboratory, where serum was separated by centrifugation at room temperature for 20 min at 1600 x *g*. Serum samples were stored at -20°C until chemical analysis.

Serum α-tocopherol concentrations were measured using a reversed-phase Phenomenex Synergi 4 µM Hydro-RP, 150×4.6 mm column and a SecurityGuard™ cartridges AQ C18 pre-column, 3.0 mm i.d. (Phenomenex, Torrance, CA) with a LC-4B amperometric electrochemical detector (Bioanalytical Systems Inc., West Lafayette, IN), following Podda et al. (1996). An isocratic mobile phase of 99:1 (v:v) methanol:water containing 0.1% (w:v) lithium perchlorate was used with a run time of 9 min and the electrochemical detector set at 500 mV. After 100 µL serum were saponified in alcoholic KOH with 1% ascorbic acid, the sample was extracted with hexane and dried, and the residue resuspended in ethanol:methanol (1:1). A 20-µL aliquot of the extract was injected into the HPLC system. Serum concentrations of cholesterol (Stanbio Cholesterol LiquiColor® Procedure No. 1010; Stanbio Laboratory, Boerne, TX), glucose (Stanbio Glucose Proc. No. 1075; Stanbio), NEFA (ACS ACOD method, WAKO Diagnostics, Richmond, VA), BHBA (Stanbio BHBA LiquiColor® Proc. No. 2440; Stanbio), urea N (Stanbio Urea Nitrogen Liqui-UV® Proc. No. 2020; Stanbio), haptoglobin (bovine-specific ELISA kit Catalog No. 2410-70; Life Diagnostics, Inc., West Chester, PA), serum amyloid A (**SAA**; multispecies ELISA kit Catalog No. KAA0021; Life Technologies, Grand Island, NY), calcium (Stanbio Total Calcium LiquiColor® Proc. No. 0150; Stanbio), magnesium (Stanbio Magnesium LiquiColor® Proc. No. 0130; Stanbio), and phosphorus (Stanbio Phosphorus Liqui-UV® Proc. No. 0830; Stanbio) were measured according to manufacturer's instructions using a FLUOstar Omega microplate autoreader (BMG Labtech Inc, San Francisco, CA).

Statistical Analysis

Data was analyzed as repeated-measures-in-time ANOVA study using the PROC MIXED procedure of SAS version 9.2 (SAS Institute, 2009). The molar ratio of α-tocopherol to cholesterol was calculated to adjust for changes in lipid transport (Traber and Jialal, 2000) and stage of lactation (Weiss, 1998). To achieve a normal distribution for their serum concentrations, concentrations of glucose, NEFA, SAA, and the α-tocopherol to cholesterol molar ratio were ln-transformed, concentrations of haptoglobin and BHBA were twice ln-transformed, concentrations of cholesterol were square-root transformed, and concentrations of phosphorus >11 mg/dL (6 samples) were set at 11 mg/dL. The variance-covariance structure of repeated measures within cow was modeled using the heterogeneous first-order autoregressive variance-covariance matrix. Fixed effects were LDA incidence (cases, control), parity (2, >2), sampling time, and the interaction between LDA incidence and sampling time. To obtain the correct degrees of freedom, the KENWARDROGER option was invoked.

To compare our results with previously published studies and identify early indicators of LDA, average serum concentrations in the last wk before calving and the first three d postpartum were calculated using the trapezoidal rule and analyzed in PROC GLM with LDA status and parity as fixed effects. In addition, we compared the results of the last blood sample before LDA diagnosis with those of control cows at d 7 postpartum in PROC GLM with LDA status and parity as fixed effects. Day 7 postpartum was chosen for comparison because most serum indicators reached at that time point their most extreme value. Potential cut-off values for detecting LDA in wk -1 or 1 postpartum were determined using Fisher's exact test. For wk 1 postpartum, we excluded samples taken at

d 0, 1, or after LDA diagnosis. Values presented in the figures and tables are least-squares means (**LSM**) and their standard errors (**SEM**) that are transformed back to their original measurement scale. All statistical tests were two-sided. Significance was declared at $P \leq 0.05$ and a tendency at 0.05 to 0.10.

RESULTS

The incidence rate of LDA in this study cohort was 4.3% (7 of 161 cows). Cows with LDA were either in their second or third parity. Left displaced abomasum was corrected at postpartum d 6, 7, 13, 13, 17, 22, and 32, respectively, using the roll and toggle method, which did not have to be repeated for any cow. Each cow had morbidities before LDA diagnosis (5 cows ketosis, 4 cows metritis, 2 cows milkfever, and 1 cow each retained placenta after twins, mastitis, or laminitis). Most cows had morbidities after LDA correction (until d 49 postpartum: four cows ketosis, one cows mastitis, and one cow died from an intestinal ulcer 2 d after LDA diagnosis at d 34 postpartum). The 10 control cows did not show signs of clinical diseases during the sampling period and had relatively normal serum concentrations of BHBA (range: 0.28 to 1.19 mM; <1.2 mM as cut off between healthy and subclinical ketosis; McArt et al., 2012), calcium (range: 6.9 to 11.8 mg/dL; >6 mg/dL as cut off between clinical milkfever and subclinical milk fever or inflammation-associated hypocalcemia; Goff, 2008; Reinhardt et al., 2011), and magnesium (range: 1.33 to 3.38 mg/dL; >1.15 mg/dL as cut off between hypomagnesemia and subclinical tetany; Goff, 2008). Before calving, all 17 cows were visually healthy.

Serum Vitamin E, Cholesterol, Body Condition and LDA

Cows that developed LDA during the first mo postpartum had, on average, lower serum α-tocopherol concentrations ($P = 0.003$; **Figure 1A**) and α-tocopherol to cholesterol molar ratios ($P = 0.03$; results not shown) than healthy cows. Sampling time affected serum α-tocopherol concentrations and α-tocopherol to cholesterol molar ratios (both $P < 0.0001$), with concentrations decreasing dramatically in the

first wk postpartum in all cows (**Figure 1A**). The nadir α-tocopherol concentrations at d 7 postpartum were lower in cases than controls (5.1 ± 1.0 μM vs. 9.2 ± 0.8 μM; $P = 0.004$). In control cows, α-tocopherol concentrations returned to prepartal concentrations (~15 μM) by 28 d postpartum. Serum α-tocopherol remained lower in cases compared with controls (<10 μM vs. 15 μM) during the entire postpartal sampling period (all $P < 0.03$; **Figure 1A**).

Serum cholesterol changed differently over time in control and case cows ($P_{Interaction} = 0.001$; **Figure 1B**). Cholesterol concentrations were similar in the two groups until d 3 postpartum, but were lower in LDA cows for the remaining sampling period (all $P< 0.05$; **Figure 1B**). A tendency to a significant interaction between LDA incidence and sampling time was observed for BCS ($P = 0.09$), which were only lower for LDA cows at 7 and 14 wk postpartum (**Figure 1C**).

Energy and Inflammation Status and LDA

Cases had on average greater NEFA concentrations than control cows($P< 0.0001$), whereas serum BHBA changed differently over time in control and case cows ($P_{Interaction} = 0.04$; **Figure 2B**). Only during the first 4 wk postpartum did cases have greater BHBA concentrations than control cows (**Figure 2B**). Sampling time affected serum NEFA and BHBA (both $P < 0.0001$). Elevated NEFA and BHBA concentrations were observed during the first wks postpartum in all cows; however, they started earlier and persisted longer for NEFA in cases (**Figures 2A, B**).

Control and LDA cows changed differently over time for serum haptoglobin ($P_{Interaction} = 0.003$) and SAA ($P_{Interaction} = 0.10$), respectively (**Figures 2C, D**). Elevated haptoglobin and SAA concentrations were observed during d 1postpartum

in all cows; however, they were greater and remained elevated for a longer period in LDA compared with control cows (**Figures 2C, D**). Fold changes between cases and controls were greater and more persistent for serum haptoglobin than for SAA (**Figures 2C, D**).

Macronutrient Status and LDA

Cows with LDA had on average lower phosphorus concentrations than control cows ($P < 0.0001$; **Figure 3A**). The LDA effect, however, was significant only for a few sampling times (d -21, -3, 0, 35, and 49 postpartum; **Figure 3A**). A tendency to a significant interaction between LDA incidence and sampling time was observed for serum urea N ($P = 0.09$), as LDA cows had lower urea N concentrations starting d 7 postpartum (**Figure 3 B**).

In contrast, a temporary decrease in serum concentrations of calcium, magnesium, and glucose was observed in cows that developed LDA (**Figure 4**). Cases had lower serum calcium concentrations ($P = 0.002$; **Figure 4A**) and magnesium concentrations ($P = 0.04$; **Figure 4B**) on average than healthy cows. The LDA effect, however, was significant only at d 14 postpartum for calcium (**Figure 4A**) and at d 7, 21, and 28 postpartum for magnesium (**Figure 4B**). No significant overall effects were observed for serum glucose concentrations; glucose concentration were, however, greater at d 0 and lower at d 28 postpartum in cases compared with controls (**Figure 4C**).

Early Serum Indicator of LDA

To identify early indicators of LDA, we compared the average serum concentrations of LDA cases and controls before LDA diagnosis (**Table 2**). Differences in α-tocopherol

concentrations and α-tocopherol to cholesterol molar ratio were apparent before calving and became greater as LDA diagnosis approached (**Table 2**). Cows that subsequently developed LDA had 24% lower serum α-tocopherol concentrations during the last wk prepartum, 33% lower concentrations during the first 3 d postpartum, and 45% lower concentrations at the last blood sampling before LDA diagnosis compared with healthy cows. A similar trend was observed for α-tocopherol to cholesterol ratios (**Table 2**). Cows that subsequently developed LDA had prepartum greater NEFA and lowerphosphorus concentrations than healthy cows (**Table 2**). After calving, BHBA, haptoglobin, and SAA concentrations were higher in cases than in control cows with the differences becoming greater as LDA diagnosis approached (**Table 2**).

Potential cut-off values for early serum indicators of LDA were evaluated and yielded no significant cut-off values for wk -1 prepartum. In contrast, significant cut-off values for serum concentrations of α-tocopherol (7 µM), BHBA (1 mM; 1 LDA cow misclassified; $P < 0.001$), haptoglobin (200 mg/L; 1 LDA cow misclassified; $P < 0.001$), and SAA (100 mg/L; 1 LDA cow and 1 control cow misclassified; $P < 0.001$) were observed for d 3 or 7 postpartum (excluding samples taken after LDA diagnosis). Six out of 7 case cows had α-tocopherol concentrations <7 µM, while 3 out of 10 control cows had α-tocopherol concentrations <7 µM during this time period ($P = 0.05$); the only case cow >7 µM decreased <7 µM 8 d before LDA diagnosis.

DISCUSSION

Our study demonstrates that serum α-tocopherol concentrations may be a useful diagnostic parameter for LDA, because group differences in α-tocopherol concentrations and α-tocopherol to cholesterol molar ratio began prepartum and became more pronounced as LDA diagnosis approached. Secondly, our study suggests that negative energy balance, inflammation, and lower vitamin E concentrations may precede LDA. Thirdly, our study indicates that cows remain in a lower nutrient status for weeks after LDA correction.

Serum α-tocopherol concentrations in healthy cows followed similar trends, as has been previously described (Meglia et al., 2006; Weiss et al., 2009), significantly decreasing in wk 1 after calving and then increasing within 3 weeks back to prepartum concentrations (**Figure 1A**). Herdt and Smith (1996) reported that lactation stage accounted for 47% of the overall variability in serum α-tocopherol concentrations. Dietary fat and α-tocopherol increased α-tocopherol concentrations in blood (Weiss et al., 1994; Weiss and Wyatt, 2003). Literature about the association between DMI and α-tocopherol concentrations in blood is limited. Goff et al. (2002) reported that mastectomized cows had greater plasma α-tocopherol concentrations than intact cows in the last 2 wk prepartum, although DMI did not differ between groups. Furthermore, the depleted α-tocopherol concentrations of mastectomized cows early postpartum, when their DMI was similar or greater than before calving, suggested that lower feed intake alone could not explain the decrease in α-tocopherol concentrations after parturition. A decrease in α-tocopherol concentrations during wk 1 postpartum even occurs after vitamin E supplementation (Meglia et al.,

2006; Weiss et al., 2009) and is thought to result from a combination of increased lipid peroxidation and production of reactive oxygen species, increased secretion of α-tocopherol into colostrum and milk, depressed feed intake, inflammation, and decreased lipid absorption and transport (Baldi, 2005). Serum α-tocopherol concentrations in this study were on the higher end of what have been previously reported (LeBlanc et al., 2004; Weiss et al., 2009; Bouwstra et al., 2010).

Cholesterol concentrations in our study (**Figure 1B**) were similar to those reported previously (Herdt and Smith, 1996; Guzelbektes et al., 2010; Stengärde et al., 2010). Blood cholesterol, which is primarily in the high density lipoprotein fraction, is considered an indicator of lipoprotein concentrations and decreased with α-tocopherol around calving (Herdt and Smith, 1996). Fat feeding and feed restriction increase cholesterol concentrations for increased lipid transport (Weiss and Wyatt, 2003; Bjerre-Harpøth et al., 2012), whereas heat and inflammation-associated diseases, in particular liver disorders, decrease cholesterol by impairing lipid transport (Bobe et al., 2004; Abeni et al., 2007; Vogel et al., 2011). Because serum lipoproteins transport α-tocopherol in blood (Traber and Jialal, 2000), α-tocopherol is usually divided by cholesterol concentrations to adjust for changes in lipid transport (Herdt and Smith, 1996).

Cows that subsequently developed LDA had 45% lower serum α-tocopherol concentrations and 39% lower α-tocopherol to cholesterol molar ratios than healthy cows at the last blood sampling prior to LDA diagnosis (**Table 2**). This study is, to our knowledge, the first report of serum α-tocopherol concentrations before LDA diagnosis. Similar differences in α-tocopherol concentrations had been previously reported for cows after LDA diagnosis (Mudron et al., 1997; Hasanpour et al., 2011) and in cows with

severe fatty liver (Hidiroglou and Hartin, 1982). In contrast, ketosis, mastitis, laminitis, and metritis are associated with non-significant or smaller changes (<20% lower) in α-tocopherol concentrations (Erskine et al., 1987; LeBlanc et al., 2004; Sivertsen et al., 2005) and, thus, cannot explain the large differences between LDA and control cows in serum α-tocopherol and α-tocopherol to cholesterol molar ratio before LDA diagnosis (**Table 2**). A lower DMI might be a probable causative factor for the lower serum α-tocopherol concentrations in LDA cows, but in the absence of DMI data in our study we cannot determine whether or not lower α-tocopherol concentrations in LDA cows were independent of feed intake. This should be addressed in future studies. Goff et al. (2002) suggested, based on their results in mastectomized cows, that besides feed intake other factors, such as increased oxidation, play a role in lower α-tocopherol in blood around calving.

A potential early indicator of LDA is serum α-tocopherol concentrations with a potential cut-off value of 7 μM. All cows that subsequently developed LDA had serum α-tocopherol concentrations <7 μM, while only 3 out of 10 control cows had α-tocopherol concentrations <7 μM during the study. Weiss et al. (1997) reported that cows with plasma α-tocopherol concentrations <3 μg/mL (equivalent to 7 μM) are 9.4 times more likely to have clinical mastitis than cows with greater α-tocopherol concentrations, and NRC dietary guidelines (2001) and Weiss (1998) suggest 3 μg/mL as target for health and immune function. In contrast, Politis et al. (2012) reported that cows with serum α-tocopherol concentrations <2 μg/mL at calving were 4 times more likely to have clinical mastitis than cows with 2 to 3 μg/mL of α-tocopherol or greater. In addition to Politis et al. (2012), many other studies reported healthy cows with α-tocopherol

concentrations <3 or even <2 μg/mL (Mudron et al., 1997; Goff et al., 2002; Weiss et al., 2009). Given the small number of LDA cows in one herd in the present study, future large, multi-herd field studies are needed to confirm serum α-tocopherol concentration as potential early indicator of LDA and determine the potential cut-off value for serum α-tocopherol.

To date, NEFA concentrations >300 to 500 μEq/L in the last wk prepartum and BHBA concentrations >1.0 to 1.4 mM during wk 1 postpartum have been confirmed as early indicators of LDA in large, multi-herd field trials (LeBlanc et al., 2005; Ospina et al., 2010; Chapinal et al., 2011), establishing negative energy balance as an early risk factor for LDA. This contention is further supported by the lower serum phosphate concentrations we observed in cows that subsequently developed LDA (**Table 2**). Low serum phosphate concentrations have been reported in cows after LDA diagnosis (Grünberg et al., 2005; Kalaitzakis et al., 2011). In contrast, the evidence for calcium concentrations as early indicators is inconsistent (LeBlanc et al., 2005; Chapinal et al., 2011). We observed similar patterns in the serum concentrations of NEFA, BHBA, and calcium in this study. Serum α-tocopherol concentrations are potentially a better indicator than NEFA or BHBA concentrations because significant group differences for α-tocopherol were detected before calving and became more pronounced as LDA diagnosis approached (**Table 2**). In contrast, elevated BHBA concentrations usually begin after calving, while many healthy cows have elevated NEFA concentrations in wk 1 postpartum (LeBlanc et al., 2005).

Another potential early indicator of LDA is elevated postpartal serum haptoglobin concentrations with a potential cut off value of 200 mg/L at d 3 or 7 postpartum.

Elevated postpartal haptoglobin concentrations have been reported as early indicator of metritis (Huzzey et al., 2009). In our study, both inflammation markers, haptoglobin and SAA, were increased postpartum before LDA diagnosis, suggesting inflammation as a potential risk factor for LDA. Both inflammation markers had greater postpartal fold-changes between cases and controls than markers of negative energy status before LDA diagnosis (**Table 2**). Results from a large, multi-herd study by Humblet et al. (2006) suggested elevated haptoglobin concentrations as a more specific disease indicator and elevated SAA as a more sensitive disease indicator in dairy cows. Our study is, to our knowledge, the first report of serum concentrations of haptoglobin and SAA prior to LDA. Previous studies reported 4.5- and 1.94-fold greater haptoglobin concentrations and 4.6-fold greater SAA concentrations in cows after LDA diagnosis compared with control cows (Gutzelbektes et al., 2010; Stengärde et al., 2010).

After LDA correction, indicators of energy and nutrient status (NEFA, phosphorus, and urea N) and serum α-tocopherol concentrations remained lower in cases than in healthy cows up to d 49. Cases had also lower BCS in wk 7 and 14 postpartum than control cows suggesting that their lower nutrient status may persist beyond d 49. Similarly, Østergaard and Gröhn (1999) reported lower BW (>30 kg) for more than 6 wk after LDA diagnosis. Unfortunately, we did not measure feed intake in this study and, thus, cannot determine whether the persistent lower nutrient status is a consequence of lower DMI, lower nutrient absorption, or a combination of both. The persistent lower energy and nutrient status in LDA cows may explain the delayed reproductive performance and increased mortality and culling rates in cows that had LDA (Raizman

and Santos, 2002). Future studies are warranted to examine why energy and nutrient status remain lower in cows that had LDA.

A strength of the current study is the intensive blood sampling schedule over 10 wk (-3 to 7 wk postpartum) that allowed us to demonstrate that lower α-tocopherol concentrations preceded LDA and remained after LDA correction. The fact that our results for previously examined indicators of LDA are consistent with the literature suggests that our findings, despite the small size of the study and the lack of cows with primary LDA, are generalizable. This is a retrospective case-control study and, thus, can only establish associations between serum vitamin E concentrations and LDA. Vitamin E alimentation trials are required to determine causality. Previous vitamin E intervention trials showed a non-significant decrease in LDA cases in one (primiparous cows: 1 of 62 in treatment group vs. 6 of 75 in control group; multiparous cows: 14 of 142 in treatment vs. 18 of 141 in control; Erskine et al., 1997) but not in another large field study (28 of 571 in treatment vs. 26 of 571 in control; LeBlanc et al., 2002). Dosage (4,470 IU vitamin E as d-α-tocopherol in Erskine et al., 1997, vs. 3,000 IU vitamin E as RRR-α-tocopheryl acetate in LeBlanc et al., 2002), baseline prepartal α-tocopherol concentrations (7.2 vs. 6.3 μM), pre- and postpartum diets (not specified in both studies), and alimentation route (i.m. vs. s.c. injection) and timing (2 vs. 1 wk prepartum) may determine the outcome of vitamin E alimentation trials. It should be noted that lower serum α-tocopherol concentrations may be a biomarker of disease, low feed intake, or both, and vitamin E supplementation may not reverse the occurence of LDA.

CONCLUSION

Serum concentrations of α-tocopherol and indicators of energy and nutrient status and inflammation were measured during -3 to 7 wk postpartum in dairy cows that developed LDA in the first month of lactation and were compared with those concentrations in healthy cows. Negative energy balance, inflammation, and lower vitamin E concentrations all preceded LDA onset and, thus, might be potential early indicators for developing LDA. Cases remained in lower nutrient status including vitamin E for at least 4 wk after LDA correction. This study was a small study with a limited number of LDA cows in a single commercial herd; larger studies under well-controlled conditions are warranted to examine the role of vitamin E in LDA.

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Figure Legends

Figure 1. Serum concentrations (LSM ± SEM) of A) α-tocopherol and B) cholesterol between day -21 and 49 postpartum and C) body condition score (BCS) between week -4 to 14 postpartum in healthy cows and cows with left displaced abomasum (LDA) between day 6 and 32 postpartum.

Figure 2. Serum concentrations (LSM ± SEM) of A) NEFA, B) BHBA, C) haptoglobin, and D) serum amyloid A between day -21 and 49 postpartum in healthy cows and cows with left displaced abomasum (LDA) between day 6 and 32 postpartum.

Figure 3. Serum concentrations (LSM ± SEM) of A) phosphorus and B) urea N between day -21 and 49 postpartum in healthy cows and cows with left displaced abomasum (LDA) between day 6 and 32 postpartum.

Figure 4. Serum concentrations (LSM ± SEM) of A) calcium, B) magnesium, and C) glucose between day -21 and 49 postpartum in healthy cows and cows with left displaced abomasum (LDA) between day 6 and 32 postpartum.

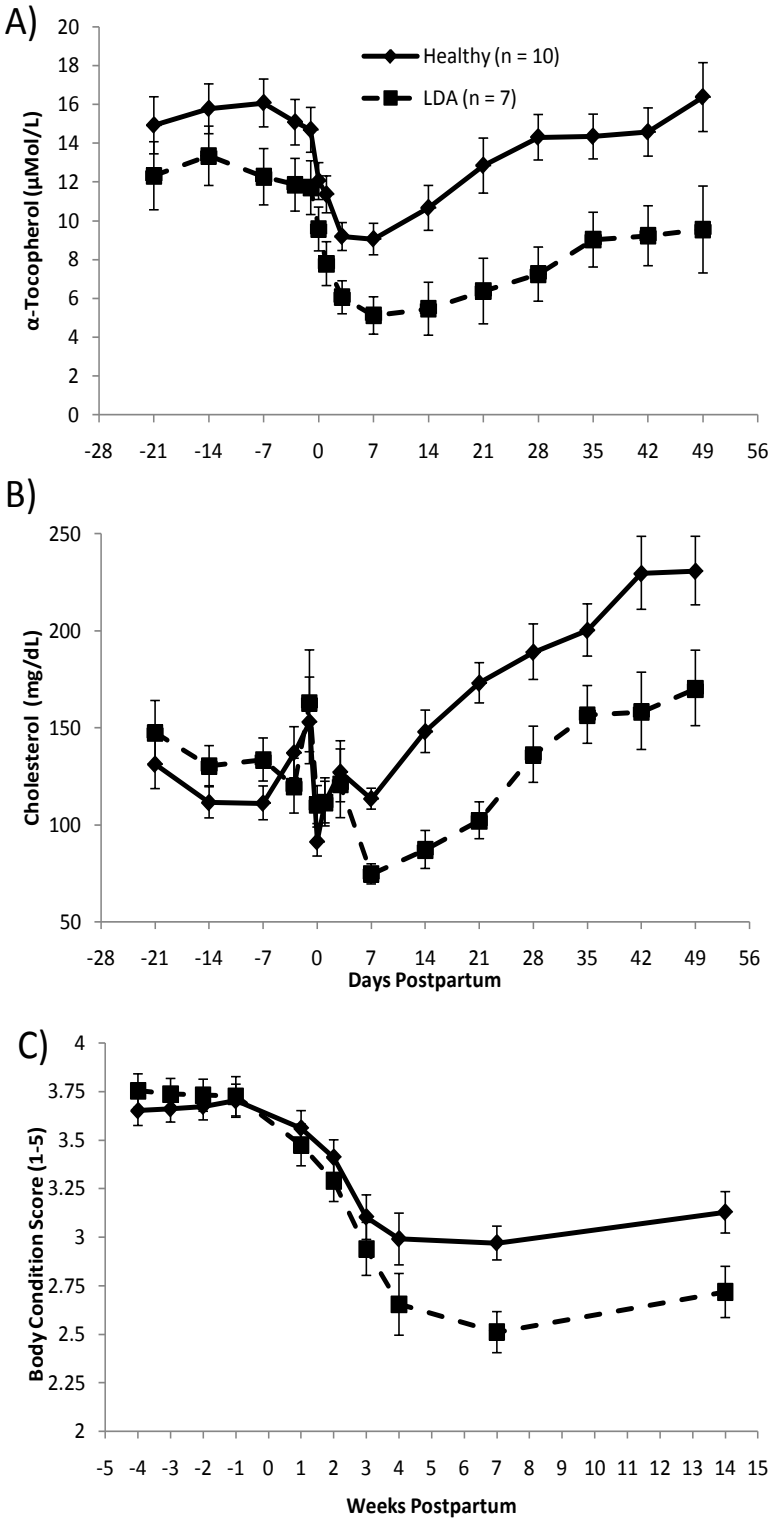
A)
Healthy (n = 10)
LDA (n = 7)
α-Tocopherol (µMol/L)
B)
Cholesterol (mg/dL)
Days Postpartum
C)
Body Condition Score (1-5)
Weeks Postpartum

Figure 1

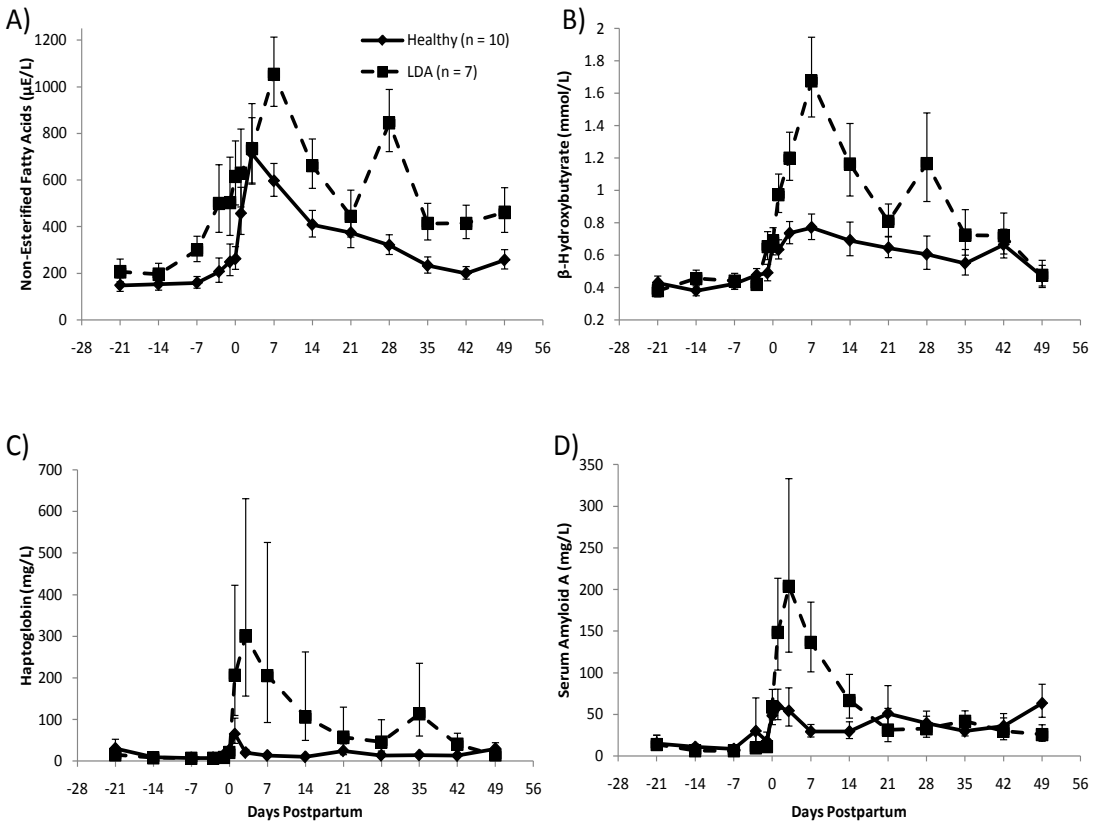
A)
Healthy (n = 10)
LDA (n = 7)
Non-Esterified Fatty Acids (μE/L)
B)
β-Hydroxybutyrate (mmol/L)
C)
Haptoglobin (mg/L)
D)
Serum Amyloid A (mg/L)
Days Postpartum
Days Postpartum

Figure 2

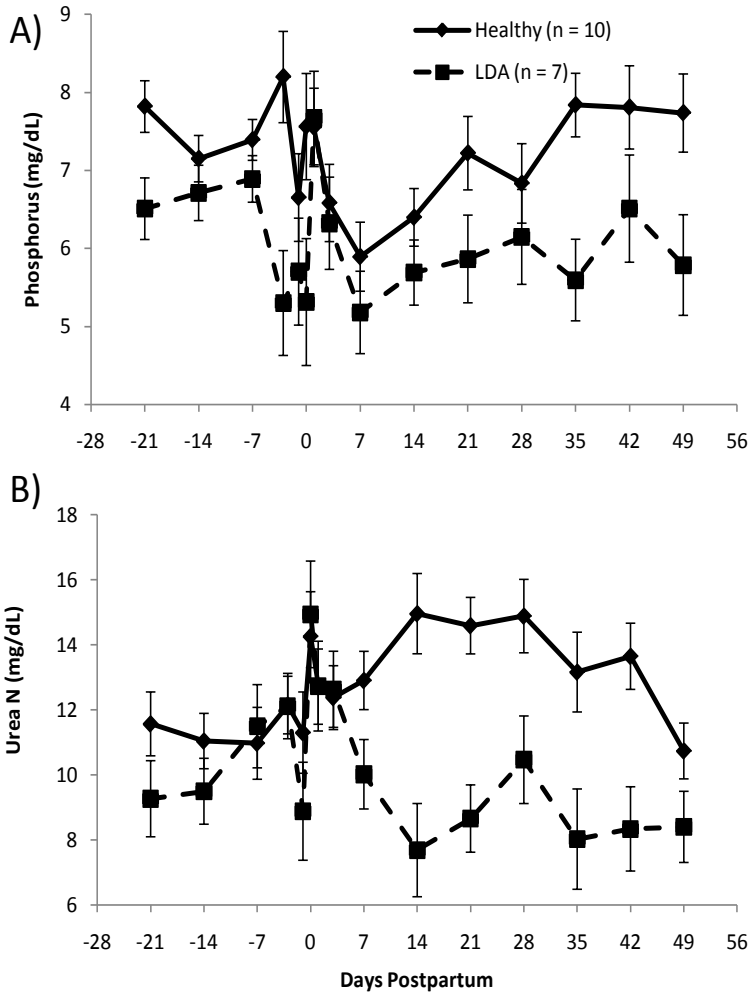
A)
Healthy (n = 10)
LDA (n = 7)
Phosphorus (mg/dL)
9
8
7
6
5
4
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
B)
Urea N (mg/dL)
18
16
14
12
10
8
6
Days Postpartum

Figure 3

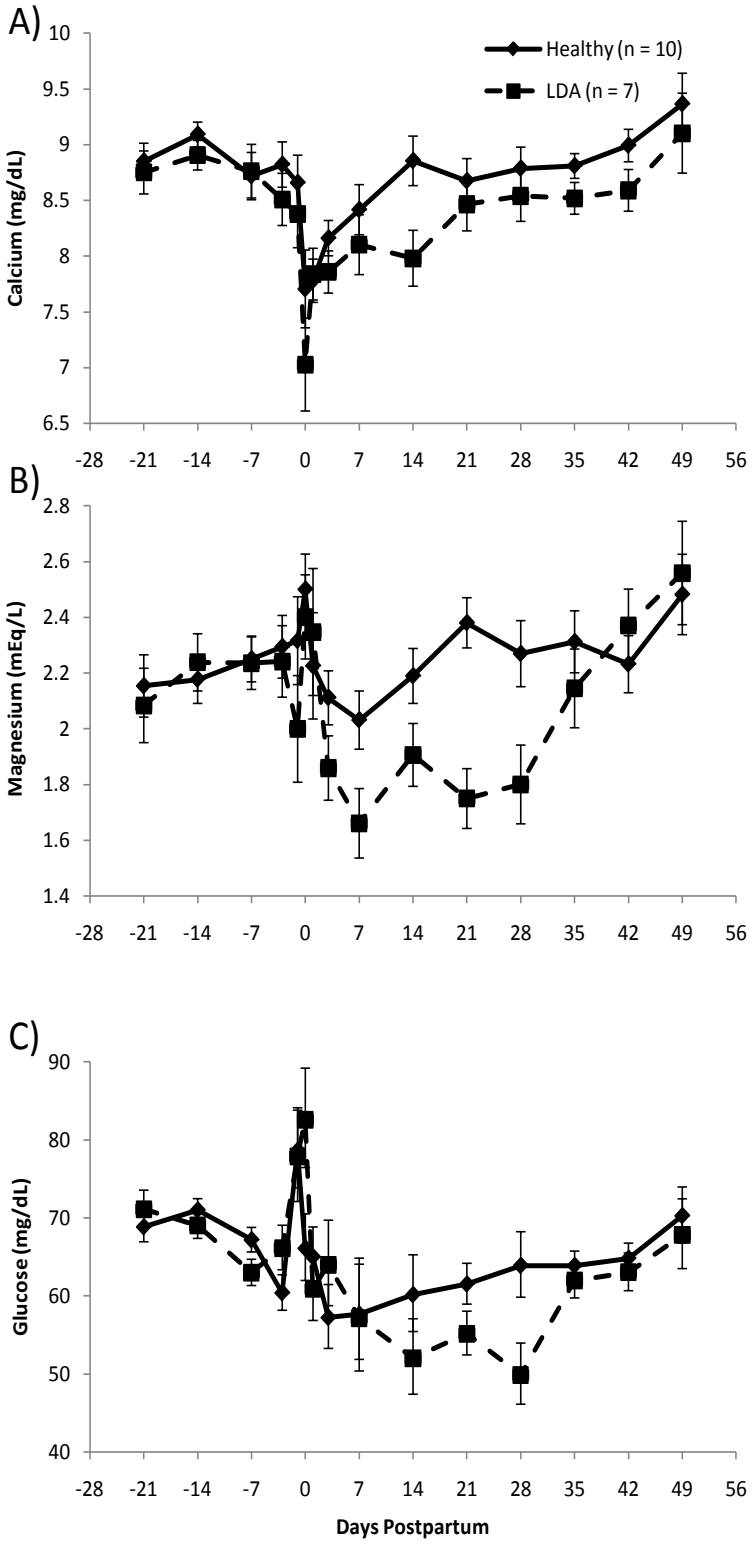
A)
Healthy (n = 10)
LDA (n = 7)
Calcium (mg/dL)
10
9.5
9
8.5
8
7.5
7
6.5
B)
Magnesium (mEq/L)
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
C)
Glucose (mg/dL)
90
80
70
60
50
40
-28 -21 -14 -7 0 7 14 21 28 35 42 49 56
Days Postpartum

Figure 4

Table 1. Feed and nutrient composition of pre- and postpartum diets

	Percent of Diet Dry Matter	
Feed Composition	Prepartum[1]	Postpartum[2]
Grass Silage		2.13
Alfalfa Hay (20% CP, 36% NDF)	13.42	19.26
Corn Silage	27.77	20.93
Triticale Hay (9% CP, 60% NDF)	13.69	
Beet Pulp	3.41	
Vitamin &Mineral Premix[1]	4.95	
Vitamin &Mineral Premix[2]		2.96
MagOx[3]	0.18	
Ground Corn	18.15	
Corn (High Moisture Ear Corn)		20.00
Corn Distillers Grain (incl. solubles)	8.06	12.33
Canola Meal	6.69	6.40
Wheat Distillers Grain (incl. solubles)		5.97
Bakery By-Product		6.39
EnerGII Regular[4]	1.82	1.74
Limestone (ground)	1.85	0.94
Sodium Bicarbonate		0.94
Analyzed Nutrient Composition		
NE_L (Mcal/kg, DM basis)	1.63	1.70
CP	13.0	18.7
ADF	27.1	16.9
NDF	36.2	27.2
Ether Extract	3.47	6.18
Magnesium	0.46	0.32
Potassium	1.30	1.23
Sodium	0.072	0.243
Iron (mg/kg)	469	570
Zinc (mg/kg)	83	115
Copper (mg/kg)	23	23
Manganese (mg/kg)	65	91
Molybdenum (mg/kg)	0.6	0.5

[1]Provides to the diet DM 6.7 g/kg Ca as calcium propionate, -carbonate, and –chloride and mono-dicalcium phosphate, 1.4 g/kg P as mono-dicalcium phosphate, 8.0 g/kg Cl as ammonium and calcium chloride, 3.4 g/kg Mg as magnesium sulfate, 30 mg/kg K, 0.99 g/kg S as magnesium, manganese, copper, cobalt, and zinc sulfate, 0.17 mg/kg Co as cobalt sulfate, 15.2 mg/kg Cu as copper sulfate, 1.012 mg/kg iodine as ethylenediaminedihydroiodide, 7.7 mg/kg Mn as manganese sulfate, 0.31 mg/kg Se as sodium selenite, 29.9 mg/kg Zn as zinc sulfate, 10.8 KIU/kg Vitamin A, 4.6 KIU/kg Vitamin D3, 167 IU/kg Vitamin E as *all rac* α-tocopheryl acetate, 1.19 g/kg Choline, 1.00 g/kg Niacin, 26.8 mg/kg Monensin

[2] Provides to the diet DM 0.30 g/kg Ca, 0.23 g/kg P from ammonium polyphosphate, 0.20 g/kg Mg, 1.23 g/kg K, 0.21 g/kg Na, 0.19 g/kg Cl, 0.26 g/kg S, 0.07 mg/kg Co as cobalt sulfate, 0.05 mg/kg Co as organic cobalt, 12.4 mg/kg Cu as copper sulfate, 4.42 mg/kg Cu as organic copper, 1.76 mg/kg I as ethylenediaminedihydroiodide, 10.7 mg/kg Mn as manganese sulfate, 0.81 mg/kg Mn as organic manganese, 0.25 mg/kg Se as sodium selenite, 59.0 mg/kg Zn as zinc sulfate, 8.00 mg/kg Zn as organic zinc, 5.01 KIU/kg Vitamin A, 1.23 KIU/kg Vitamin D3, 24.5 IU/kg Vitamin E as *all rac* α-tocopheryl acetate, 0.25 g/kg Methionine

[3]Guaranteed to contain no less than 56% Mg

[4]Contains (DM Basis) 90.4% total fat and 9.6% Ca as calcium salts of long chain fatty acids from Inman (Clackamas, OR)

Table 2. Concentrations of serum indicators prior to diagnosis of left displaced abomasum (LDA)

	Last week Prepartum			First 3 days Postpartum			Last Sample Pre LDA Diagnosis		
Indicator	Control	LDA	P-value	Control	LDA	P-value	Control	LDA	P-value
α-tocopherol (μM)	16.0 ± 1.3	12.1 ± 1.5	0.10	10.9 ± 1.0	7.3 ± 1.1	0.04	9.1 ± 0.7	5.0 ± 0.9	0.004
Cholesterol (mg/dL)	129± 10	127± 12	0.91	112 ± 10	112 ± 11	0.98	110 ± 7	104 ± 8	0.61
ATOC/cholesterol[1]	5.07 ± 0.52	3.76 ± 0.48	0.09	3.78 ± 0.44	2.62 ± 0.36	0.07	3.09 ± 0.26	1.90 ± 0.19	0.003
NEFA (μEq/L)	191± 36	406± 91	0.03	508 ± 105	742 ± 181	0.27	591 ± 79	1043 ± 165	0.02
BHBA (mMol/L)	0.47 ± 0.04	0.47 ± 0.04	0.99	0.67 ± 0.05	1.05 ± 0.10	0.003	0.74 ± 0.10	1.95 ± 0.37	<0.001
Haptoglobin (mg/L)	9.76 ± 0.79	7.30 ± 0.61	0.03	45 ± 17	278 ± 200	0.03	12 ± 3	440 ± 298	<0.001
SAA (mg/L)[2]	17± 7	10± 5	0.44	60 ± 22	197 ± 88	0.06	23 ± 6	146 ± 44	<0.001
Phosphorus (mg/dL)	7.36 ± 0.31	6.15± 0.37	0.03	7.27 ± 0.40	6.95 ± 0.48	0.63	5.72 ± 0.39	6.27 ± 0.47	0.39
Urea N (mg/dL)	11.2 ± 1.0	11.0 ± 1.2	0.92	12.9 ± 0.9	13.0 ± 1.0	0.98	12.4 ± 0.8	12.5 ± 0.9	0.96
Calcium (mg/dL)	8.82± 0.16	8.53± 0.19	0.28	7.90 ± 0.13	7.71 ± 0.15	0.37	8.43 ± 0.24	8.05 ± 0.28	0.34
Magnesium (mg/dL)	2.56± 0.10	2.72 ± 0.12	0.34	2.66 ± 0.14	2.65 ± 0.17	0.96	2.37 ± 0.14	1.97 ± 0.17	0.11
Glucose (mg/dL)	68.6 ± 2.3	67.1 ± 2.6	0.68	64.1 ± 3.2	65.2 ± 3.9	0.84	58.7 ± 4.0	41.0 ± 3.3	0.006

[1]ATOC/cholesterol = α-tocopherol to cholesterol molar ratio (μM/mM)

[2]SAA = serum amyloid A

CHAPTER 3 DEPLETED SERUM VITAMIN E CONCENTRATIONS PRECEDE RETAINED PLACENTA IN MULTIPAROUS DAIRY COWS

INTERPRETIVE SUMMARY: **Depleted serum vitamin E concentrations precede retained placenta in multiparous dairy cows.** By *Qu et al.* To determine the association between vitamin E status and retained placenta, serum vitamin E concentrations were measured between -3 and 7 weeks postpartum in multiparous cows that developed retained placenta in early lactation and compared to those in visually healthy cows and cows with other diseases. In comparison to visually healthy cows, Lower vitamin E concentrations preceded and persisted after retained placenta, indicating lower serum α-tocopherol concentrations as a potential early indicator for developing retained placenta.

DEPLETED SERUM VITAMIN E CONCENTRATIONS PRECEDE RETAINED PLACENTA IN MULTIPAROUS DAIRY COWS

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Accepted:

ABSTRACT

Retained placenta (RP), defined as fetal membranes not being expelled within 24 hours after calving, is a costly disease in multiparous dairy cows that is associated with perturbations in metabolism of long-chain, polyunsaturated fatty acids. Vitamin E is an antioxidant that can alter metabolism of long-chain, polyunsaturated fatty acids and has been shown to prevent RP. We hypothesized that serum vitamin E (α-tocopherol) concentrations are depleted before and after calving in cows that will develop RP. The objective of this study was to evaluate if and for how long serum concentrations of α-tocopherol, metabolites, acute phase proteins, and macrominerals are different between multiparous dairy cows that were either healthy, developed RP, or developed other diseases after calving. Using a nested case-control design, blood samples, taken at day -21, -14, -7, -3, -1, 0, 1, 3, 7, 14, 21, 28, 35, 42, and 49 postpartum from 96 multiparous Holstein cows (32 cows per group) that a) were visually healthy (Healthy), b) developed RP (Retained Placenta), or c) developed other diseases (Other Diseases), such as mastitis, metritis, laminitis, or ketosis, were analyzed for serum concentrations of α-tocopherol, indicators of energy and nutrient status and inflammation. Besides indicators of negative energy balance and inflammation, cows that developed RP had prepartum 30% lower prepartal serum α-tocopherol concentrations (8.7 ± 0.6 vs. 12.5 ± 0.6 μM; $P< 0.001$) and 23% lower α-tocopherol to cholesterol molar ratios (3.12 vs. 4.03 μM/mM; $P< 0.001$) compared to visually healthy cows. These group differences were already significant three weeks before calving for α-tocopherol concentrations (8.3 ± 0.7 vs. 11.9± 0.7 μM; $P< 0.001$) and α-tocopherol to cholesterol molar ratios (2.68 vs. 3.66 μM/mM; $P = 0.001$). Up to day 28 postpartum,

serum α-tocopherol concentrations remained lower in RP compared with visually healthy cows (<10 vs. ~13 μM; all $P < 0.001$). Serum concentrations of α-tocopherol, metabolites, acute phase proteins, and macrominerals. These findings suggest lower serum α-tocopherol concentrations are a potential early indicator for the development of RP in multiparous cows.

Key Words: dairy cow, retained placenta, vitamin E.

INTRODUCTION

Retention of fetal membranes (retained placenta) is defined as failure to expel fetal membranes within 12 or within 24 h after parturition; the latter is used in this study. Normalexpulsion occurs within 8 h after calf delivery. Retained placenta (**RP**) is an economically important disease that affects approximately 7.8% (range: 1.3 -39.2%) of U.S. dairy cows (Kelton et al., 1998; USDA, 2009). The average cost, including treatment costs, milk loss, and increased days open, is estimated to be $285 per case (Kelton et al., 1998). Not included in the cost estimate are that cows with RP have increased culling rates and incidence of other metabolic and infectious diseases, especially metritis (Laven and Peters, 1996). The primary risk factors associated with RP are premature births, multiple calves, dystocia (calving problems), abortion, still births, uterine infections, age of cow, milk fever, antioxidant (e.g., selenium, vitamin E, and β-carotene) deficiency, and season (reviewed in Laven and Peters, 1996; Drillich, 2011).

Multiple physical, endocrine, and cellular factors are involved in the expulsion of fetal membranes. Several causes for RP have been proposed in recent years: a) uterine atony (<2 % of cases); b) edema of the chorionic villi as consequence of physical damage associated with birthing complications, caesarian section, or twisted uterus; c) cellular dysfunction and necrosis related to uterine infections; d) incomplete breakdown of extracellular matrix by collagenase and matrix metalloproteinases related to steroid hormone imbalances; e) decreased innate and humoral immune responses; and f) oxidative damage associated with insufficient antioxidants (McNaughton and Murray, 2009; Drillich, 2011). α-Tocopherol, the most abundant and biologically active form of vitamin E, is a lipid soluble antioxidant that can prevent PUFA oxidation and can also

alter steroid hormone metabolism and signal transduction of cytokine expression (as reviewed by Cook-Mills and McCary, 2010; Molano and Meydani, 2012; Traber, 2014). The NRC recommends supplementation of dairy cows with 1.6 IU vitaminE/kg BW (approximately 80 IU/kg DMI) during the dry period and 0.8 IU vitamin E/kg BW (approximately 20 IU/kg DMI) during lactation (NRC, 2001). Some experts recommend additional α-tocopherol supplementation in the late dry period for supporting the increased requirements and decreased intake of α-tocopherol during early lactation (Weiss, 1998; Baldi, 2005; Politis, 2012).

We and others previously documented that cows are in the first weeks after calving in a depleted vitamin E status (Weiss et al., 2009; Qu et al., 2013). A meta-analysis by Bourne et al. (2007) documented that vitamin E alimentation during the last month before calving decreases the risk in dairy cows to develop RP by 53%. Using data from a large vitamin E supplementation trial in the Guelph, Canada, area, LeBlanc et al. (2004) showed that cows that developed RP had in the last wk postpartum lower serum α-tocopherol concentrations than cows that did not develop RP. Little is known, however, if and for how long circulating α-tocopherol concentrations are lower before and after RP in non-supplemented cows and whether the observed changes are specific to RP or also occur in cows with other diseases. We hypothesized that the lower α-tocopherol status is not specific to the time span when cows have RP. The objective of this study was to compare serum α-tocopherol concentrations between -3 to 7 weeks postpartum of multiparous dairy cows that were either a) visually healthy, b) developed RP, or c) developed other diseases after calving.

MATERIALS AND METHODS

Animals and Study Design

All procedures involving animals were approved by the Oregon State University Institutional Animal Care and Use committee. The research was conducted on a 1,000-head commercial dairy farm in Oregon's Central Willamette Valley during Spring and Summer 2010. The cohort consisted of 161 multiparous Holstein cows (parity 2 to 7). Thirty two cows (2 to 5 parities) that were diagnosed with RP (retained for 1 to 11 d), 32 healthy control cows (not visible signs of diseases during the first 28 d postpartum), and 32 disease control cows (visible diseased and treated during the first 28 d postpartum) that were similar in parity, calving month, and age, were selected for this nested case-control study.

During the last 4 wk before expected calving, cows were housed in a straw-bedded free stall barn and were fed once in the morning (7:30) a TMR based on corn, corn silage, and alfalfa and triticale hay, which met NRC guidelines (NRC, 2001) and contained supplemental vitamin E at 167 IU/kg DM (**Table 1**). After calving, healthy cows stayed the first 2 d in the hospital pen, and then for 4 wk in the early lactation pen, and then based on body size in 3 mid-lactation pens. Cows diagnosed with diseases were moved back to the hospital pen for treatment. Cows from the hospital, early lactation, and the mid-lactation pen were fed at 7:00, 9:00, 10:00, respectively, and 13:30 for all cows, a TMR based on corn, corn silage, and alfalfa hay, which met NRC guidelines (NRC, 2001) and contained supplemental vitamin E at 24.5 IU/kg DM (**Table 1**).

Starting 28 d before predicted calving date, BCS of cows were scored weekly until 4 wk postpartum and then at wk 7 and 14 postpartum (Edmonson et al., 1989). During the

study period, cows were monitored daily for flakes in the milk, gait, appetite, general appearance, alertness, vaginal discharge, and retained placenta. Uterine discharge was checked twice a week, and urinary ketones and body temperature were checked if cow appeared not healthy. Medical treatments were administered based on the standard operating procedures of the dairy farm, which was for RP: cows with RP remained in the hospital pen and were infused with 57 g tetracycline HCl powder (324 g tetracycline/lb; IVX Animal Health, Inc., St. Joseph, MO) in 1 L of water every 4 to 8 d until the placenta was expelled. To prevent infections, cows were injected i.m. for up to 7 d with penicillin (40 mL/d, Penicillin G Procaine; Aspen Veterinary Resources® Ltd), followed by treatment for up to 7 d with sulfadimethoxine (30 g/d, Sulfasol soluble powder; Med-Pharmex®, Pomona, CA).

Blood Collection and Analysis

Blood samples were taken at d -21 (-24 to -18), -14 (-17 to -11), -7 (-10 to -5), -3 (-4 or -3), -1 (-2 or -1), 0, 1, 3, 7, 14, 21, 28, 35, 42, and 49 postpartum within 10 min after morning feeding. Blood (5 to 8 mL) was obtained from the coccygeal vein or artery in 10 mL serum vacutainer tubes (BD Vacutainer® Plus Plastic Serum Tubes, BD Diagnostics, Franklin Lakes, NJ), placed on ice, and transported to the laboratory, where serum was separated by centrifugation at room temperature for 20 min at 1600 x *g*. Serum samples were stored at -20°C until chemical analysis.

Serum α-tocopherol concentrations were measured using a reversed-phase Phenomenex Synergi 4 µM Hydro-RP, 150×4.6 mm column and a SecurityGuard™ cartridges AQ C18 pre-column, 3.0 mm i.d. (Phenomenex, Torrance, CA) with a LC-4B amperometric electrochemical detector (Bioanalytical Systems Inc., West Lafayette, IN),

following Podda et al. (1996). An isocratic mobile phase of 99:1 (v:v) methanol:water containing 0.1% (w:v) lithium perchlorate was used with a run time of 9 min and the electrochemical detector set at 500 mV. After 100 µL serum were saponified in alcoholic KOH with 1% ascorbic acid, the sample was extracted with hexane and dried, and the residue resuspended in ethanol:methanol (1:1). A 20-µL aliquot of the extract was injected into the HPLC system. Serum concentrations of cholesterol (Stanbio Cholesterol LiquiColor® Procedure No. 1010; Stanbio Laboratory, Boerne, TX), glucose (Stanbio Glucose Proc. No. 1075; Stanbio), NEFA (ACS ACOD method, WAKO Diagnostics, Richmond, VA), BHBA (Stanbio BHBA LiquiColor® Proc. No. 2440; Stanbio), urea N (Stanbio Urea Nitrogen Liqui-UV® Proc. No. 2020; Stanbio), haptoglobin (bovine-specific ELISA kit Catalog No. 2410-70; Life Diagnostics, Inc., West Chester, PA), calcium (Stanbio Total Calcium LiquiColor® Proc. No. 0150; Stanbio), magnesium (Stanbio Magnesium LiquiColor® Proc. No. 0130; Stanbio), and phosphorus (Stanbio Phosphorus Liqui-UV® Proc. No. 0830; Stanbio) were measured according to manufacturer's instructions using a FLUOstar Omega microplate autoreader (BMG Labtech Inc, San Francisco, CA).

Statistical Analysis

Data was analyzed as repeated-measures-in-time ANOVA study using the PROC MIXED procedure of SAS version 9.2 (SAS Institute, 2009). The molar ratio of α-tocopherol to cholesterol was calculated to adjust for changes in lipid transport (Traber and Jialal, 2000) and stage of lactation (Weiss, 1998). To achieve a normal distribution for their serum concentrations, concentrations of glucose, NEFA, and the α-tocopherol to cholesterol molar ratio were ln-transformed, concentrations of haptoglobin and BHBA

were twice ln-transformed, concentrations of cholesterol were square-root transformed, and concentrations of phosphorus >11 mg/dL were set at 11 mg/dL. The variance-covariance structure of repeated measures within cow was modeled using the heterogeneous first-order autoregressive variance-covariance matrix. Fixed effects were RP incidence (healthy, RP, other diseases), parity (2, >2), sampling time, and the interaction between RP incidence and sampling time. To obtain the correct degrees of freedom, the KENWARDROGER option was invoked.

To compare our results with previously published studies and identify early indicators of RP, average serum concentrations in the last 3 wk and in the last wk before calving were calculated using the trapezoidal rule and analyzed in PROC GLM with RP status and parity as fixed effects. In addition, we compared changes in concentrations between the average of 3 and 2 wk and 1 wk prepartum and concentrations at d 0 between groups in PROC GLM with RP status and parity as fixed effects. Values presented in the figures and tables are least-squares means (**LSM**) and their standard errors (**SEM**) that are transformed back to their original measurement scale. All statistical tests were two-sided. Significance was declared at $P \leq 0.05$ and a tendency at 0.05 to 0.10.

RESULTS

The incidence rate of RP in this study cohort was 19.9% (32 of 161 cows). Cows with RP were in their second to fifth parity. Cows retained the placenta for 1 to 11 d (3, 2, 1, 2, 4, 4, 4, 6, 4, 1, and 1 cow for 1 to 11 d, respectively). Of the 32 RP cows, 22 cows had calved at least 5 d early (range -5 to -19 d before predicted calving date), 16 cows had twins, 5 cows had dystocia (hard pull; one cowhad a twisted uterus), and 8 cows appeared sluggish after calving. Except for one RP cow, all other cows displayed one or more of the previously described symptoms. In the following 28 days, all RP cows were treated for metritis, 4 cows for laminitis, 3 cows for mastitis, 2 cows for ketosis, 2 cows for milk fever, and 1 cow for left displaced abomasum. The 32 healthy cows did not show signs of clinical diseases during the first 28 days postpartum. The 32 cows with other diseases were treated for the following diseases during the first 28 days postpartum: 11 cows were treated for laminitis, 8 cows for ketosis, and 7 cows each for metritis and for mastitis.

Serum Vitamin E, Cholesterol, and Retained Placenta

Cows that developed RP had prepartum 31% lower prepartal serum α-tocopherol concentrations (8.7 ± 0.6 vs. 12.5 ± 0.56 μM; $P< 0.001$) and 29% lower α-tocopherol to cholesterol molar ratios (3.12 vs. 4.03 μM/mM; $P< 0.001$) compared to healthy cows (**Figure 1A**). These group differences were already significant 3 weeks before calving for α-tocopherol concentrations (8.3 ± 0.7 vs. 11.9± 0.7 μM; $P< 0.001$) and α-tocopherol to cholesterol molar ratios (2.68 vs. 3.66 μM/mM; $P = 0.001$). Sampling time affected serum α-tocopherol concentrations and α-tocopherol to cholesterol molar ratios (both $P < 0.0001$), with concentrations decreasing dramatically in the first wk postpartum

in all cows (**Figure 1A**). The nadir α-tocopherol concentrations at d 7 postpartum were lower in RP than in healthy cows (5.1 ± 0.3 μM vs. 7.2 ± 0.3 μM; $P < 0.001$). Up to d 28 postpartum, serum α-tocopherol concentrations remained lower in RP than in visually healthy cows (<10 vs. ~13 μM; all $P < 0.001$; **Figure 1A**). Serum α-tocopherol concentrations of cows with other diseases were between those of healthy and RP cows but, except for d -21 prepartum (lower in RP cows for α-tocopherol) and for d 3 and 49 postpartum (higher in RP cows for α-tocopherol to cholesterol molar ratios), were not significantly different from RP cows (**Figure 1A**).

Serum cholesterol changed differently over time in the three cow groups ($P_{Interaction} < 0.001$; **Figure 1B**). Until calving, cholesterol concentrations were similar in the 3 groups; starting d 7 postpartum, cholesterol concentrations were lower in RP than in healthy cows (all $P < 0.003$). Between d 1 and 21 postpartum, cholesterol concentrations were lower in RP cows than in cows with other diseases (all $P < 0.06$; **Figure 1B**).

Energy Status and Retained Placenta

Serum NEFA and BHBA concentrations changed differently over time in the three cow groups ($P_{Interaction} < 0.001$; **Figures 2A, B**). Before RP onset, RP cows had higher NEFA concentrations (d -21 and d -7 to 1 postpartum) and higher BHBA concentrations (d -14 to -1 postpartum) than healthy cows. After RP onset, RP cows had lower NEFA concentrations (d 3 to 14 postpartum) than RP cows. Group differences between RP cows and cows with other diseases were observed only at d d -3 (higher in RP cows) and d 14 postpartum (lower in RP cows) for NEFA (**Figure 2A**) and at d -21 and -7 postpartum (higher in RP cows) for BHBA (**Figure 2B**).

Inflammation Status and Retained Placenta

Cows with RP had on average higher haptoglobin concentrations than healthy cows ($P = 0.007$); group differences were significant directly after RP onset (d 1 and 7) and at d 21 postpartum; **Figure 3A**). Group differences between RP cows and cows with other diseases were observed only at d -21 prepartum (higher in RP cows; **Figure 3A**).

Overall, the three groups did not differ in serum glucose concentrations; however, RP cows had at calving, which is directly before RP onset, greater glucose concentrations than healthy cows (d 0 to 3 postpartum) and than cows with other diseases (d 0 and 3 postpartum; **Figure 3B**). Before calving, serum glucose concentrations were or tended to be lower in RP than in healthy cows (all $P \leq 0.08$; **Figure 3B**). At d 14 postpartum, RP cows had higher glucose concentrations than cows with other diseases compared with disease controls (**Figure 3B**).

Serum urea N changed differently over time in the three cow groups ($P_{\text{Interaction}} < 0.001$; **Figure 3C**). At calving, RP cows had higher urea N concentrations (d 0 and 1 postpartum) than healthy cows. Later in the postpartum period, RP cows had higher urea N concentrations (between d 14 and 42 postpartum) than healthy cows. No significant group differences were observed between RP cows and cows with other diseases (**Figure 3C**).

Macromineral Status and RP

Lower serum concentrations of calcium, magnesium, and phosphorus were observed in RP cows during the peripartal period (**Figure 4**). On average, RP cows had lower serum calcium concentrations ($P = 0.03$) than healthy cows and tended to

have lower serum calcium concentrations ($P = 0.07$) than cows with other diseases (**Figure 4A**). Prepartum, group differences were significant only at d -21 (lower in RP cows than in cows with other diseases). Postpartum, group differences were significant only at d 7 and 28 postpartum (lower in RP than in healthy cows; **Figure 4A**).

On average, cases had lower serum magnesium concentrations ($P< 0.001$) than healthy cows and tended to have lower serum magnesium concentrations ($P = 0.07$) than cows with other diseases (**Figure 4B**). Prepartum, RP cows had lower magnesium concentrations at d -21 compared with healthy cows or cows with other diseases and at d -7 and -3 compared with cows with other diseases. Postpartum, RP cows had lower magnesium concentrations between 3 and 28 days than healthy cows and at d 3, 5, and 21 postpartum compared with cows with other diseases (**Figure 4B**).

On average, cows with RP had lower serum phosphorus concentrations than healthy cows ($P= 0.03$; **Figure 4C**). Prepartum, RP cows had lower phosphorus concentrations at d -21 compared with healthy cows or cows with other diseases and at d -7 compared with cows with other diseases (**Figure 4C**).

Early Serum Indicator of Retained Placenta

To identify early indicators of RP, we compared averaged serum concentrations of the three groups until calving (**Table 2**). Differences in α-tocopherol concentrations and α-tocopherol to cholesterol molar ratio were apparent already 2 to 3 weeks before calving between healthy cows and cows that would subsequently become sick with RP or other diseases (**Table 2**). Compared with healthy cows, cows that subsequently developed RP

had 32% lower serum α-tocopherol concentrations during 2 and 3 wk prepartum, 32% lower concentrations during the last wk postpartum, and 31% lower concentrations at d 0. A similar trend was observed for α-tocopherol to cholesterol ratios (**Table 2**).

Compared with healthy cows, cows that subsequently developed RP had elevated serum NEFA and BHBA concentrations during the last 3 wk prepartum. At calving, serum NEFA and BHBA concentrations were elevated also in healthy cows, so that group differences were smaller for serum NEFA concentrations or not significant for BHBA concentrations (**Table 2**). Cows with other diseases had NEFA and BHBA concentrations between healthy and RP cows (**Table 2**).

Compared with healthy cows, cows that subsequently developed RP had lower glucose concentrations during the last 3 wk prepartum (**Table 2**). At calving, serum concentrations of glucose were greater in RP cows compared with healthy cows or cows with other diseases. Serum urea N concentrations did not differ between RP and healthy cows until calving, when urea N were greater in RP than in healthy cows. Cows with other diseases had intermediate values between RP and healthy cows for serum glucose and urea N concentrations (**Table 2**). Cows that subsequently developed diseases (RP and other diseases combined) tended to have higher haptoglobin concentrations in the last wk prepartum and at calving (both $P = 0.06$), which were significant between healthy cows and cows with other diseases (both $P = 0.04$).

Compared with healthy cows, cows that subsequently developed RP had lower magnesium concentrations during the last 3 wk prepartum (**Table 2**). Cows with other diseases had intermediate values between RP and healthy cows for magnesium concentrations (**Table 2**). During 2 and 3 wk postpartum, cows that subsequently

developed RP had lower phosphorus concentrations than both other groups; no group differences were observed during the last wk postpartum or at calving (**Table 2**). The only significant group differences for serum calcium concentrations were observed during 2 and 3 wk postpartum between cows that subsequently developed RP compared with other diseases, the latter having higher values (**Table 2**).

DISCUSSION

Our study demonstrates that serum α-tocopherol concentrations may be a useful diagnostic parameter for RP, as group differences in α-tocopherol concentrations and α-tocopherol to cholesterol molar ratio began already 3 wk prepartum. Secondly, our study suggests that besides lower vitamin E status, negative energy balance and inflammation precede RP. Thirdly, our study indicates that RP cows remain lower in vitamin E, nutrient status, and macromineral status for the first four weeks after calving.

Serum α-tocopherol concentrations in healthy cows followed similar trends, as has been previously described (Meglia et al., 2006; Weiss et al., 2009), significantly decreasing in wk 1 after calving and then increasing within 3 weeks back to prepartum concentrations (**Figure 1A**). Herdt and Smith (1996) reported that lactation stage, specifically the transition period, accounted for most of the variability in serum α-tocopherol concentrations. A potential reason is that hormonal changes around calving, specifically a spike in estrogen, may decrease lipoprotein synthesis and transport (Goff and Horst, 1997; Katoh, 2002). Feeding supplemental fat and α-tocopherol alimentation increased α-tocopherol concentrations in blood (Weiss et al., 1994; Weiss and Wyatt, 2003). A decrease in α-tocopherol concentrations during wk 1 postpartum even occurs after vitamin E supplementation

(Meglia et al., 2006; Weiss et al., 2009) and is thought to result from a combination of depressed feed intake, decreased lipid absorption and transport, inflammation, increased lipid peroxidation and production of reactive oxygen species, and increased secretion of α-tocopherol into colostrum and milk (reviewed by Baldi, 2005). Serum α-tocopherol concentrations in this study were on the higher end of what have been previously reported in healthy cows, which might be related to the α-tocopherol content of the TMR (LeBlanc et al., 2004; Weiss et al., 2009; Bouwstra et al., 2010).

Cholesterol concentrations in our study (**Figure 1B**) were similar to those reported previously (Herdt and Smith, 1996; Guzelbektes et al., 2010; Stengärde et al., 2010). Blood cholesterol, which is, as is α-tocopherol, primarily in the HDL fraction (approximately 80% of total cholesterol), is considered an indicator of lipoprotein concentrations and decreased with α-tocopherol around calving (Herdt and Smith, 1996). Fat feeding and feed restriction increase cholesterol concentrations for increased lipid transport (Weiss and Wyatt, 2003; Bjerre-Harpøth et al., 2012), whereas heat and inflammation-associated diseases, in particular liver disorders, decrease cholesterol by impeding cholesterol efflux from cells (Abeni et al., 2007; Vogel et al., 2011). Because cholesterol and α-tocopherol are transported together in blood (Traber and Jialal, 2000), α-tocopherol is usually divided by cholesterol concentrations to adjust for changes in lipid transport (Herdt and Smith, 1996).

Cows that subsequently developed RP had prepartum 30% lower serum α-tocopherol concentrations and 23% lower α-tocopherol to cholesterol molar ratios than healthy cows at the last blood sampling prior to RP diagnosis. Similarly, LeBlanc et al. (2004) reported lower α-tocopherol concentrations in cows with versus without RP in the last

wk before calving; however, that study did not distinguish between healthy cows and cows with other diseases. Our study indicates that cows that developed mastitis, metritis, ketosis, or laminitis after calving had prepartum on average 22% lower serum α-tocopherol concentrations than healthy cows, which decreased from -20% in wk 2 and 3 prepartum to -38% at the day of calving. Lower prepartal DMI in cows that will become sick after calving may be the reason for the lower prepartal serum α-tocopherol concentrations in cows. Serum indicators of energy status in this study (e.g., elevated prepartal NEFA and BHBA concentrations and lower prepartal glucose concentrations in cows that will develop diseases in early lactation) but not indicators of nutrient status (e.g., similar prepartal cholesterol and urea N concentrations before calving) would support this hypothesis. In the absence of DMI data in our study we cannot determine whether or not lower α-tocopherol concentrations in cows with RP or other diseases were independent of feed intake. Based on their results in mastectomized and intact cows, Goff et al. (2002) suggested that feed intake alone cannot explain the changes in α-tocopherol concentrations around calving. Future studies have to address this question.

The question arises whether α-tocopherol depletion causes RP and other diseases. Vitamin E alimentation trials are required to determine causality. A meta-analysis by Bourne et al. (2007) of previous vitamin E alimentation trials showed that alimentation during the last month before calving decreases the risk in dairy cows to develop RP by 53%. A meta-analysis by Moyo et al. (2004) indicated that vitamin E alimentation decreases mastitis. It is possible that vitamin E status may influence molecular pathways involved in RP, specifically steroid hormone synthesis and metabolism, cytokine expression, and immune function (reviewed by Cook-Mills and McCary, 2010; Molano

and Meydani, 2012; Traber, 2014). Vitamin E supplementation improves neutrophil function in cows (Politis et al., 2004; Weiss et al., 2009). It should be noted that, besides vitamin E, also selenium, vitamin C, and β-carotene, are involved in these pathways. Our study is a retrospective case-control study and, thus, can only establish associations between serum vitamin E concentrations and RP and other diseases. Lower serum α-tocopherol concentrations may be a biomarker of disease, low feed intake, or both, and vitamin E supplementation may not reverse the occurence of RP and other diseases in early lactation. Independent of whether the lower α-tocopherol concentrations are caused by low DMI alone or also by other factors, our data suggest serum α-tocopherol as a potential predictive indicator of RP and other diseases in early lactation.

To date, NEFA concentrations >300 to 500 μEq/L have been confirmed as early indicators of RP in large, multi-herd field trials in the last wk prepartum (LeBlanc et al., 2004; Ospina et al., 2010; Chapinal et al., 2011), establishing negative energy balance as an early risk indicator of RP. Negative energy balance in late gestation is associated with several risk factors of RP, for examples as multiples, age of cow, and obesity. However, in several smaller studies, no differences in prepartal NEFA concentrations have been observed between cows with or without RP (Kaneene et al., 1997; Quiroz-Rocha et al., 2009; Seifi et al., 2011). We also observed elevated prepartal BHBA concentrations in cows with RP compared to healthy cows. In contrast, previous studies did not report BHBA differences between cows with and without RP (Quiroz-Rocha et al., 2009; Chapinal et al., 2011; Seifi et al., 2011). Choice of the comparison group (e.g., healthy cows vs. cows without RP) may explain the differences in the results, as cows with other

diseases had also higher prepartal NEFA and BHBA concentrations than our healthy cows.

One objective of the study was to evaluate for how long before calving cows that subsequently develop RP have lower α-tocopherol concentrations and elevated NEFA and BHBA concentrations. Group differences for α-tocopherol, NEFA, and BHBA were stable for the last three weeks precalving, suggesting that all three indicators could be used early to identify cows at increased risk for diseases in early lactation. This would be useful for experimental studies to identify cows at increased risk for diseases in early lactation or to block cows to treatments according to their predicted disease risk. In the field, it could help producers to stratify cows based on their risk profile to different preventive strategies. Future studies are warranted to examine the causal factors, including feed intake, that resulted in the negative energy balance and lower vitamin E status already present 3 wk prepartum.

Haptoglobin concentrations have been reported as indicator of RP (Skinner et al., 1991; Crawford et al., 2005; Mordak, 2009), whereas, to our knowledge, the association between prepartal haptoglobin concentrations and subsequent RP diagnosis has not been investigated. In our study, haptoglobin concentrations were elevated after but not before calving in cows with RP compared to healthy cows. Other studies also did not report that prepartal haptoglobin concentrations are elevated in cows that subsequently develop diseases (Huzzey et al., 2011). However, we observed that cows with other diseases had higher haptoglobin concentrations in the last wk before calving than healthy cows (P = 0.04), suggesting that inflammation may start before calving in cows that calve with infectious diseases already present (Sabedra, 2012).

Serum concentrations of glucose and urea N were increased at calving and the first days thereafter. Elevated concentrations of glucose and urea N and decreased concentrations of α-tocopherol in blood have been reported in the first phase of the acute phase response to bacterial lipopolysaccharide infection (Waldron et al., 2005; Silanikova et al., 2011; Vernay et al., 2012). As haptoglobin responses to bacterial infections are time delayed by approximately 24 h (Jacobson et al., 2004), our data suggest that inflammation may precede RP onset. The short time span between acute phase response and RP onset makes inflammation markers unsuitable as predictive indicators of RP.

After RP, serum concentrations of α-tocopherol, nutrient status (cholesterol, urea N), and macromineral status (calcium, magnesium, and phosphorus) remained lower between 2 and 4 weeks after calving in RP than in healthy cows. Unfortunately, we did not measure feed intake in this study and, thus, cannot determine whether the persistent lower nutrient and macromineral status is a consequence of lower DMI, lower nutrient absorption, or a combination of both. However, energy status, as indicated by serum NEFA and BHBA concentrations, was not adversely affected. Future studies are warranted to examine why nutrient and macromineral status but not energy status remained lower in cows that had RP.

A strength of the current study is the intensive blood sampling schedule over 10 wk (-3 to 7 wk postpartum) that allowed us to demonstrate that lower α-tocopherol concentrations preceded RP and remained after RP. The fact that our results for previously examined indicators of RP are consistent with the literature suggests that ours, despite the small size of the study, are generalizable. Our incidence rate for RP was with 19.9% at the high end of what was previously reported (range: 1.3 -

39.2%; Kelton et al., 1998). The relative high RP incidence rate can be explained by the high number of cows with multiples, that only multiparous cows were in the cohort, and that most of the births occurred during spring and early summer, when the incidence of RP is greater (Joosten et al., 1987; Laven and Peters, 1996; Drillich, 2011).

CONCLUSION

Serum concentrations of α-tocopherol and indicators of energy and nutrient status and inflammation were measured during -3 to 7 wk postpartum in dairy cows that had RP after calving and were compared with those concentrations in healthy cows and cows with other diseases, such mastitis, metritis, laminitis, and ketosis. Elevated NEFA and BHBA concentrations and lower vitamin E concentrations all preceded RP onset for 3 weeks before calving and, thus, might be potential early indicators for developing RP. Diseased cows remained in lower nutrient status, including vitamin E, for the first 4 wk after calving. This study was a small study in a single commercial herd; larger studies under well-controlled conditions are warranted to examine the role of vitamin E in RP.

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Figure Legends

Figure 1. Serum concentrations (LSM ± SEM) of A) α-tocopherol and B) cholesterol between day -21 and 49 postpartum in visually healthy cows (Healthy), cows with other diseases (Other Diseases), and cows with retained placenta (Retained Placenta).

Figure 2. Serum concentrations (LSM ± SEM) of A) NEFA and B) BHBA between day -21 and 49 postpartum in visually healthy cows (Healthy), cows with other diseases (Other Diseases), and cows with retained placenta (Retained Placenta).

Figure 3. Serum concentrations (LSM ± SEM) of A) haptoglobin, B) glucose, and C) urea N between day day -21 and 49 postpartum in visually healthy cows (Healthy), cows with other diseases (Other Diseases), and cows with retained placenta (Retained Placenta).

Figure 4. Serum concentrations (LSM ± SEM) of A) calcium, B) magnesium, and C) phosphorus between day -21 and 49 postpartum in visually healthy cows (Healthy), cows with other diseases (Other Diseases), and cows with retained placenta (Retained Placenta).

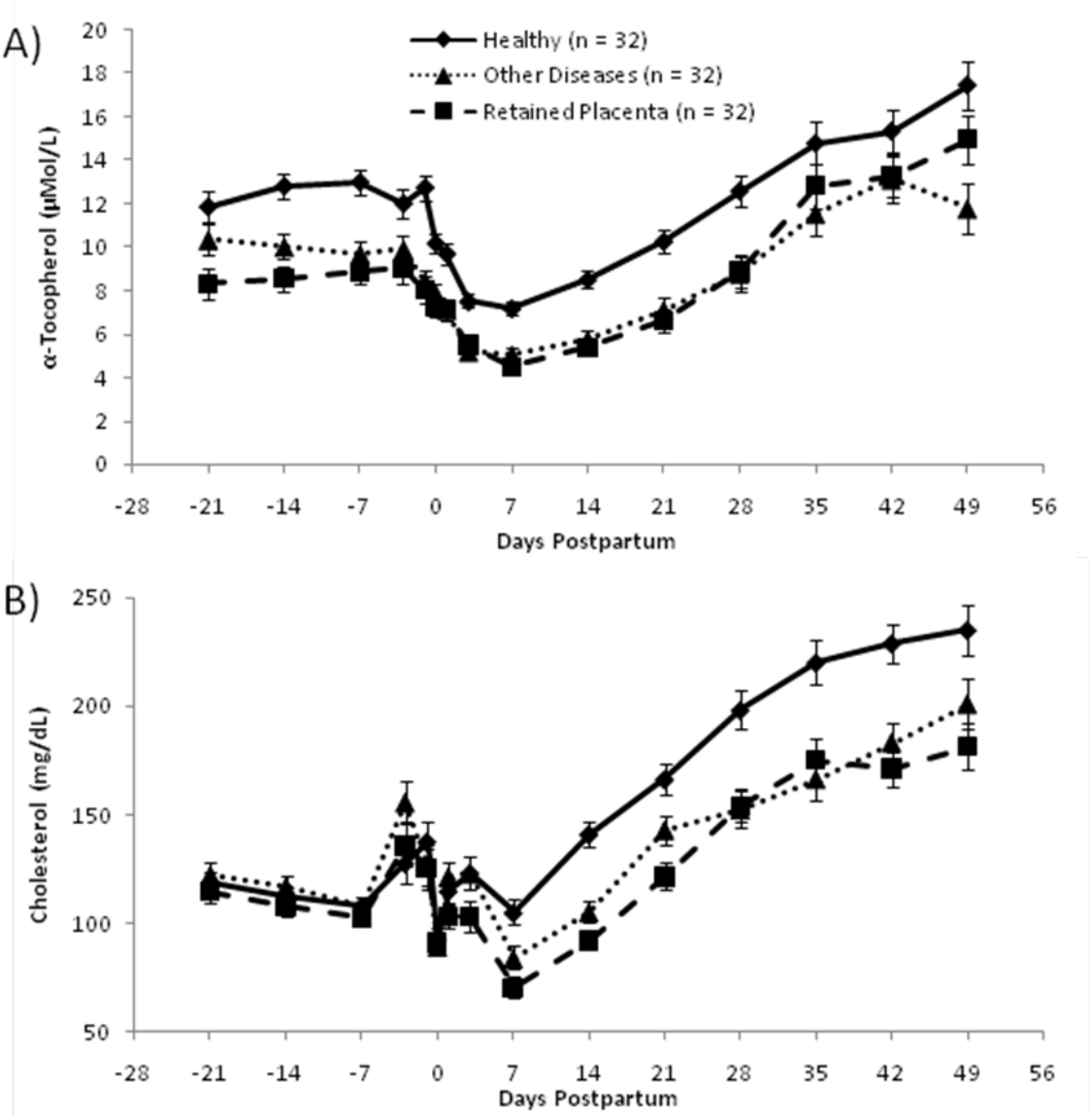
A)
Healthy (n = 32)
Other Diseases (n = 32)
Retained Placenta (n = 32)
α-Tocopherol (µMol/L)
Days Postpartum
B)
Cholesterol (mg/dL)
Days Postpartum

Figure 1

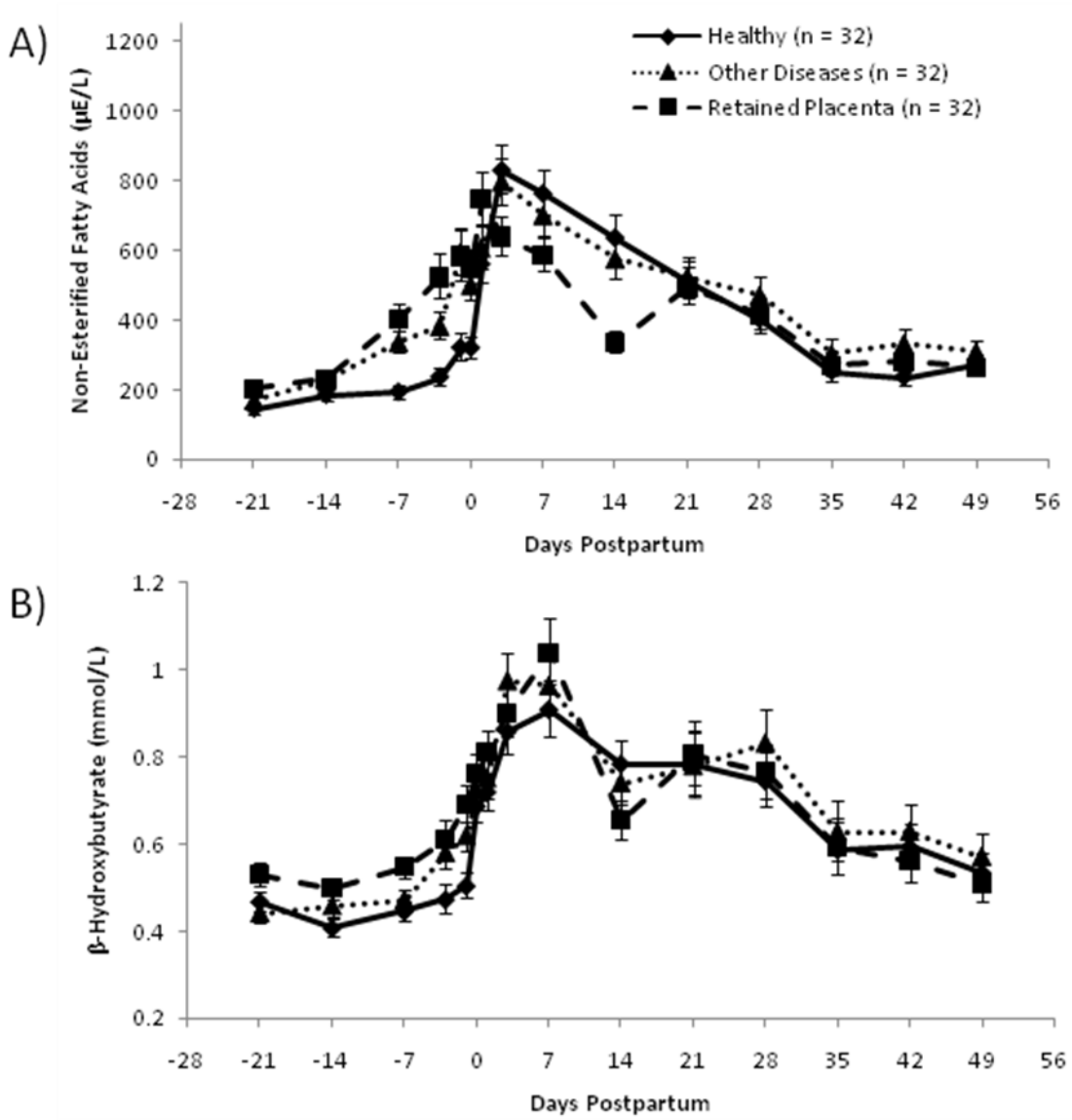
A)
Healthy (n = 32)
Other Diseases (n = 32)
Retained Placenta (n = 32)
Non-Esterified Fatty Acids (µE/L)
1200
1000
800
600
400
200
0
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days Postpartum
B)
β-Hydroxybutyrate (mmol/L)
1.2
1
0.8
0.6
0.4
0.2
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days Postpartum

Figure 2

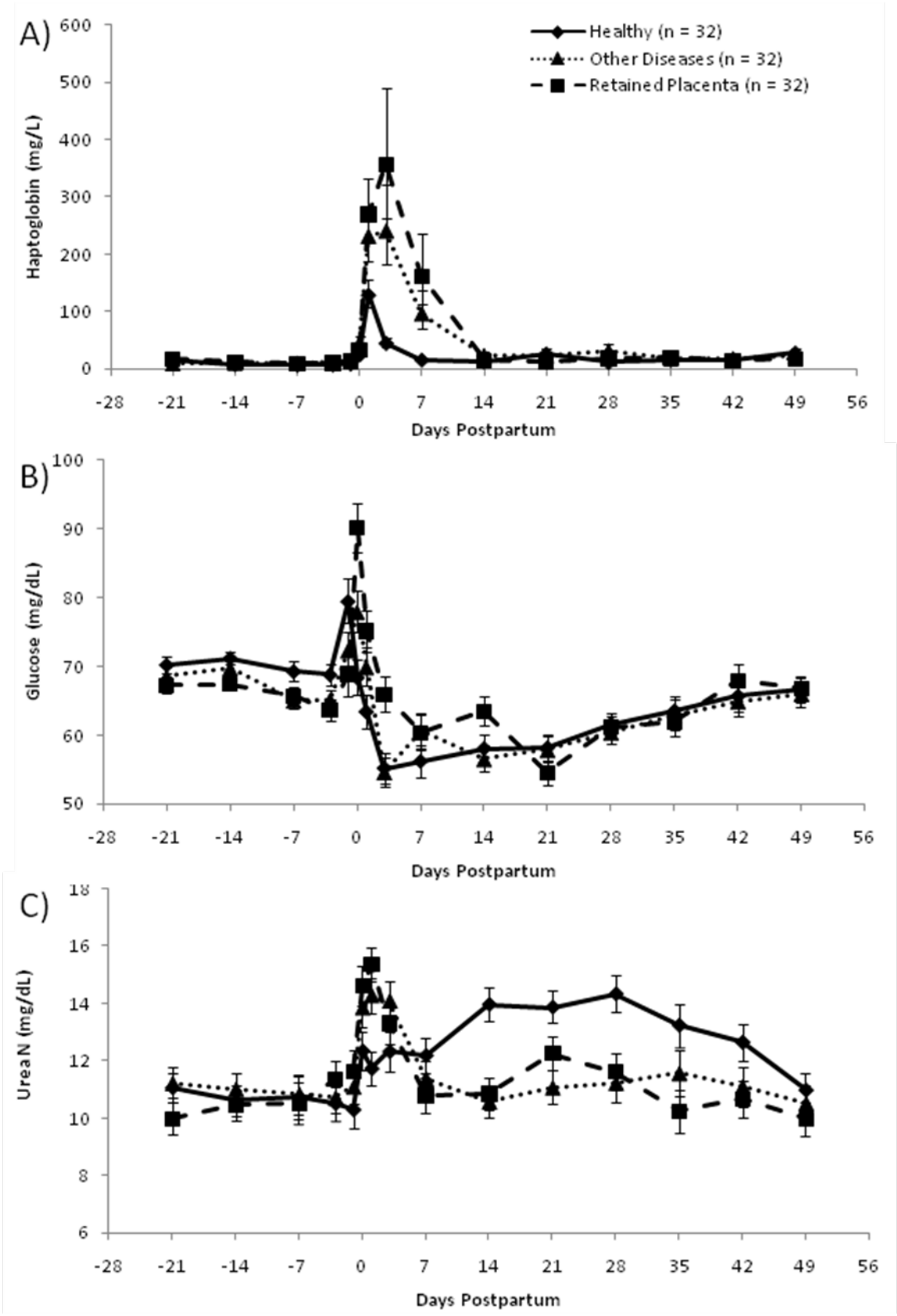
A)
Haptoglobin (mg/L)
600
500
400
300
200
100
0
Healthy (n = 32)
Other Diseases (n = 32)
Retained Placenta (n = 32)
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days Postpartum
B)
Glucose (mg/dL)
100
90
80
70
60
50
Days Postpartum
C)
Urea N (mg/dL)
18
16
14
12
10
8
6
Days Postpartum

Figure 3

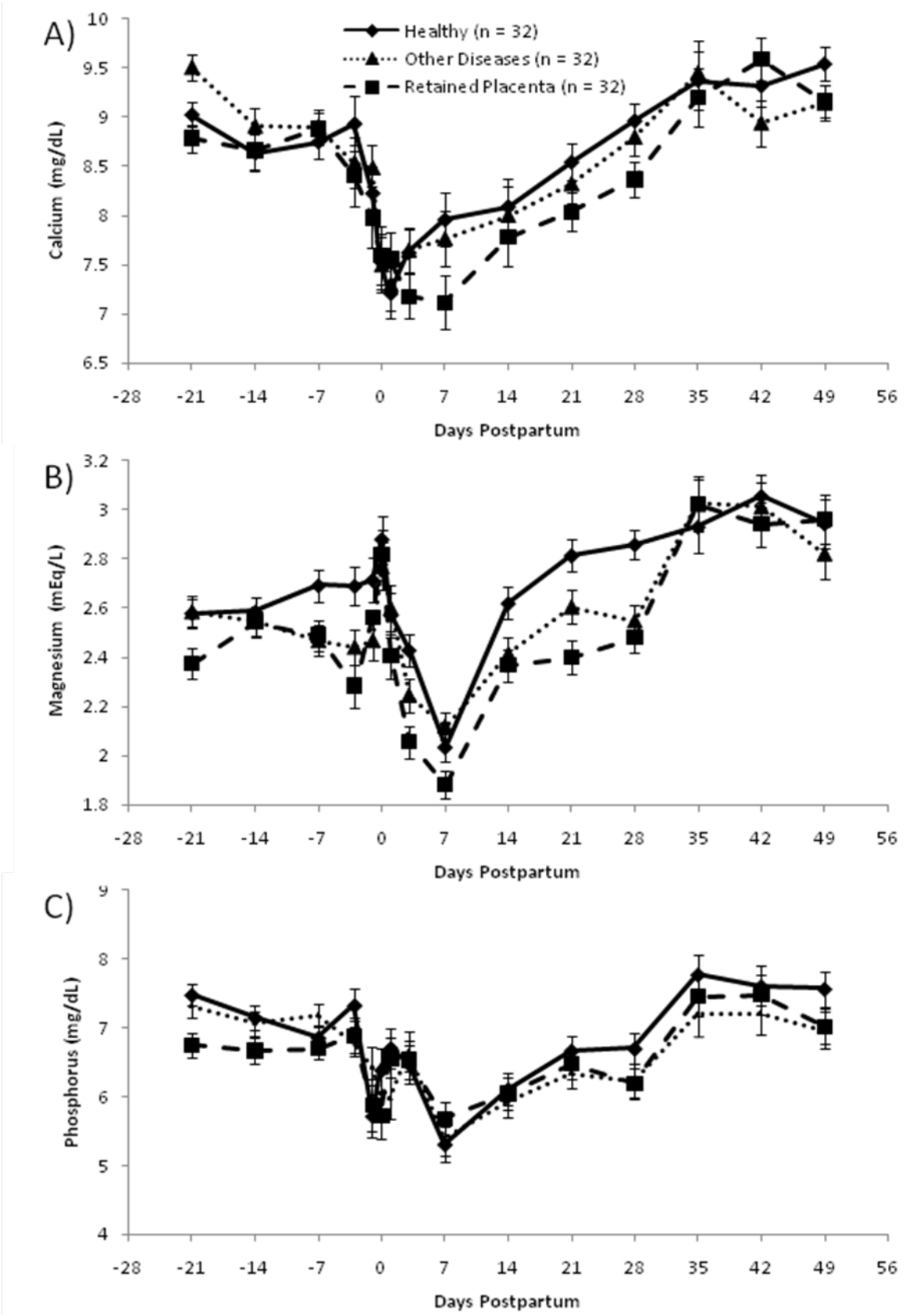
A)
Healthy (n = 32)
Other Diseases (n = 32)
Retained Placenta (n = 32)
Calcium (mg/dL)
10
9.5
9
8.5
8
7.5
7
6.5
-28 -21 -14 -7 0 7 14 21 28 35 42 49 56
Days Postpartum
B)
Magnesium (mEq/L)
3.2
3
2.8
2.6
2.4
2.2
2
1.8
-28 -21 -14 -7 0 7 14 21 28 35 42 49 56
Days Postpartum
C)
Phosphorus (mg/dL)
9
8
7
6
5
4
-28 -21 -14 -7 0 7 14 21 28 35 42 49 56
Days Postpartum

Figure 4

Table 1. Feed and nutrient composition of pre- and postpartum diets

	Percent of Diet Dry Matter	
Feed Composition	Prepartum[1]	Postpartum[2]
Grass Silage		2.13
Alfalfa Hay (20% CP, 36% NDF)	13.42	19.26
Corn Silage	27.77	20.93
Triticale Hay (9% CP, 60% NDF)	13.69	
Beet Pulp	3.41	
Vitamin &Mineral Premix[1]	4.95	
Vitamin &Mineral Premix[2]		2.96
MagOx[3]	0.18	
Ground Corn	18.15	
Corn (High Moisture Ear Corn)		20.00
Corn Distillers Grain (incl. solubles)	8.06	12.33
Canola Meal	6.69	6.40
Wheat Distillers Grain (incl. solubles)		5.97
Bakery By-Product		6.39
EnerGII Regular[4]	1.82	1.74
Limestone (ground)	1.85	0.94
Sodium Bicarbonate		0.94
Analyzed Nutrient Composition		
NE_L (Mcal/kg, DM basis)	1.63	1.70
CP	13.0	18.7
ADF	27.1	16.9
NDF	36.2	27.2
Ether Extract	3.47	6.18
Magnesium	0.46	0.32
Potassium	1.30	1.23
Sodium	0.072	0.243
Iron (mg/kg)	469	570
Zinc (mg/kg)	83	115
Copper (mg/kg)	23	23
Manganese (mg/kg)	65	91
Molybdenum (mg/kg)	0.6	0.5

[1]Provides to the diet DM 6.7 g/kg Ca as calcium propionate, -carbonate, and –chloride and mono-dicalcium phosphate, 1.4 g/kg P as mono-dicalcium phosphate, 8.0 g/kg Cl as ammonium and calcium chloride, 3.4 g/kg Mg as magnesium sulfate, 30 mg/kg K, 0.99 g/kg S as magnesium, manganese, copper, cobalt, and zinc sulfate, 0.17 mg/kg Co as cobalt sulfate, 15.2 mg/kg Cu as copper sulfate, 1.012 mg/kg iodine as ethylenediaminedihydroiodide, 7.7 mg/kg Mn as manganese sulfate, 0.31 mg/kg Se as sodium selenite, 29.9 mg/kg Zn as zinc sulfate, 10.8 KIU/kg Vitamin A, 4.6 KIU/kg Vitamin D3, 167 IU/kg Vitamin E as *all rac* α-tocopheryl acetate, 1.19 g/kg Choline, 1.00 g/kg Niacin, 26.8 mg/kg Monensin

[2] Provides to the diet DM 0.30 g/kg Ca, 0.23 g/kg P from ammonium polyphosphate, 0.20 g/kg Mg, 1.23 g/kg K, 0.21 g/kg Na, 0.19 g/kg Cl, 0.26 g/kg S, 0.07 mg/kg Co as cobalt sulfate, 0.05 mg/kg Co as organic cobalt, 12.4 mg/kg Cu as copper sulfate, 4.42 mg/kg Cu as organic copper, 1.76 mg/kg I as ethylenediaminedihydroiodide, 10.7 mg/kg Mn as manganese sulfate, 0.81 mg/kg Mn as organic manganese, 0.25 mg/kg Se as sodium selenite, 59.0 mg/kg Zn as zinc sulfate, 8.00 mg/kg Zn as organic zinc, 5.01 KIU/kg Vitamin A, 1.23 KIU/kg Vitamin D3, 24.5 IU/kg Vitamin E as *all rac* α-tocopheryl acetate, 0.25 g/kg Methionine

[3]Guaranteed to contain no less than 56% Mg

[4]Contains (DM Basis) 90.4% total fat and 9.6% Ca as calcium salts of long chain fatty acids from Inman (Clackamas, OR)

Table 2. Concentrations of serum indicators prior to diagnosis of retained placenta (RP)

Indicator	Group			Contrast (P-value)	
	Healthy (H)	Other Diseases (OD)	Retained Placenta (RP)	H vs. RP	OD vs. RP
	N = 32	N = 32	N = 32		
2/3 Weeks Prepartum:					
α-tocopherol (µM)	12.4 ± 0.6	10.0 ± 0.6	8.5± 0.6	<0.001	0.07
Cholesterol (mg/dL)	116± 4	114± 4	105 ± 4	0.09	0.15
ATOC/cholesterol[1]	4.01 ± 0.22	3.32 ± 0.18	3.12±0.17	0.001	0.40
NEFA (µEq/L)	184± 16	253± 23	300±27	<0.001	0.15
BHBA (mMol/L)	0.44 ± 0.02	0.57 ± 0.02	0.62 ±0.02	<0.001	0.008
Haptoglobin (mg/L)	13.4± 2.5	11.0± 2.0	14.3±2.8	0.79	0.25
Glucose (mg/dL)	70.4 ± 1.0	68.3 ±1.0	67.2±1.0	0.03	0.42
Urea N (mg/dL)	10.9 ± 0.4	10.9 ± 0.5	10.2±0.5	0.29	0.25
Calcium (mg/dL)	8.82 ± 0.11	9.12 ± 0.11	8.78±0.11	0.80	0.03
Magnesium (mg/dL)	2.61 ± 0.04	2.52 ± 0.05	2.48 ±0.05	0.05	0.48
Phosphorus (mg/dL)	7.20 ± 0.11	7.15 ± 0.12	6.70 ±0.12	0.003	0.005
Last Week Prepartum:					
α-tocopherol (µM)	12.6 ± 0.6	9.4 ± 0.6	8.6 ± 0.6	<0.001	0.32
Cholesterol (mg/dL)	122± 6	131± 6	117 ± 6	0.57	0.10
ATOC/cholesterol[1]	3.91 ± 0.23	2.78 ± 0.17	2.88 ±0.17	<0.001	0.66
NEFA (µEq/L)	247± 23	455± 44	497 ±48	<0.001	0.49
BHBA (mMol/L)	0.48 ± 0.02	0.57 ± 0.02	0.61 ±0.03	<0.001	0.20
Haptoglobin (mg/L)	8.9± 1.2	12.8± 2.0	12.2±1.9	0.21	0.38
Glucose (mg/dL)	71.2 ± 1.3	67.3 ±1.3	65.3 ±1.3	0.002	0.24
Urea N (mg/dL)	10.6 ± 0.5	10.7± 0.6	11.0±0.6	0.60	0.74
Calcium (mg/dL)	8.78± 0.17	8.64 ± 0.17	8.64 ±0.17	0.56	0.99
Magnesium (mg/dL)	2.68 ± 0.06	2.47 ± 0.06	2.46 ±0.06	0.009	0.84
Phosphorus (mg/dL)	6.74 ± 0.16	6.83 ± 0.17	6.66 ±0.17	0.71	0.44
First Day after Calving:					
α-tocopherol (µM)	10.3 ± 0.5	7.7 ± 0.5	7.1 ± 0.5	<0.001	0.41
Cholesterol (mg/dL)	94 ± 4	88 ± 4	91 ± 4	0.31	0.20
ATOC/cholesterol[1]	4.15 ± 0.22	3.22 ± 0.18	2.94 ± 0.16	<0.001	0.79
NEFA (µEq/L)	325 ± 31	496 ± 49	545 ± 54	<0.001	0.47
BHBA (mMol/L)	0.69 ± 0.04	0.71 ± 0.04	0.75 ± 0.04	0.33	0.48
Haptoglobin (mg/L)	24.2 ± 5.0	44.5 ± 11.4	34.1 ± 8.0	0.58	0.23
Glucose (mg/dL)	69.2 ± 2.6	75.1 ± 2.9	85.7 ± 3.3	<0.001	0.01
Urea N (mg/dL)	12.2± 0.7	13.7± 0.8	14.4±0.8	0.05	0.48
Calcium (mg/dL)	7.53 ±0.28	7.55 ±0.28	7.64 ±0.28	0.77	0.80
Magnesium (mg/dL)	2.89 ±0.10	2.75 ±0.10	2.77 ±0.10	0.43	0.88
Phosphorus (mg/dL)	6.27 ± 0.3	6.28 ± 0.31	5.97 ±0.31	0.51	0.47

[1]ATOC/cholesterol = α-tocopherol to cholesterol molar ratio (µM/mM)

CHAPTER 4DEPLETED SERUM VITAMIN E CONCENTRATIONS PRECEDE MILK FEVER IN MULTIPAROUS DAIRY COWS

INTERPRETIVE SUMMARY: **Depleted serum vitamin E concentrations milk fever in multiparous dairy cows.** By *Qu et al.* To determine the association between vitamin E status and retained placenta, serum vitamin E concentrations were measured between -3 and 7 weeks postpartum in multiparous cows that milk fever in early lactation and compared to those in healthy cows. Lower vitamin E concentrations preceded and persisted after milk fever, indicating lower serum α-tocopherol concentrations as a potential early indicator for developingmilk fever.

DEPLETED SERUM VITAMIN E CONCENTRATIONS PRECEDE MILK FEVER IN MULTIPAROUS DAIRY COWS

Qu, Y., A. N. Fadden, M. G. Traber, and G. Bobe.

Received

Accepted

ABSTRACT

Milk fever (MF), defined as the clinical manifestation of hypocalcemia within the first 48 hours after calving, is a costly disease in multiparous dairy cows that is associated with perturbations in calcium transport. We previously documented that depleted serum vitamin E (α-tocopherol) concentrations precede left displaced abomasum. We hypothesized that serum α-tocopherol concentrations are depleted before calving in cows that will develop MF, a gateway disorder for left displaced abomasum. Our objective was to compare prepartal serum α-tocopherol concentrations of multiparous dairy cows that either a) were healthy after calving, b) developed MF, or c) developed after calving other diseases on a commercial dairy herd. Using a nested case-control design, blood samples, taken at day -21, -14, -7, -3, -1, 0, 1, 3, 7, 14, 21, 28, 35, 42, and 49 postpartum from multiparous Holstein cows that a) were healthy during the sampling period (n=10), b) developed clinical signs of MF (n=9), or c) developed in the first 28 days after calving other diseases (n=31), such as mastitis, metritis, laminitis, or ketosis, were analyzed for serum concentrations of α-tocopheroland indicators of energy and nutrient status and inflammation. Cows that later developed MF had 37% lower prepartal serum α-tocopherol concentrations (9.0 ± 0.9 vs. 14.2 ± 0.8 μM; $P< 0.001$) and 35% lower α-tocopherol to cholesterol molar ratios (3.08 vs. 4.78 μM/mM; $P< 0.001$) compared to healthy cows. These group differences were already significant three weeks before calving for α-tocopherol concentrations (8.3 ± 0.9 vs. 13.8± 0.8 μM; $P< 0.001$) and α-tocopherol to cholesterol molar ratios (2.86vs. 4.18 μM/mM; $P = 0.003$). Up to day 28postpartum, serum α-tocopherol concentrations remained lower in MF versus healthy cows (<9 vs. ~13 μM; all $P < 0.002$). These

findings suggest lower serum α-tocopherol concentrations as potential early indicator for developing of MF in multiparous cows.

Key Words: dairy cow, milk fever, vitamin E.

INTRODUCTION

Clinical milk fever (**MF**) is defined as the clinical manifestation of hypocalcemia within the first 48 hours after calving. Insufficient concentrations of soluble calcium and magnesium impair motor and nerve function in the cows, decrease feed intake, and suppress the immune system (Goff, 2008). Milk fever is an economically important disease that affects approximately 4.9% (range 0.03 to 22.3%) of US dairy cows (Kelton et al., 1998; USDA, 2009). The average cost, including treatment costs, milk loss, and increased days open, is estimated to be $335 per case (Kelton et al., 1998). Not included in the cost estimate are that MF cows have increased incidence rates of other metabolic and infectious diseases (Curtis et al., 1983). The primary risk factors associated with milk fever is diet, parity (more prevalent in older cows), obesity, and season(Goff, 2008).

α-Tocopherol, the most abundant and biologically active form of vitamin E, is a lipid soluble antioxidant that prevents PUFA oxidation (as reviewed by Baldi, 2005; Traber and Atkinson, 2007; Politis, 2012). Current NRC recommendations for supplemental α-tocopherol in dairy cows are 1.6 IU/kg BW (approximately 80 IU/kg DMI) during the dry period and 0.8 IU/kg BW (approximately 20 IU/kg DMI) during lactation (NRC, 2001). Dietary vitamin E requirements are elevated in early lactation because lipid peroxidation is increased (Castillo et al., 2006; Sordillo and Aitken, 2009) and significant amounts of vitamin E are secreted in the colostrum (Weiss et al., 2009). Depressed feed intake, inflammation, and low lipid absorption and transport may decrease dietary vitamin E utilization (Baldi, 2005).

The role of vitamin E in the etiology of MF is not known. Goff et al. (1990) reported for Jersey cows and Sivertsen et al. (2005) for Norwegian dairy cows reported that cows

with or without MF had similar plasma ATOC concentrations. Furthermore, prepartal ATOC alimentation did not affect incidence of MF (Erskine et al., 1997; LeBlanc et al., 2002). However, based on the association between depleted α-tocopherol concentrations in cows with diseases that often follow MF, we hypothesized that depleted α-tocopherol concentrations precede MF and remain after MF, which, to our knowledge, has not been examined. Thus, the objective of this study was to determine serum α-tocopherol concentrations of multiparous dairy cows with secondary MF during the first mo of lactation and without disease between -3 to 7 wk postpartum.

MATERIALS AND METHODS

Animals and Study Design

All procedures involving animals were approved by the Oregon State University Institutional Animal Care and Use committee. The research was conducted on a 1,000-head commercial dairy farm in Oregon's Central Willamette Valley during Spring and Summer 2010. The cohort consisted of 161 multiparous Holstein cows (parity 2 to 7). Nine cows (5 cows in 3rd parity, 3 cows in 4th parity, and 1 cow in 5th parity) that were treated for MF and had serum calcium concentrations below 6 mg/dL within 48 h after calving (Milkfever), 10 healthy cows that were not treated for diseases during the sampling period (Healthy), and 31 cows with other diseases (Other Diseases; treated for ketosis, metritis, laminitis, or mastitis during the first 28 d postpartum) that were similar in calving month and age, were selected for this nested case-control study.

During the last 4 wk before expected calving, cows were housed in a straw-bedded free stall barn and were fed once in the morning (7:30) a TMR based on corn, corn silage, and alfalfa and triticale hay, which met NRC guidelines (NRC, 2001) and contained supplemental vitamin E at 167 IU/kg DM (**Table 1**). After calving, healthy cows stayed the first 2 d in the hospital pen, and then for 4 wk in the early lactation pen, and then based on body size in 3 mid-lactation pens. Cows from the hospital, early lactation, and the mid-lactation pen were fed at 7:00, 9:00, 10:00, respectively, and 13:30 for all cows, a TMR based on corn, corn silage, and alfalfa hay, which met NRC guidelines (NRC, 2001) and contained supplemental vitamin E at 24.5 IU/kg DM (**Table 1**).

Starting 28 d before predicted calving date, BCS of cows were scored weekly until 4 wk postpartum and then at wk 7 and 14 postpartum (Edmonson et al., 1989). During the study period, cows were monitored daily for flakes in the milk, gait, appetite, general appearance, alertness, vaginal discharge, and retained placenta. Uterine discharge was checked twice a week, and urinary ketones and body temperature were checked if cow appeared not healthy. Medical treatments were administered based on the standard operating procedures of the dairy farm. For milk fever: if a cow appeared lethargic, the cow remained in the hospital pen and received i.v. 0.5 L CMPK (calcium-magnesium-phosphorus-potassium-dextrose solution; Aspen Veterinary Resources® Ltd, Liberty MO) and 0.5 L of dextrose (50% dextrose; Veterinary Resources® Ltd, Liberty MO) and orally a 10 gal drench [2 lbs Fresh Cow Drench (TPi, Madera CA) and 8 ounces of propylene glycol dissolved in 10 gal water]. If a cow could not stand (only one cow was recumbent), the cow received in addition i.v. 0.5 L of Milk Fever CP™ (calcium borogluconate, 26% w/v; dextrose: 15% w/v; magnesium borogluconate: 6% w/v; Aspen Veterinary Resources® Ltd, Liberty MO).

Blood Collection and Analysis

Blood samples were taken at d -21 (-24 to -18), -14 (-17 to -11), -7 (-10 to -5), -3 (-4 or -3), -1 (-2 or -1), 0, 1, 3, 7, 14, 21, 28, 35, 42, and 49 postpartum within 10 min after morning feeding. Blood (5 to 8 mL) was obtained from the coccygeal vein or artery in 10 mL serum vacutainer tubes (BD Vacutainer® Plus Plastic Serum Tubes, BD Diagnostics, Franklin Lakes, NJ), placed on ice, and transported to the laboratory, where serum was separated by centrifugation at room temperature for 20 min at 1600 x *g*. Serum samples were stored at -20°C until chemical analysis.

Serum α-tocopherol concentrations were measured using a reversed-phase Phenomenex Synergi 4 µM Hydro-RP, 150×4.6 mm column and a SecurityGuard™ cartridges AQ C18 pre-column, 3.0 mm i.d. (Phenomenex, Torrance, CA) with a LC-4B amperometric electrochemical detector (Bioanalytical Systems Inc., West Lafayette, IN), following Podda et al. (1996). An isocratic mobile phase of 99:1 (v:v) methanol:water containing 0.1% (w:v) lithium perchlorate was used with a run time of 9 min and the electrochemical detector set at 500 mV. After 100 µL serum were saponified in alcoholic KOH with 1% ascorbic acid, the sample was extracted with hexane and dried, and the residue resuspended in ethanol:methanol (1:1). A 20-µL aliquot of the extract was injected into the HPLC system. Serum concentrations of cholesterol (Stanbio Cholesterol LiquiColor® Procedure No. 1010; Stanbio Laboratory, Boerne, TX), glucose (Stanbio Glucose Proc. No. 1075; Stanbio), NEFA (ACS ACOD method, WAKO Diagnostics, Richmond, VA), BHBA (Stanbio BHBA LiquiColor® Proc. No. 2440; Stanbio), urea N (Stanbio Urea Nitrogen Liqui-UV® Proc. No. 2020; Stanbio), haptoglobin (bovine-specific ELISA kit Catalog No. 2410-70; Life Diagnostics, Inc., West Chester, PA), calcium (Stanbio Total Calcium LiquiColor® Proc. No. 0150; Stanbio), magnesium (Stanbio Magnesium LiquiColor® Proc. No. 0130; Stanbio), and phosphorus (Stanbio Phosphorus Liqui-UV® Proc. No. 0830; Stanbio) were measured according to manufacturer's instructions using a FLUOstar Omega microplate autoreader (BMG Labtech Inc, San Francisco, CA).

Statistical Analysis

Data was analyzed as repeated-measures-in-time ANOVA study using the PROC MIXED procedure of SAS version 9.2 (SAS Institute, 2009). The molar ratio of α-

tocopherol to cholesterol was calculated to adjust for changes in lipid transport (Traber and Jialal, 2000) and stage of lactation (Weiss, 1998). To achieve a normal distribution for their serum concentrations, concentrations of glucose, NEFA, and the α-tocopherol to cholesterol molar ratio were ln-transformed, concentrations of haptoglobin and BHBA were twice ln-transformed, concentrations of cholesterol were square-root transformed, and concentrations of phosphorus >11 mg/dL were set at 11 mg/dL. The variance-covariance structure of repeated measures within cow was modeled using the heterogeneous first-order autoregressive variance-covariance matrix. Fixed effects were MF incidence (Healthy, Other Diseases, Milkfever), sampling time, and the interaction between MF incidence and sampling time. To obtain the correct degrees of freedom, the KENWARDROGER option was invoked.

To compare our results with previously published studies and identify early indicators of MF, average serum concentrations in the last 3 wk and in the last wk before calving were calculated using the trapezoidal rule and analyzed in PROC GLM with MF status as fixed effect. In addition, we compared changes in concentrations between the average of 3 and 2 wk and 1 wk prepartum and concentrations at d 0 between groups in PROC GLM with MF status as fixed effect. Values presented in the figures and tables are least-squares means (**LSM**) and their standard errors (**SEM**) that are transformed back to their original measurement scale. All statistical tests were two-sided. Significance was declared at $P \leq 0.05$ and a tendency at 0.05 to 0.10.

RESULTS

The incidence rate of MF in this study cohort was 5.6% (9 of 161 cows). Cows with MF were either their third (5 cows), fourth (3 cows), or sixth parity (1 cow) and showed signs of MF either in the first 24 h of calving (8 cows) and in the following 24 h (1 cow). During the first 28 d after calving, 3 cows were treated for metritis, 3 cows for mastitis, 3 cows for unspecified reasons, 2 cows for retained placenta, 1 cow for laminitis, 1 cow for ketosis, and 1 cow for left displaced abomasum. Except for one MF cow, all other cows developed 1 or more diseases after MF. The 10 healthy cows did not show signs of clinical diseases during the sampling period and had relatively normal serum concentrations of BHBA (<1.2 mM as cut off between healthy and subclinical ketosis; McArt et al., 2012), calcium (>6 mg/dL as cut off between clinical milkfever and subclinical milk fever or inflammation-associated hypocalcemia; Goff, 2008; Reinhardt et al., 2011), and magnesium (>1.15 mg/dL as cut off between hypomagnesemia and subclinical tetany; Goff, 2008). Cows with other diseases had all calcium concentrations above 6 mg/dL during the first 48 hafter calving and were treated for diseases during the first 28 days postpartum: 11 cows were treated for laminitis, 8 cows for ketosis, and 7 cows each for metritis and for mastitis.

Serum Vitamin E, Cholesterol, and Milkfever

Cows that later developed MF had 37% lower prepartal serum α-tocopherol concentrations (9.0 ± 0.9 vs. 14.2 ± 0.8 µM; $P< 0.001$) and 35% lower α-tocopherol to cholesterol molar ratios (3.08 vs. 4.78 µM/mM; $P< 0.001$) compared to healthy cows (**Figure 1A**). These group differences were already significant 3 weeks before calving

for α-tocopherol concentrations (8.3 ± 0.9 vs. 13.8± 0.8 μM; $P< 0.001$) and α-tocopherol to cholesterol molar ratios (2.86vs. 4.18 μM/mM; $P = 0.003$). Sampling time affected serum α-tocopherol concentrations and α-tocopherol to cholesterol molar ratios (both $P < 0.0001$), with concentrations decreasing dramatically in the first wk postpartum in all cows (**Figure 1A**). The nadir α-tocopherol concentrations at d 7 postpartum were lower in cases than healthy controls (4.2 ± 0.5 μM vs. 7.9 ± 0.5 μM; $P < 0.001$). Up to day 28postpartum, serum α-tocopherol concentrations remained lower in MF versus healthy cows (<9 vs. ~13 μM; all $P < 0.002$; **Figure 1A**). Serum α-tocopherol concentrations of cows with other diseases were between those of healthy control and of MF cows but, except for the day of calving, were not significantly different from MF cows (**Figure 1A**).

Serum cholesterol changed differently over time in healthy cows, MF cows, and cows with other diseases ($P_{Interaction}< 0.001$; **Figure 1B**). Cholesterol concentrations were similar in the 3 groups until calving, but were lower in MFor disease control than in healthy control cowsbetween d 7 and 21 postpartum (all $P< 0.01$; **Figure 1B**).

Energy Status and Milkfever

Cows with MF had on average higher NEFA concentrations ($P = 0.005$) than healthy cows ($P= 0.03$; **Figure 2A**). The MF effect, however, was significant only for d -14, -1, and 0 postpartum. Higher BHBA concentrations were only observed in cows with MF at d -1 postpartum than in healthy controls (**Figure 2B)**. Group differences between cows with other diseases and with MF were observed only at d -21 postpartum for NEFA (**Figure 2A**).

Inflammation and Milkfever

Cows with MF had higher haptoglobin concentrations than healthy cows between d 3 and 21 postpartum (all $P < 0.03$; **Figure 3A**). Group differences between cows with other diseases and MF were only observed at d 28 postpartum for haptoglobin (**Figure 3A**). No significant overall effects were observed for serum glucose concentrations; glucose concentration were, however, greater at d 0 to 3 postpartum in cases compared with healthy cows and greater at d -7, 3, and 14 in cases compared with cows with other diseases (**Figure 3B**). Serum urea N was not significantly affected by MF (**Figure 3C**). The only significant differences were observed between MF and healthy cows at d 1 postpartum and between cases and disease controls at d 28 postpartum (**Figure 3C**).

Macromineral Status and Milkfever

Cases had on average lower serum calcium concentrations ($P = 0.001$) than healthy cows and tended to have lower serum calcium concentrations ($P = 0.08$) than cows with other diseases (**Figure 4A**). The only significant differences wer observed between healthy and MF cows at d 0, 3, and 7 postpartum and between MF cows and cows with other diseases at d 0 postpartum (**Figure 4A**). Serum magnesium N was not significantly affected by MF (**Figure 4B**). Cows with MF had on average lower phosphorus concentrations than healthy cows ($P< 0.001$) and cows with other diseases ($P = 0.04$; **Figure 4C**). The MF effect, however, was significant only for d -3, 0, 7 to 28, and 49 postpartum compared to healthy cows and at d1 postpartum compared with cows with other diseases ($P= 0.02$; **Figure 4C**).

Early Serum Indicator of Milkfever

To identify early indicators of MF, we compared the average serum concentrations of MF cases and healthy and disease controls before MF diagnosis (**Table 2**). Differences in α-tocopherol concentrations and α-tocopherol to cholesterol molar ratio were apparent already 2 to 3 weeks before calving between healthy cows and cows with other diseases and became more pronounced until calving (**Table 2**). Cows that subsequently developed MF had 35% lower serum α-tocopherol concentrations during 2 and 3 wk prepartum, 39% lower concentrations during the last wk postpartum, and 47% lower concentrations during the first 24 h after calving compared with healthy cows. At calving, MF cows had also lower α-tocopherol concentrations than cows with other diseases. Similar trends were also observed for α-tocopherol to cholesterol ratios (**Table 2**).

Besides difference in α-tocopherol and α-tocopherol to cholesterol ratios, no significant group differences between MF cases and controls were observed 2 to3 wk prepartum for other blood parameters (**Table 2**). In the last wk prepartum and at the day of calving, MF cows had greater NEFA and lower phosphorus concentrations than control cows, which were also observed for NEFA at the day of calving (**Table 2**). In addition, MF cows had higher glucose and lower calcium concentrations at the day of calving (**Table 2**). Between 2 and 3 wk prepartum and the last wk prepartum, increases in NEFA concentrations were observed in MF and disease control cows but not in healthy cows (**Table 2**).

DISCUSSION

Our study demonstrates that serum α-tocopherol concentrations may be a useful diagnostic parameter for MF, because group differences in α-tocopherol concentrations and α-tocopherol to cholesterol molar ratio began prepartum and became more pronounced until calving. Secondly, our study suggests that negative energy balance and lower vitamin E and phosphorus concentrations may precede MF. Thirdly, our study indicates that cows remain in a lower vitamin E, calcium, and phosphorus status for the first 4 weeks after calving.

Serum α-tocopherol concentrations in healthy cows followed similar trends, as has been previously described (Meglia et al., 2006; Weiss et al., 2009), significantly decreasing in wk 1 after calving and then increasing within 3 weeks back to prepartum concentrations (**Figure 1A**). Circulating lipoprotein concentrations, including α-tocopherol, are lower around calving (Herdt and Smith, 1996). Hormonal changes around calving and inflammation suppress lipid transport (Katoh, 2002). In contrast, dietary fat and α-tocopherolalimentation increase α-tocopherol concentrations in blood (Weiss et al., 1994; Weiss and Wyatt, 2003). A decrease in α-tocopherol concentrations during wk 1 postpartum even occurs after vitamin E supplementation (Meglia et al., 2006; Weiss et al., 2009) and is thought to result from a combination of increased lipid peroxidation and production of reactive oxygen species, increased secretion of α-tocopherol into colostrum and milk, depressed feed intake, inflammation, and decreased lipid absorption and transport (Baldi, 2005). Serum α-tocopherol concentrations in this study were on the higher end of what have been previously reported (LeBlanc et al., 2004; Weiss et al., 2009; Bouwstra et al., 2010).

Cholesterol concentrations in our study (**Figure 1B**) were similar to those reported previously (Herdt and Smith, 1996; Guzelbektes et al., 2010; Stengärde et al., 2010). Blood cholesterol, which is primarily in the HDL fraction, is considered an indicator of lipoprotein concentrations and decreased with α-tocopherol around calving (Herdt and Smith, 1996). Fat feeding and feed restriction increase cholesterol concentrations for increased lipid transport (Weiss and Wyatt, 2003; Bjerre-Harpøth et al., 2012), whereas heat and inflammation-associated diseases, in particular liver disorders, decrease cholesterol by impairing lipid transport (Bobe et al., 2004; Abeni et al., 2007; Vogel et al., 2011). Because serum lipoproteins transport α-tocopherol in blood (Traber and Jialal, 2000), α-tocopherol is usually divided by cholesterol concentrations to adjust for changes in lipid transport (Herdt and Smith, 1996).

Cows with MF had 47% lower serum α-tocopherol concentrations and 46% lower α-tocopherol to cholesterol molar ratios than healthy cows at the day of calving. This study is, to our knowledge, the first report of serum α-tocopherol concentrations in Holstein cows with or without MF. No significantly differences in α-tocopherol concentrations had been previously reported for cows with or without MF in Jersey (Goff et al., 1990) and Norwegian cattle (Sivertsen et al., 2005). Similar differences in α-tocopherol concentrations had been previously reported for cows after LDA diagnosis (Mudron et al., 1997; Hasanpour et al., 2011) and in cows with severe fatty liver (Hidiroglou and Hartin, 1982). A lower DMI might be a probable causative factor for the lower serum α-tocopherol concentrations in MF cows, but in the absence of DMI data in our study we cannot determine whether or not lower α-tocopherol concentrations in LDA cows were independent of feed intake. This should be addressed in future studies. Goff et al. (2002)

suggested, based on their results in mastectomized cows, that besides feed intake other factors, such as increased oxidation, play a role in lower α-tocopherol in blood around calving.

Similar to our results, elevated prepartal NEFA concentrations have been previously reported in cows that will develop MF (Oikawa and Katoh, 2002; Melendez et al., 2009, Moyes, 2013), establishing negative energy balance as another prognostic indicator of MF. In addition, lower serum concentrations of cholesterol, calcium, and phosphorus have been reported in cows with MF (Oikawa and Katoh, 2002; Stearič and Zadnik, 2010). Our results suggest that lower phosphorus concentrations start before calving and are getting more pronounced as calving nears. Ménard and Thompson (2007) that low phosphorus concentrations is an indicator for poor treatment response. Future studies are needed to examine the relationship between blood phosphorus status and MF.

Elevated postpartal haptoglobin concentrations have been reported as indicator of MF in one study (Crawford et al., 2005) but not in another study (Skinner et al., 1991). We observed elevated haptoglobin concentrations in MF cows in the first 2 weeks after calving. No differences between MF and healthy cows in haptoglobin concentrations were observed before calving. Our study is, to our knowledge, the first report of serum concentrations of haptoglobin prior to MF.

After MF treatment, serum concentrations of α-tocopherol and nutrient status (cholesterol, calcium, and phosphorus) remained lower in cows that had MF than in healthy cows between 2 and 4 weeks after calving in this study. Those are similar with previous studies that the low situation of cholesterol, calcium and posphours after calving in MF cows (Oikawa and Katoh, 2002). Unfortunately, we did not measure feed intake

in this study and, thus, cannot determine whether the persistent lower nutrient status is a consequence of lower DMI, lower nutrient absorption, or a combination of both.

A strength of the current study is the intensive blood sampling schedule over 10 wk (-3 to 7 wk postpartum) that allowed us to demonstrate that lower α-tocopherol concentrations preceded MF and remained after MF correction. This is a retrospective case-control study and, thus, can only establish associations between serum vitamin E concentrations and MF and other diseases. It should be noted that lower serum α-tocopherol concentrations may be a biomarker of disease, low feed intake, or both.

CONCLUSION

Serum concentrations of α-tocopherol and indicators of energy and nutrient status and inflammation were measured during -3 to 7 wk postpartum in dairy cows with MF and were compared with those concentrations in healthy cows. Negative energy balance and lower α-tocopheroland phosphorus concentrations all preceded MF onset and, thus, might be potential early indicators for developing MF. Cases remained in lower nutrient status including vitamin E for at least 4 wk after MF correction. This study was a small study with a limited number of MF cows in a single commercial herd; larger studies under well-controlled conditions are warranted to examine the role of vitamin E in MF.

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Figure Legends

Figure 1. Serum concentrations (LSM ± SEM) of A) α-tocopherol and B) cholesterol between day -21 and 49 postpartum in healthy cows (Healthy), cows with other diseases (Other Diseases), and cows with milk fever (Milkfever).

Figure 2. Serum concentrations (LSM ± SEM) of A) NEFA and B) BHBA between day -21 and 49 postpartum in visually healthy cows (Healthy), cows with other diseases (Other Diseases), and cows with milk fever (Milkfever).

Figure 3. Serum concentrations (LSM ± SEM) of A) haptoglobin, B) glucose, and C) urea N between day day -21 and 49 postpartum in visually healthy cows (Healthy), cows with other diseases (Other Diseases), and cows with milk fever (Milkfever).

Figure 4. Serum concentrations (LSM ± SEM) of A) calcium, B) magnesium, and C) phosphorus between day -21 and 49 postpartum in visually healthy cows (Healthy), cows with other diseases (Other Diseases), and cows with milk fever (Milkfever).

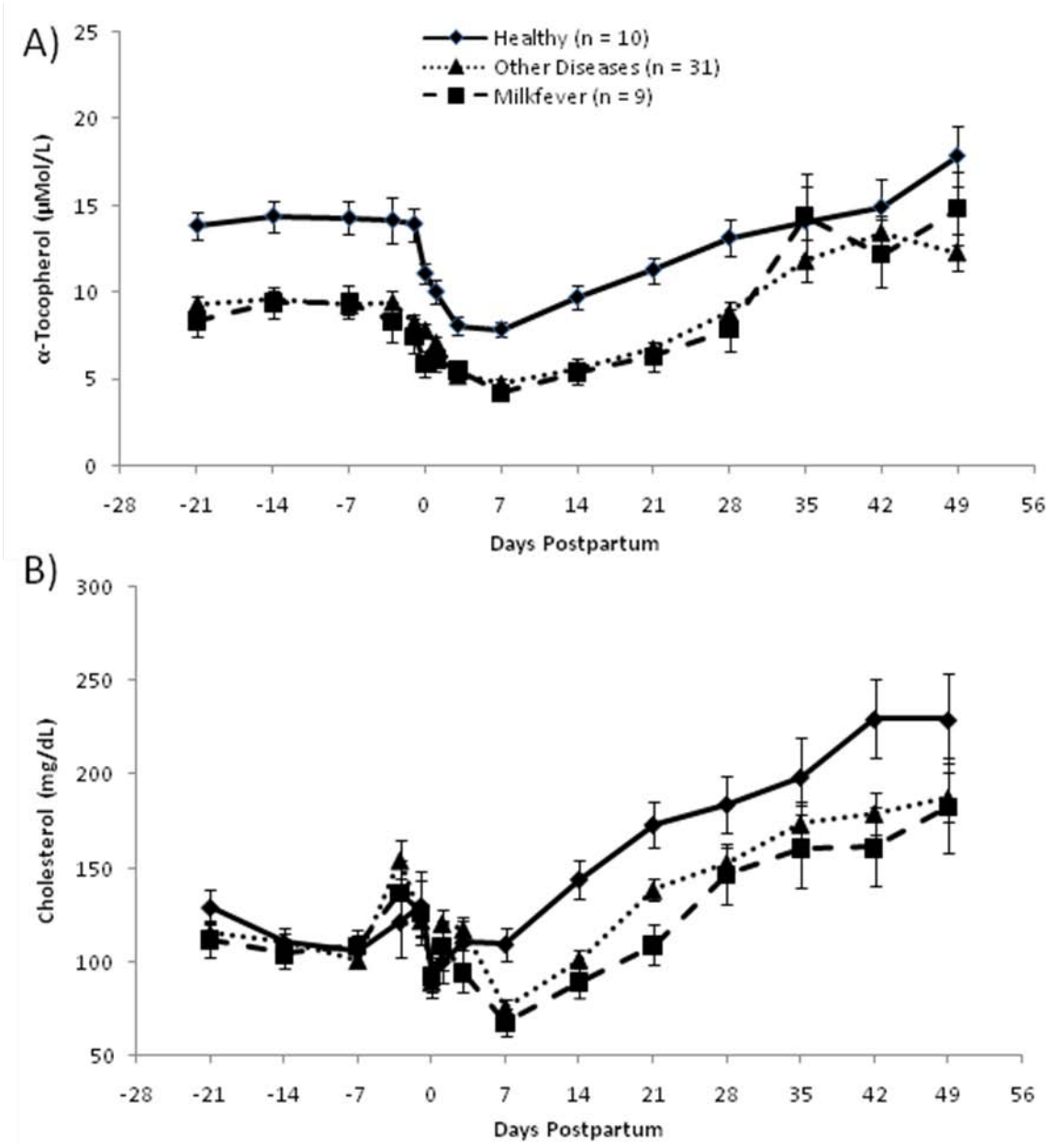
A)
Healthy (n = 10)
Other Diseases (n = 31)
Milkfever (n = 9)
α-Tocopherol (µMol/L)
25
20
15
10
5
0
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days Postpartum
B)
Cholesterol (mg/dL)
300
250
200
150
100
50
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days Postpartum

Figure 1

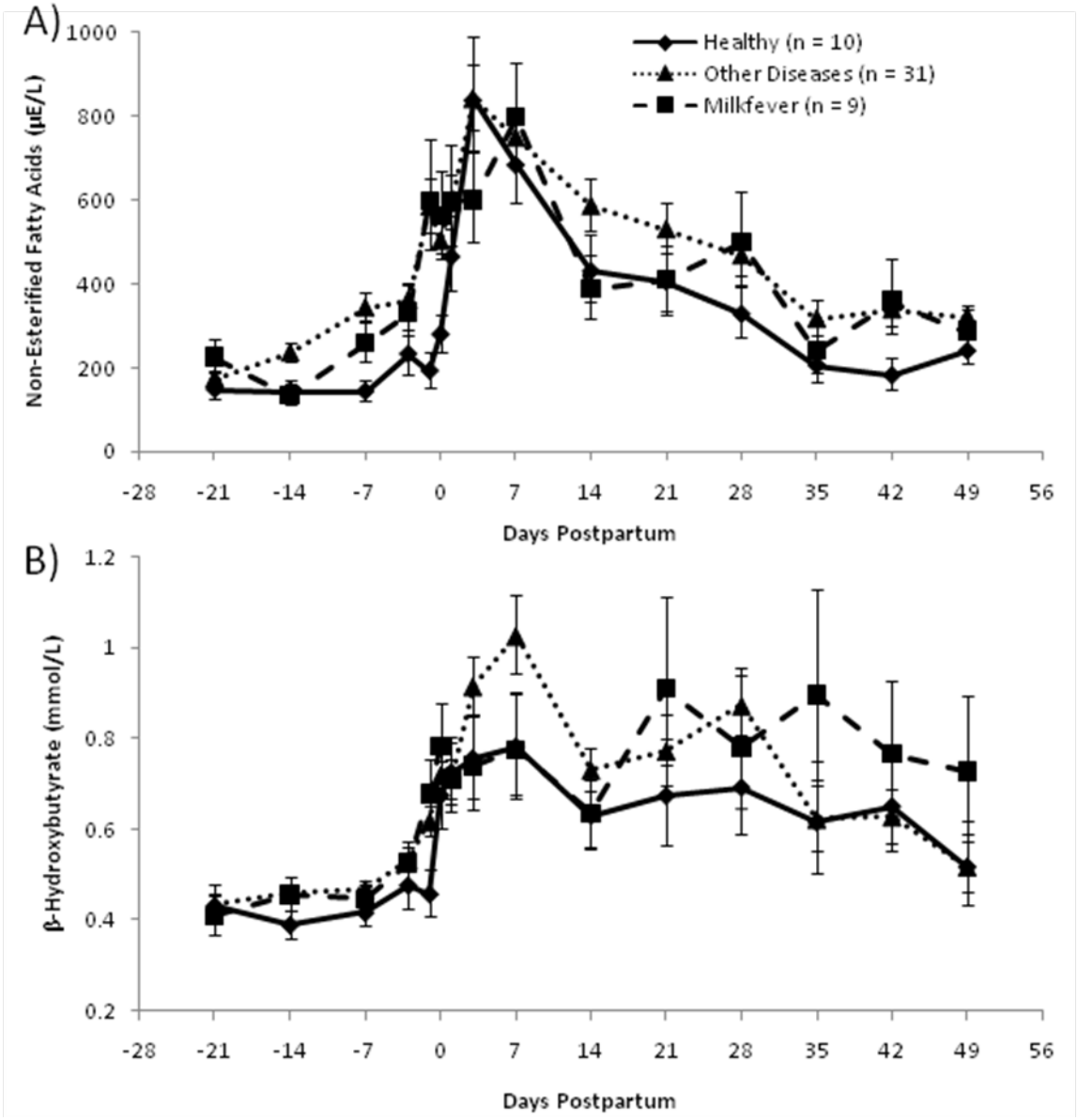
A)
Non-Esterified Fatty Acids (µE/L)
1000
800
600
400
200
0
Healthy (n = 10)
Other Diseases (n = 31)
Milkfever (n = 9)
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days Postpartum
B)
β-Hydroxybutyrate (mmol/L)
1.2
1
0.8
0.6
0.4
0.2
Days Postpartum

Figure 2

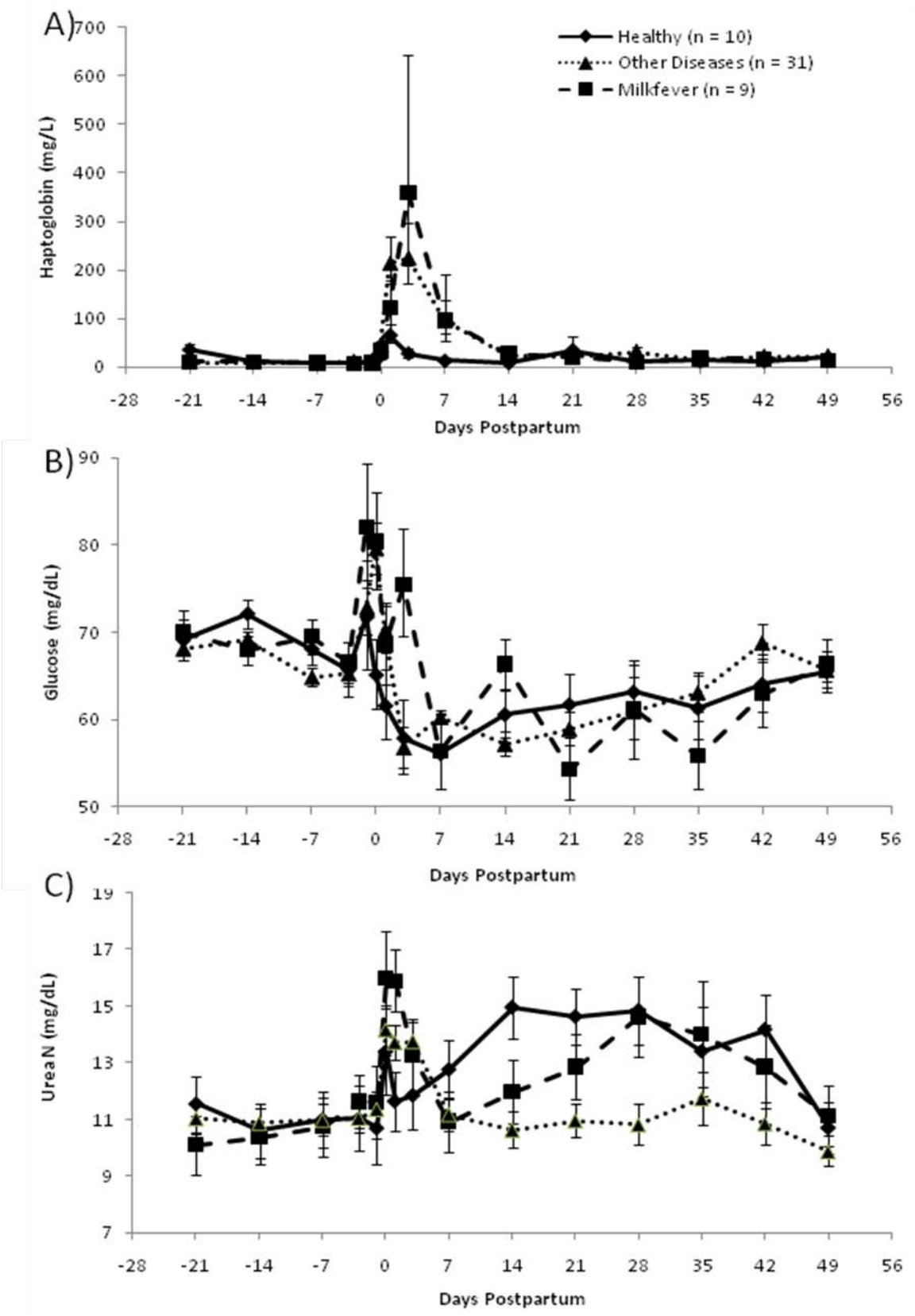
A)
Healthy (n = 10)
Other Diseases (n = 31)
Milkfever (n = 9)
Haptoglobin (mg/L)
0
100
200
300
400
500
600
700
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days Postpartum
B)
Glucose (mg/dL)
50
60
70
80
90
Days Postpartum
C)
Urea N (mg/dL)
7
9
11
13
15
17
19
Days Postpartum

Figure 3

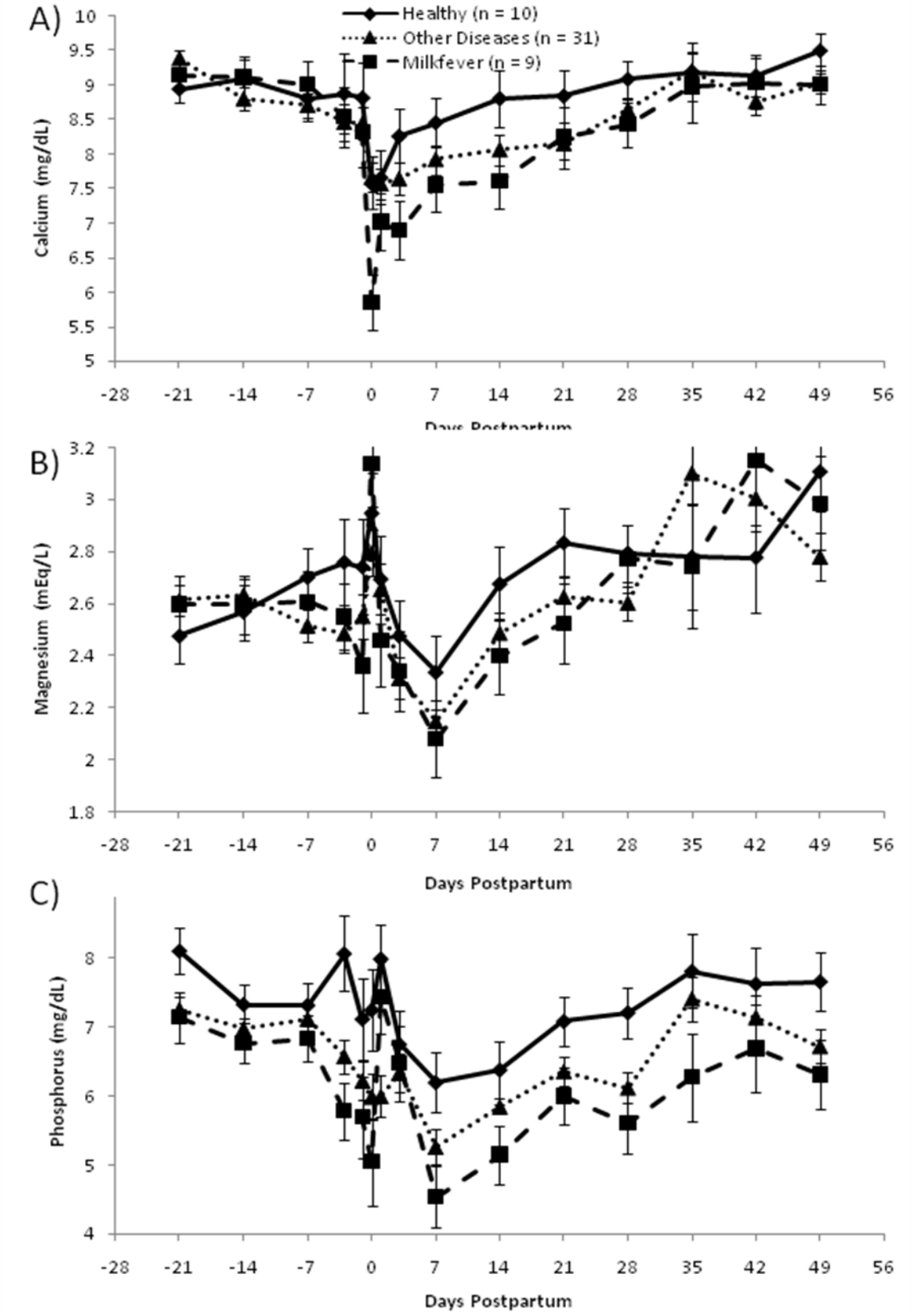
A)
Healthy (n = 10)
Other Diseases (n = 31)
Milkfever (n = 9)
Calcium (mg/dL)
10
9.5
9
8.5
8
7.5
7
6.5
6
5.5
5
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
B)
Magnesium (mEq/L)
3.2
3
2.8
2.6
2.4
2.2
2
1.8
Days Postpartum
C)
Phosphorus (mg/dL)
8
7
6
5
4
Days Postpartum

Figure 4

Table 1. Feed and nutrient composition of pre- and postpartum diets

	Percent of Diet Dry Matter	
Feed Composition	Prepartum[1]	Postpartum[2]
Grass Silage		2.13
Alfalfa Hay (20% CP, 36% NDF)	13.42	19.26
Corn Silage	27.77	20.93
Triticale Hay (9% CP, 60% NDF)	13.69	
Beet Pulp	3.41	
Vitamin &Mineral Premix[1]	4.95	
Vitamin &Mineral Premix[2]		2.96
MagOx[3]	0.18	
Ground Corn	18.15	
Corn (High Moisture Ear Corn)		20.00
Corn Distillers Grain (incl. solubles)	8.06	12.33
Canola Meal	6.69	6.40
Wheat Distillers Grain (incl. solubles)		5.97
Bakery By-Product		6.39
EnerGII Regular[4]	1.82	1.74
Limestone (ground)	1.85	0.94
Sodium Bicarbonate		0.94
Analyzed Nutrient Composition		
NE_L (Mcal/kg, DM basis)	1.63	1.70
CP	13.0	18.7
ADF	27.1	16.9
NDF	36.2	27.2
Ether Extract	3.47	6.18
Magnesium	0.46	0.32
Potassium	1.30	1.23
Sodium	0.072	0.243
Iron (mg/kg)	469	570
Zinc (mg/kg)	83	115
Copper (mg/kg)	23	23
Manganese (mg/kg)	65	91
Molybdenum (mg/kg)	0.6	0.5

[1]Provides to the diet DM 6.7 g/kg Ca as calcium propionate, -carbonate, and –chloride and mono-dicalcium phosphate, 1.4 g/kg P as mono-dicalcium phosphate, 8.0 g/kg Cl as ammonium and calcium chloride, 3.4 g/kg Mg as magnesium sulfate, 30 mg/kg K, 0.99 g/kg S as magnesium, manganese, copper, cobalt, and zinc sulfate, 0.17 mg/kg Co as cobalt sulfate, 15.2 mg/kg Cu as copper sulfate, 1.012 mg/kg iodine as ethylenediaminedihydroiodide, 7.7 mg/kg Mn as manganese sulfate, 0.31 mg/kg Se as sodium selenite, 29.9 mg/kg Zn as zinc sulfate, 10.8 KIU/kg Vitamin A, 4.6 KIU/kg Vitamin D3, 167 IU/kg Vitamin E as *all rac* α-tocopheryl acetate, 1.19 g/kg Choline, 1.00 g/kg Niacin, 26.8 mg/kg Monensin

[2] Provides to the diet DM 0.30 g/kg Ca, 0.23 g/kg P from ammonium polyphosphate, 0.20 g/kg Mg, 1.23 g/kg K, 0.21 g/kg Na, 0.19 g/kg Cl, 0.26 g/kg S, 0.07 mg/kg Co as cobalt sulfate, 0.05 mg/kg Co as organic cobalt, 12.4 mg/kg Cu as copper sulfate, 4.42 mg/kg Cu as organic copper, 1.76 mg/kg I as ethylenediaminedihydroiodide, 10.7 mg/kg Mn as manganese sulfate, 0.81 mg/kg Mn as organic manganese, 0.25 mg/kg Se as sodium selenite, 59.0 mg/kg Zn as zinc sulfate, 8.00 mg/kg Zn as organic zinc, 5.01 KIU/kg Vitamin A, 1.23 KIU/kg Vitamin D3, 24.5 IU/kg Vitamin E as *all rac* α-tocopheryl acetate, 0.25 g/kg Methionine

[3]Guaranteed to contain no less than 56% Mg

[4]Contains (DM Basis) 90.4% total fat and 9.6% Ca as calcium salts of long chain fatty acids from Inman (Clackamas, OR)

Table 2. Concentrations of serum indicators prior to diagnosis of milk fever (MF)

Indicator	Group			Contrast (P-value)	
	Healthy Control (HC)	Disease Control (DC)	Milk Fever (MF)	HC vs. MF	DC vs. MF
	N = 10	N = 31	N = 9		
2/3 Weeks Prepartum:					
α-Tocopherol (µM)	14.1 ± 0.8	9.4±0.5	9.1±0.9	<0.001	0.76
Cholesterol (mg/dL)	115 ± 11	109 ± 4	108 ± 8.0	0.55	0.91
ATOC/cholesterol[1]	4.76± 0.39	3.29± 0.15	2.69±0.31	0.001	0.84
NEFA (µEq/L)	151±23	261 ±21	208 ±33	0.12	0.18
BHBA (mMol/L)	0.42± 0.03	0.46 ± 0.02	0.44±0.03	0.49	0.61
Haptoglobin (mg/L)	22.4± 9.0	11.4± 1.8	10.9±3.4	0.08	0.88
Glucose (mg/dL)	70.0 ± 1.6	67.8 ±0.9	69.1± 1.7	0.69	0.49
Urea N (mg/dL)	11.0 ± 0.8	11.0± 0.4	10.4±0.8	0.64	0.56
Calcium (mg/dL)	8.95 ±0.19	8.95 ± 0.11	9.10 ±0.20	0.59	0.50
Magnesium (mg/dL)	2.58± 0.09	2.58 ± 0.05	2.60±0.10	0.90	0.88
Phosphorus (mg/dL)	7.57 ± 0.22	7.08 ± 0.12	6.95 ±0.23	0.06	0.61
Last Week Prepartum:					
α-Tocopherol (µM)	14.2 ± 1.0	9.0± 0.5	8.7±1.0	<0.001	0.76
Cholesterol (mg/dL)	117± 11	127± 6	125±12	0.64	0.89
ATOC/cholesterol[1]	4.63 ± 0.51	2.78 ± 0.17	2.69±0.31	<0.001	0.80
NEFA (µEq/L)	183± 31	447± 42	351±63	0.006	0.21
BHBA (mMol/L)	0.45± 0.03	0.54± 0.02	0.53±0.04	0.15	0.75
Haptoglobin (mg/L)	8.8± 2.2	13.3 ± 2.1	8.7 ±2.3	0.99	0.14
Glucose (mg/dL)	68.5± 1.6	67.4±1.2	71.3±2.3	0.35	0.11
Urea N (mg/dL)	11.0± 0.9	11.0 ± 0.5	11.4 ±1.0	0.77	0.76
Calcium (mg/dL)	8.88± 0.30	8.50± 0.17	8.64±0.32	0.57	0.71
Magnesium (mg/dL)	2.69± 0.11	2.51 ± 0.06	2.55±0.11	0.37	0.77
Phosphorus (mg/dL)	7.43± 0.30	6.63± 0.17	6.26±0.32	0.01	0.31
First Day after Calving:					
α-tocopherol (µM)	11.1 ±0.8	7.8±0.5	5.9±0.9	<0.001	0.05
Cholesterol (mg/dL)	91±11	91±6	92±11	0.93	0.89
ATOC/cholesterol[1]	4.57±0.46	3.26±0.18	2.45±0.26	<0.001	0.02
NEFA (µEq/L)	279±50	507±49	582± 110	0.003	0.49
BHBA (mMol/L)	0.67±0.08	0.71±0.05	0.76±0.09	0.45	0.58
Haptoglobin (mg/L)	24.2±9.8	40.2±9.9	34.3±16.8	0.49	0.72
Glucose (mg/dL)	65.1±4.1	78.1±2.7	79.7±5.2	0.03	0.78
Urea N (mg/dL)	13.4±1.7	13.8±1.0	15.1±1.8	0.48	0.50
Calcium (mg/dL)	7.58±0.35	7.70±0.20	5.85±0.37	0.002	<0.001
Magnesium (mg/dL)	2.95±0.16	2.78±0.09	3.14±0.17	0.43	0.07
Phosphorus (mg/dL)	7.25±0.55	6.00±0.31	5.04±0.58	0.009	0.16

[1]ATOC/cholesterol = α-tocopherol to cholesterol molar ratio (µM/mM)

CHAPTER 5 SUMMARY

Results shown in Chapters 2 to 4 demonstrate that low serum ATOC concentrations precede MFA, RP, and LDA and, thus, could be used as a potential diagnostic indicator for these 3 diseases in early lactation multiparous cows. In addition, low serum ATOC concentrations remained for several weeks after the disease, indicating that vitamin E alimentation as part of the treatment regimen may be beneficial. These findings suggest potential benefits of vitamin E alimentation for prevention and treatment of diseases in early lactation multiparous dairy cows, which will be the focus of future studies

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