EFFECT OF FEEDING SYNTHETIC ANTIOXIDANTS AND PREPARTUM EVAPORATIVE COOLING ON PERFORMANCE OF PERIPARTURIENT HOLSTEIN COWS DURING SUMMER IN FLORIDA

By

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To my husband, Zheng Fu who loves me, encourages me, and supports me

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LIST OF ABBREVIATIONS

ADF	acid detergent fiber
AO	antioxidants
ASP	acid soluble protein
BCS	body condition score
BHA	butylated hydroxyanisole
BHBA	beta-hydroxy butyric acid
BHT	butylated hydroxytoluene
BUN	blood urea nitrogen
Con A	concanavalin A
DHR	dihydrorhodamine 123
DIM	days in milk
DMI	dry matter intake
ELISA	enzyme-linked immunosorbent assays
GPx	glutathione peroxidase
GSH	glutathione
GSSG	glutathione disulfide
Hb	hemaglobin
Hp	haptoglobin
IgG	immunoglobulin G
I.m.	intramascularly
LCFA	long chain fatty acids
MDA	malondialdehyde
MFI	mean fluorescence intensity
NDF	neutral detergent fiber

NEFA	non esterified fatty acid
NIR	near infrared
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer solution
PCV	packed cell volume
RBC	red blood cell
ROS	reactive oxygen species
SCC	somatic cell count
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
TBA	thiolbarbituric acid
TBARS	thiolbarbituric acid reactive substances
THI	temperature humidity index
WBC	white blood cell

Abstract of Thesis Presented to the Graduate School
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By

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The objective of this study was to evaluate the effect of supplementation with 0 or 250 mg of synthetic antioxidants (**AO**, Agrado Plus, Novus International, MO) per kg of dietary DM and prepartum evaporative cooling on periparturient Holstein cows (n = 35) from 21 days before through 49 days after parturition in a 2 by 2 factorial design. Uterine health was evaluated via metricheck at 7, 16, and 25 days in milk (DIM). Blood was collected at -15, 1, 8, 15, and 29 DIM for oxidative markers. Phagocytosis and oxidative burst of neutrophils were measured in whole blood collected at -15, 0, 7, and 14 DIM. Acute-phase proteins were measured in plasma collected three times weekly. A uterine horn was flushed at 40 ± 2 DIM for diagnosis of subclinical endometritis. Rectal temperature of cooled cows was lower prepartum (39.2 vs. 39.6°C). Prepartum cooling resulted in greater mean concentration of milk fat during 7 wk (3.54 vs. 3.32%) and mean production of 3.5% FCM during the first 4 wk postpartum (26.5 vs. 23.0 kg/d). Cooling reduced the concentration of circulating WBC postpartum (7864 vs. 10,199 per µL of blood) and of circulating lymphocytes (3463 vs. 5432 per µL of blood) and increased proportion of neutrophils undergoing oxidative burst (83 vs. 77%) isolated

from cows fed the control diet. Prepartum cooling of multiparous cows resulted in less oxidative stress as evidenced by lower activity of GPx in RBC (8,854 vs. 12,247 nmol/min/mL) and of SOD in RBC (2503 vs. 3111 U/mL). Feeding AO increased concentration of milk true protein (3.07 vs. 2.94%) but decreased concentration of milk fat (3.25 vs. 3.61%) resulting in less production of milk fat (0.88 vs. 1.04 kg/d) and of 3.5% FCM (26.2 vs. 29.5 kg/d). In addition, cows fed AO had a greater incidence of endometritis (60 vs. 27%) at 25 DIM and of subclinical endometritis at 40 DIM (80 vs. 33%). Feeding AO to prepartum cooled cows reduced plasma concentration of TBARS (1.78 vs. 2.33 nmol/mL), proportion of neutrophils undergoing oxidative burst (77 vs. 83%), and mean florescence intestity of phagocytosis of primiparous cows postpartum (36 vs. 57%).

CHAPTER 1
INTRODUCTION

Oxygen and Oxidative Stress

Animals do not use energy in feed directly for requirements of maintenance, activity, pregnancy, and productive purposes. Carbohydrates, lipids, and amino acids must undergo oxidative phosphorylation as the final stage to generate ATP. During oxidative phosphorylation, molecular oxygen is reduced by accepting 4 electrons to produce two molecules of water. The electrons which reduce oxygen to water are derived from metabolism of feed. This process is called cellular respiration.

However electrons which fail to be incorporated into the terminal acceptor of the transport chain may cause problems. About 1 to 2% of consumed oxygen is not completely reduced due to the escape of electrons from the intermediate complex in the respiratory chain (Levine, 1985). For example, the passage of electrons from reduced ubiquinone to complex III involves the radical $Q^{\cdot}$ which could pass an electron to oxygen to form a superoxide radical (O_2^{-}). Another example is the generation of O_2^{-} during the hydroxylation reactions catalyzed by Cytochrome P-450 (Nelson, 2008). Anything which increases metabolic demands such as parturition, lactation, heat stress, and disease or disorders could increase oxygen requirements, number of electrons transferred, and production of O_2^{-} (Sordillo and Aitken, 2009).

Superoxide radicals can be reduced to hydrogen peroxide (H_2O_2) by acceptance of a second electron. With acceptance of a third electron, oxygen can be reduced to hydroxyl radical (OH^{-}). The generation of OH^{-} may cause two types of damage depending on the location. On the one hand, OH^{-} may attack the peroxidative chain which can damage cellular and subcellular membranes (Gutteridge, 1994). On the other

hand, OH^- is produced at the site where Fe is associated with a macromolecule such as DNA or protein causing damage of DNA and protein (Casciola-Rosen et al., 1997). The term “reactive oxygen species (ROS)” refers to oxygen-derived free radicals such as O_2^-, H_2O_2 and OH^-.

Oxidative Status and Health of Dairy Cattle

Cells normally are protected against the harmful effects of ROS by antioxidant defenses. But when the generation of ROS exceeds the capacity of defensive systems to eliminate ROS, the oxidation-antioxidation system is imbalanced. This process is defined as oxidative stress. Levine and Kidd (1985) elucidated that the progression from oxidative stress to chronic disease can be divided into 4 stages (Levine, 1985). In the first stage, the individual is healthy and able to deal with oxidative stress. In the second stage, due to chronic deficiency of antioxidant nutrients or exposure to oxidants, the individual adapts to oxidative stress. In the third stage, continued oxidative stress depletes antioxidants so that the individual is subjected to oxidative damage. The third stage can become more severe so that absorption of antioxidant nutrients is influenced. In the fourth stage, the rate of deterioration of antioxidant defense exceeds the rate of recovery resulting in subclinical or clinical diseases.

The effects of antioxidants on oxidative status and health of dairy cows have been examined in recent years. Supplementation with vitamin E and Se usually reduced incidence of retained fetal membranes and mastitis (Miller et al., 1993; Allison and Laven, 2000). The supplementation of antioxidants also has been reported to improve oxidative status (Brzezinska-Slebodzinska et al., 1994; Vazquez-Anon et al., 2008; Sahoo et al., 2009).

The objectives of the literature review (Chapter 2) are the following: 1) to introduce the classes of antioxidants namely **enzymatic antioxidants** including superoxide dismutase (SOD) and glutathione peroxidase (GPx), **nonenzymatic antioxidants** including glutathione (GSH), vitamin A, β-carotene, vitamin E and Se; and **synthetic antioxidants** including ethoxyquin, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT), 2) to summarize the effectiveness of antioxidants on performance, immune system, and oxidative status of animals. In addition the effect of heat stress on performance and immune system of animals is reviewed. In the subsequent research (Chapter 3), the effects of feeding synthetic antioxidants and prepartum cooling on the performance of periparturient Holstein cows was investigated.

CHAPTER 2
LITERATURE REVIEW

Reactive Oxygen Species Formation

Free radicals are molecules within the animals' body that have at least an unpaired electron in the outer orbit. They can accept or donate electrons from other molecules to generate a more stable molecule through oxidation and reduction reactions (Gitto et al., 2002; Halliwell, 2007a; Sordillo and Aitken, 2009). Reactive oxygen species (**ROS**) is a collective term to classify oxygen-derived free radicals, including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) (Gitto et al., 2002). Normally, ROS are produced either through oxidative phosphorylation within the inner membrane of mitochondria or phagocytosis of pathogens to stimulate NADPH oxidase in neutrophils (Paape et al., 2003; Sordillo and Aitken, 2009). Animals and human beings may undergo metabolic and physiological adaptations during the transition from pregnancy to lactation accompanied by elevated requirements for oxygen. This increased oxygen demand augments the production and accumulation of ROS in tissues.

Oxidative Stress and Oxidative Damage

Oxidative stress refers to an imbalance between production of free radicals and antioxidant mechanisms (Halliwell, 2007a). It can result from dietary imbalances, pregnacy, environmental pollutants, solar radiation, or heat stress (Gitto et al., 2002, Miller et al., 1993). Oxidative stress can result in oxidative damage to molecules, cells, and tissues which may subsequently develop to certain kinds of diseases or disorders such as endometriosis, heart failure, diabetes and so on in different species (West, 2000; Sun et al., 2002; Jackson et al., 2005). For example, oxidative stress could

indirectly lead to Ca^{2+} overload which activates phospholipase A_2 and C. These enzymes lead to membrane phospholipid hydrolysis (Gitto et al., 2002; Halliwell, 2007a). Another example is that oxidative stress could lead to membrane lipid peroxidation which gives rise to peroxyl radicals.

Lipid Peroxidation

Lipid peroxidation is a chain reaction (initiation, propagation, and termination). This reaction is initiated by attack of reactive hydroxyl radicals on polyunsaturated fatty acids in plasma membranes. Propagation of lipid peroxidation gives rise to a lipid radical ($L^{\cdot}$) which interacts with oxygen under aerobic conditions to produce a peroxyl radical ($LOO^{\cdot}$). The reaction between peroxyl radicals generates nonradical products which terminates this chain reaction (Burton and Ingold, 1984, Halliwell, 2007a).

Antioxidant Defenses

Antioxidants can be defined as any substance that helps protect cells by delaying, preventing or removing oxidative damage (Halliwell, 2007b). Antioxidants can be classified based on their chemical and physical characteristics, namely enzymatic antioxidants, such as superoxide dismutase (**SOD**) and glutathione peroxidase (**GPx**), nonenzymatic antioxidants such as glutathione, ascorbic acid, vitamin E, β-carotene, and ubiquinone, and synthetic antioxidants such as ethoxyquin, butylated hydroxyanisole (**BHA**), and butylated hydroxytoluene (**BHT**).

1) Enzymatic Antioxidants

Enzymatic antioxidants work most efficiently and directly reduce ROS.

a) Superoxide Dismutase

Superoxide dismutase catalyzes the dismutation of superoxide to H_2O_2 and O_2 (Miller et al., 1993; Mates et al., 1999).

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

The three forms of SOD are the following: Cu/Zn-SOD in cytosols, Mn-SOD in mitochondria, and Fe-SOD. Cu/Zn-SOD plays an important role in the first defense against ROS (Mates and Sanchez-Jimenez, 1999; Bernabucci et al., 2005). The activity of Cu/Zn-SOD in milk was similar to that in bovine erythrocytes (Przybylska et al., 2007). Mn-SOD, which is a homotetramer, functions to remove superoxide produced in the electron transport chain in mitochondria. It can be induced and depressed by cytokines (Mates et al., 1999). Experiments using gene knock-out mice demonstrated that Mn-SOD is essential for life, but that Cu/Zn-SOD is not (Mates and Sanchez-Jimenez, 1999). The Fe-SOD are found only in prokaryotes such as *Escherichia coli* (Fridovich, 1975). It seems to provide a defense against exogenous superoxide.

Superoxide dismutase activity has been used to evaluate oxidative status in animals. Cows in the last 3 wk of pregnancy had an incremental increase in plasma SOD activity and reached peak at 4 d after calving (Bernabucci et al., 2005). The same laboratory reported in 2002 that cows giving birth during summer had greater erythrocyte SOD activity compared with the cows calving in spring. The activity of SOD in placental tissues was higher in cows with retained placental membranes than those without retained placental membranes (Kankofer et al., 1996).

b) Glutathione Peroxidase

Glutathione peroxidase can be classified as Se-dependent and Se-independent. The Se-dependent enzyme catalyzes the reduction of hydroperoxides to water, accompanying the oxidation of glutathione (GSH) to glutathione disulfide (GSSG).

$$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O$$

Glutathione can be regenerated by reducing equivalents from $NADPH_2$ (Mates et al., 1999, Miller et al., 1993).

Glutathione peroxides are composed of at least 5 isoenzymes (GPx 1 to 5) in mammals (Przybylska et al., 2007). Glutathione peroxidase 1 (GPx1) which is comprised of four identical SeCys-containing subunits is the most predominant. It is found in the cytoplasm of erythrocytes, kidney, and liver. The preferred substrates are hydrogen peroxide and a wide range of organic hydroperoxides. Glutathione peroxidase 4 (GPx4), also called phospholipid hydroperoxide glutathione peroxidase, is distributed in both cytosol and the membrane fraction, whose preferred substrate is phospholipid and cholesterol hydroperoxides (Thomas et al., 1990). Glutathione peroxidase 2 (GPx2) is a cytosolic enzyme, whereas glutathione peroxidase 3 (GPx3) is extracellular, especially abundant in plasma. The GPx3 metabolizes phospholipid hydroperoxides and plays a direct role in protection of membranes (Takahashi et al., 1987). Glutathione peroxidase 5 (GPx5), being Se-independent, is newly discovered in mouse epididymis (Przybylska et al., 2007). Glutathione peroxidase has been detected in bovine milk and its activity was strongly and positively correlated with Se concentration (Przybylska et al., 2007).

Glutathione peroxidase activity can be used to evaluate oxidative status. Generally speaking, the activity of this enzyme could be elevated during parturition and lactation, by heat stress, by BW loss, by diseases, or by consumption of different types of feedstuffs. When blood samples were collected from dairy cows at 21 ± 7 d before calving, calving, and 21 DIM, the GPx activity in peripheral blood mononuclear cells (PBMC) increased at calving and at 21 DIM compared with the prepartum period

(Sordillo et al., 2007). Similarly, weekly blood samples collected from 30 d before calving to 30 d after calving indicated that plasma GPx activity began to increase a week before calving and was greater after calving than before calving (Bernabucci et al., 2005). The same authors also reported that cows giving birth during summer had erythrocytes of greater GPx activity at 21 d before calving compared to that of cows giving birth during spring (Bernabucci et al., 2002). However, GPx activity in plasma did not differ during the transition period between cows calving in the summer and spring season. Burke et al. (2007) reported that PBMC of heifers had less GPx activity when housed under high temperature-humidity indices (THI) compared to neutral THI. Brennan conducted a study to determine the effect of BW loss of beef cows which could increase the production of ROS through fat mobilization on the antioxidant activity of GPx and antioxidant mRNA levels (Brennan et al., 2009). Total RNA was isolated from skeletal muscle, glutathione peroxidase 1 and GPx4 target gene mRNA was measured using real-time reverse transcription-PCR. Erythrocyte GPx activity was measured to determine oxidative status. No differences were found in GPx1 activity between cows losing or maintaining BW. Therefore BW loss did not influence the GPx activity of erythrocytes. However, abundance of GPx4 mRNA in skeletal muscle was 1.4-fold greater during BW loss. Activity of plasma GPx was greater in patients who were diagnosed with ulcerative colitis or Crohn's disease compared with healthy individuals who didn't have any sign, symptom or previous records of inflammatory bowel disease (Tuzun et al., 2002). The activity of GPx in placental tissues was greater in cows with retained placental membranes than those without retained placenta membranes (Kankofer et al., 1996). Cows fed grain or a mixed diet had a greater muscle GPx

activity than cows consuming only pasture (Mercier et al., 2004; Descalzo and Sancho, 2008), probably due to the different concentrations of Se in different feedstuffs (Ammerman, 1975). Lambs fed brown seaweed extract exhibited greater GPx activity in erythrocyte and white blood cell compared with animals not fed the extract, indicating that brown seaweed extract improved lamb antioxidant status by increasing antioxidant capacity (Saker et al., 2004). 2) Nonenzymatic Antioxidants

a) Glutathione (GSH)

Glutathione, a low-molecular-weight thiol, is abundant in animal cells and plasma. Majority of the cellular GSH (85 to 90%) is distributed in the cytosol, with the rest located in many organelles including the mitochondria, nuclear matrix, and peroxisomes. On average, the concentration of GSH ranges from 0.5 to 10 mmol/L, with the exception of bile acid which contains more than 10 mmol/L. The roles of glutathione include the following: 1) antioxidant defense including the scavenging of free radicals and other reactive species, removal of hydrogen and lipid peroxides, and prevention of oxidation of biomolecules; 2) metabolic roles such as serving as a substrate for synthesis of leucotriene C4, a substrate to convert formaldehyde to formate, and for storage and transport of cysteine; 3) aids in regulation of cytokine production, immune responses, and mitochondrial function and integrity (Wu et al., 2004). Usually the concentration ratio of GSH: GSSG is used as an index of the cellular oxidative status, which is greater than 10 under normal physiological conditions (Griffith, 1999). The GSH:GSSG ratio in PBMC of heifers was less under heat stress, which indicated either increased production of ROS or decreased antioxidant status (Burke et al., 2007). Cows exposed to heat stress and fed endophyte-infected tall fescue had a lower whole-blood GSH concentration compared with those under heat stress without endophyte-infected

tall fescue in the diet (Lakritz et al., 2002). The addition of GSH did not alter the proliferative response of lymphocyte isolated from the blood of Holstein cows at 38.5°C (neutral) or 42°C (heat stress) (Kamwanja et al., 1994).

b) Vitamin A and *B*-carotene

B-carotene is the major dietary precursor of vitamin A in dairy cattle (LeBlanc et al., 2004; Przybylska et al., 2007). *B*-carotene that escapes from the rumen is absorbed and converted to retinol in the intestinal mucosa and transported to the liver with fat (LeBlanc et al., 2004). Experiments indicate that *β*-carotene is neither a peroxide-decomposing antioxidant such as catalase and GPx nor a conventional chain-breaking antioxidant such as vitamin E (Burton and Ingold, 1984). It functions as an unusual antioxidant at low oxygen pressures (Burton and Ingold, 1984) and a scavenger of free radicals produced from unsaturated long chain fatty acid (**LCFA**) peroxidation (Hino et al., 1993).

The bioavailability of vitamin A for cattle is considerably limited due to its destruction by ruminal microbes (Rode et al., 1990; Weiss et al., 1995). Hino et al. (1993) did an *in vitro* study to examine whether the addition of *β*-carotene could alleviate the inhibition of bacterial growth caused by LCFA. The results demonstrated that the addition of *β*-carotene increased bacterial growth with the presence of LCFA. The active form of vitamin A could be different for different functions (Hemken and Bremel, 1982). Alosilla et al. (2007) reported that feeding different commercial vitamin A sources to yearling beef cattle led to different retinol concentrations in liver, indicating that some supplemental vitamin A sources had greater amounts of vitamin A reaching the duodenum for absorption and storage than others.

Vitamin A plays an important role in resistance to infectious disease, especially mastitis (NRC, 2001). Johnston and Chew (1984) studied the peripartum concentrations of vitamin A and β-carotene in Holstein cows with or without mastitis. They reported that plasma concentrations of vitamins A and β-carotene decreased rapidly before calving and reached their lowest point at calving (vitamin A) or on d 4 to d 6 postpartum (β-carotene). In addition, concentration of vitamin A in plasma was lower in mastitic cows from d 0 to 7 and at wk 2 and 4 than nonmastitic cows, whereas β-carotene was greater in mastitic cows from prepartum to d 7. When feeding lactating Holstein cows β-carotene at a rate of 0 or 300 mg/d from 3 to 98 d postpartum, concentration of β-carotene in serum declined in both control and carotene-supplemented groups between 1 and 2 wk postpartum, but increased to 225 μg/dl after 3 wk in supplemented group, whereas the concentration stayed the same in control group. Additionally, feeding β-carotene did not affect the length of first estrous cycle or peak concentration of progesterone in the first estrous cycle. However, the incidence of mastitis was less for the supplemented group (Wang et al., 1988). In a review paper Hemken and Bremel (1982) indicated that a deficiency of vitamin A was associated with a number of reproductive problems such as retained placenta and abortions. Consumption of vitamin A to meet requirement could improve conception rate or reduce days open for cows.

Pregnant Holstein cows were given 0, 300, or 600 mg/d of β-carotene or 120,000 IU/d of vitamin A from 4 wk before expected calving date to 4 wk postpartum (Michal et al., 1994). Blood lymphocyte proliferation in response to 5 μg/mL of concanavalin A (**Con A**) was greater at 1 wk before calving and at 2 wk postpartum in cows fed 600 mg/d of β-carotene compared to unsupplemented group. The phagocytic ability of

Staphylococcus aureus by neutrophils was enhanced in cows fed 300 mg/d of *β*-carotene at 1 wk after calving compared with cows in other treatments. Tjoelker and co-workers (1990) conducted a trial to evaluate the impact of supplementation of vitamin A and β-carotene on neutrophil and lymphocyte function of dairy cows in the nonlactating period. Cows were assigned randomly to one of 3 treatments: 53,000 IU of vitamin A, 213,000 IU of vitamin A, or 53,000 IU vitamin A plus 400 mg of *β*-carotene per d from 6 wk before to 2 wk after dry-off. Phagocytosis and bacterial killing ability of *S. aureus* by neutrophils were not different among treatments, but lymphocyte blastogenesis was stimulated by 10 μg/mL of Con A on wk 2 for cows fed 53,000 IU of vitamin A but did not change in other treatments throughout the experiment.

Whether supplemental vitamin A exerts these effects through its role as an antioxidant is unknown.

c) Vitamin E

Vitamin E is the term for a class of lipid-soluble tocopherols (*α, β, γ, δ*) and tocotrienols (*α, β, γ, δ*), of which *α*-tocopherol has the highest biological activity (Brigelius-Flohe and Traber, 1999; NRC, 2001). Vitamin E acts as a chain-breaking antioxidant that limits the propagation of peroxidation by trapping free radicals (Nockels et al., 1996; Brigelius-Flohe and Traber, 1999; Goupy P., 2007; Gobert et al., 2009). It also exerts other functions involving cellular signaling, immunity and reproductive function. Vitamin E is absorbed in the small intestine and enters the circulation via the lymphatic system. It is absorbed with lipids, transported from the small intestine to the liver, packed in lipoproteins, and distributed via plasma to the rest of the body (Herdt and Smith, 1996). Although found in feedstuffs, the concentration and activity of vitamin

E is easy to lose during processing and storage of feedstuffs. Therefore, supplementation of vitamin E in the diet is necessary. The common form of supplemental vitamin E fed to dairy cattle is DL-α-tocopheryl acetate.

Plasma concentration of α-tocopherol was reduced during the last month prepartum and at 1 or 2 wk postpartum (LeBlanc et al., 2002; Rezamand et al., 2007) in dairy cows. Goff et al. (2002) reported a similar pattern in that the concentration of vitamin E declined from 1 wk before calving to 3 DIM. A steady vitamin E state may be reached by supplementation of 3000 IU/d for 2 wk prepartum to pregnant heifers (Bouwstra et al., 2008). Oxidative damage during lipid peroxidation and its prevention by vitamin E can be analyzed by quantification of 8-isoprostane (F_2-isoprostanes). 8-isoprostane concentration was reduced when overweight patients received 800 IU/d of vitamin E for 3 mon and 1200 IU/d for another 3 mon compared with a placebo group (Sutherland et al., 2007), indicating that supplementation of vitamin E may improve oxidative status in obese humans. Two studies (Rimm et al., 1993, Stampfer et al., 1993) in human species reported that high intakes of vitamin E reduced the risk of cardiovascular diseases. Supplementation or injection of vitamin E to animals/humans has reduced oxidative damage in the body.

d) Selenium

Many selenoproteins have Se in their structures to participate in the antioxidant defense system of cells (Cerri et al., 2009). Considerable evidence exists that Se functions by a similar mechanism as vitamin E in lipid peroxidation (Hamilton and Tappel, 1963). Selenium is an integral part of the enzyme GPx which functions to prevent oxidative damage to tissues or cells.

Dietary supplementation of 2 mg/d of Se in the form of sodium selenite increased concentration of Se and GPx activity in blood of dairy cows during the periparturient period compared to cows not supplemented with Se (Grasso et al., 1990). However, feeding Se in the form of sodium selenite at 0.3 mg/kg of dietary DM from 25 d before to 70 d after calving did not influence incidence of postpartum diseases (metritis, ketosis, and mastitis) and ovarian responses compared with cows fed Se yeast at the same rate (Cerri et al., 2009) due to the lack of effects of source of dietary Se on Se status. A similar dietary supplementation of Se study was conducted by Silvestre (2006), in which they reported Se yeast reduced the risk of some postpartum uterine problems compared with that of a supplemental inorganic source of Se. The disparity of effects of supplementation of Se on health was likely due to differences in Se status due to location. Cows on the study of Silvestre (2006) were managed in Florida using forages grown on Se-deficient soils whereas cows used by Cerri et al. (2009) were managed in California on Se-adequate soils. This was reflected by different blood concentrations of Se.

Erskine et al. (1987) reported that somatic cell count (**SCC**) obtained from 9 dairy herds decreased as concentration of plasma Se increased in cows. The GPx activity was positively correlated with Se intake but negatively with SCC. Injection of 1 mg/kg of BW of Se at 21 d prior to estimated calving date for dairy cows reduced the incidence of mastitis by 12% compared to cows not receiving Se injection (Smith et al., 1984). The effect of supplementation or injection of vitamin E and Se on mammary health has been reviewed by Smith et al. (1997) who pointed out that vitamin E and Se deficiency were

associated with greater incidence of mastitis and greater SCC. Therefore alleviating deficiencies of vitamin E and Se can enhance mammary health.

3) Synthetic Antioxidants

Three commonly used synthetic antioxidants are ethoxyquin, butylated hydroxyanisole (**BHA**), and butylated hydroxytoluene (**BHT**). These synthetic antioxidants are used mainly by the feed industry to delay the peroxidation of feed lipids and to stabilize the formulation of vitamin A and vitamin D_3 in premixes and feeds. In addition, Kahl (1984) summarized that synthetic antioxidants have been associated with a wide variety of molecular, cellular, and organ activity, roughly divided into three categories: 1) modulation of growth, macromolecule synthesis and differentiation; 2) modulation of immune response; and 3) interference with O_2 activation.

Ethoxyquin is used widely as an antioxidizing agent in food formulation for animals including fish, livestock and pets. It functions as a scavenger of free radicals which are formed during lipid peroxidition (Yamashita, 2009). The FDA approved feeding rate of ethoxyquin for use in animal feeds is 150 ppm (FDA, 2010). It has been used effectively to promote color retention and preserve fat-soluble vitamins. However, its toxic effect *in vitro* on phagocytosis by leukocytes isolated from swim bladder of tilapia was reported by Yamashita et al. (2009) when concentration of ethoxyquin in media was > 0.1 mg/L. They also reported phagocytic activity by inflammatory leucocytes isolated from fish fed ethoxyquin at a rate of 150 mg/kg for 30 d was reduced compared with the activity in fish fed no ethoxyquin.

Two additional synthetic antioxidants, BHA and BHT, are used as food additives by the feed industry. Butylated hydroxyanisole is effective to preserve animal fats but not vegetable oils. It is approved for human and animal use. Butylated hydroxytoluene

has similar properties. Björkhem et al. (1991) reported that BHT had an antiatherogenic effect in cholesterol-fed rabbits. However, organ proliferation and histopathological changes were induced by BHT and BHA in rat liver (Kahl, 1984).

More studies of the effects and mechanism of action of synthetic antioxidants in animals need to be investigated in the future.

Overview of Immune Function

The immune system can be divided simply into two systems: innate and adaptive systems. In the innate system, foreign bodies are destroyed and/or neutralized by an array of cells and molecules that initiate immediate responses without any "memory" about that foreign body. In the adaptive system, immune responses are the result of a previous memory obtained by exposure to a particular antigen. Therefore, adaptive immunity provides life-long protection against reinfection by the same pathogens (Janeway, 2004). Both innate immunity and adaptive immunity responses depend upon the activities of WBC or leukocytes. Neutrophils, macrophages (the mature form of monocytes), and eosinophils are the primary cells to arrive at the infection site and phagocytize pathogens or parasites without requiring memory (innate immunity). Phagocytosis by macrophages and neutrophils is triggered by the binding of ligand to the receptors, and subsequent destruction of pathogens takes place by complement or by the generation of toxic chemicals, such as superoxide radicals, hydrogen peroxide, and nitric oxide (Calder, 2007). Adaptive immune responses rely upon lymphocytes, i.e. B cells and T cells. Therefore, the components of the immune system communicate and work with each other to help hosts defend against infectious agents from the environment.

Acute-phase proteins, as their names imply, are synthesized by the liver and secreted into the blood to protect the host from local inflammation or stress. These proteins mimic the action of antibodies but are nonspecific. Acid-soluble protein is one acute-phase protein which contains mainly α1-acid glycoprotein. It is an anti-inflammatory agent that controls inappropriate or extended activation of the immune system (Jafari et al., 2006). Acid-soluble protein has a dual immune-modulatory effect in that it causes immune activation of macrophages to secrete cytokines or immune suppression to control immune response. Haptoglobin (**Hp**) is another acute-phase protein which binds to hemoglobin and so inhibits bacterial proliferation by reducing the availability of iron (Wassell, 2000; Huzzey, 2009) and functions as an antioxidant by virtue of its ability to prevent hemoglobin-driven oxidative damage of tissues (Melamed-Frank et al., 2001). Many studies (Hirvonen et al., 1996; Wittum et al., 1996; Huzzey, 2009) have reported that an increase in plasma concentration of acute-phase proteins, especially haptoglobin, is an indicator of severity or chronicity of sickness in cattle.

Effect of Feeding Antioxidants on the Immune System

Numerous studies have been conducted to evaluate the effects of supplementation of antioxidants on neutrophil functions, acute-phase proteins and immune challenge. A review of effects of vitamin E supplementation on health and fertility of dairy cattle (Allison and Laven, 2000) indicated that Holstein calves receiving up to 500 IU/d of dietary vitamin E increased the blastogenic responses of T cells and B cells (Reddy et al., 1987). Injection of 3000 IU of vitamin E at 10 to 5 d before calving increased the killing ability of bacteria by neutrophils at calving (Hogan et al., 1992). Weiss and Hogan (2005) reported that supplementing Se to provide 0.3 mg/kg of dietary DM from either sodium selenate or Se-yeast with 500 IU of vitamin E did not

affect the percentage of neutrophils that phagocytized *E. coli* in Holstein heifers or cows, but bacterial killing ability by neutrophils tended to be increased for cows fed selenite. Similarly, supplementation of vitamin E (400 to 600 mg/d) or Se (0.3 mg/kg of dietary DM) alone to 21 multiparous Holstein cows increased the proportion of bacteria killed by neutrophils, but did not influence phagocytic ability (Hogan et al., 1990). Grasso et al. (1990) reported that cows supplemented with 2 mg/d of sodium selenite during the transition period had greater bacterial killing ability by neutrophils in milk and increased viability of neutrophils when challenged with *S. aureus* compared with cows not supplemented with Se. The percentage of neutrophils that phagocytized and killed *Candida albicans* was greater for cows that received sufficient Se (0.1 ppm of dietary DM) than cows given a deficient Se diet (Boyne and Arthur, 1979). However, the percentage of neutrophils that phagocytized *C. albicans* did not differ between cows fed the two dietary treatments. Ascorbic acid (vitamin C) is considered to be the most abundant and important water-soluble antioxidant. Functions of neutrophils (the proportion of neutrophils that phagocytized bacteria and number of intracellular bacteria per neutrophil) isolated from whole blood were not influenced by supplementation with either 0 or 30 g/d of vitamin C starting from 2 wk before calving through 7 DIM (Weiss and Hogan, 2007). Yamashita et al. (2009) evaluated the effect of a synthetic antioxidant, ethoxyquin, on immunity of tilapia. The phagocytic activity of leucocytes *in vitro* was lower in leucocytes exposed to 0.1 mg/L of ethoxyquin than leucocytes not exposed to ethoxyquin. In summary, supplementation of natural antioxidants to dairy cows improved killing ability by neutrophils measured as oxidative burst.

Bull calves were assigned randomly to one of three supplementation rates of vitamin E (285, 570, and 1140 IU/d, respectively) for 21 d. They were vaccinated with 4 mL of ovalbumin (2 mg/mL of PBS). A linear increase in IgG concentration with increased dietary supplementation of vitamin E was detected at 21 d after ovalbumin ingestion (Rivera et al., 2002). Similar to this finding, calves vaccinated with 125 IU of vitamin E at 7 wk of age had greater IgG values compared with those receiving no vitamin E (Reddy et al., 1987). The enhancement of serum IgG to *Pasteurella haemolytica* was detected in steers injected i.m. with 25 mg of Se and 340 IU of vitamin E (Droke and Loerch, 1989).

Heifer calves were fed 2000 IU of vitamin E for 0, 7, 14, or 28 d (Carter et al., 2002). No differences were detected in Hp concentrations in plasma among treatments on any sampling day. However plasma concentrations of acid-soluble protein were lower in calves fed 7, 14, or 28 d of vitamin E on d 7 of experimental period compared to concentration of calves not receiving vitamin E. The effects of supplementation of vitamin E on responses of antibody and acute-phase proteins were not consistant possibly due to differing initial concentrations of vitamin E that may have made the animals more or less susceptible to respond to additional vitamin E supplementation.

Effect of Feeding Synthetic Antioxidants on Performance

Few experiments have been conducted to examine the effects of supplementation of synthetic antioxidants on the performance of dairy cattle. Feeding lactating Holstein cows a basal diet containing distillers grains (15% of dietary DM) supplemented without or with (0 or 0.02% of dietary DM) a blend of ethoxyquin, BHA and BHT (Agrado Plus, Novus International, St. Louis, MO) did not affect DMI (26.6 ± 0.5 kg/d) or milk yield (49.8 ± 1.7 kg/d) (Preseault, 2008). Although milk fat depression occured in both control

and Agrado Plus - supplemented treatment groups, the extent of milk fat depression was less for cows fed Agrado Plus (3.22 vs. 3.32%). In another abstracted study (He, 2008), milk yield of dairy cows was not influenced by adding Agrado Plus (0 or 0.025% of dietary DM) to six diets containing 1 of 5 vegetable oils (control, palm, high-oleic safflower, high-linoleic safflower, linseed, or corn oil) at 5% of dietary DM.

However Bowman et al. (2008) reported that dairy cows fed Agrado Plus at 250 mg/kg of dietary DM increased DMI (22.2 vs. 22.7 kg/d) and tended to increase milk yield ($P < 0.10$) (Bowman, 2008). Vázquez-Añón et al. (2008) also detected increased DMI (20.2 vs. 20.9 kg/d, SEM = 0.22) and increased production of 3.5% FCM (27.3 vs. 28.3 kg/d, SEM = 0.36) when dairy cows (171 ± 10 DIM) were fed Agrado Plus at 200 mg/kg of dietary DM.

An *in vitro* study using continuous cultures (Vazquez-Anon et al., 2008) was conducted to investigate the effect of presence or absence of Agrado Plus on nutrient digestibility, microbial N and fatty acid metabolism. This study was a 2 x 2 factorial design in which two types of oil (oxidized vs. unoxidized mixture of unsaturated oil) and supplementation or not of Agrado Plus at 200 mg/kg of dietary DM were combined. Feeding Agrado Plus increased NDF and ADF digestion, and increased conversion of feed N to microbial N, but tended to reduce the outflow of 18:3 in the effluent.

Few experiments have been conducted to investigate the effect of supplementary synthetic antioxidants on performance of species other than dairy cattle. In poultry, feeding laying hens ethoxyquin at 250 mg/kg of food from 32- to 88-wk of age did not affect weight gain and egg production compared with hens fed control diet which contained 5 mg of vitamin E and 125 mg of ethoxyquin/kg of food (Bartov et al., 1991).

Similar results have been reported for chicks in that dietary ethoxyquin fed at 0, 125, 500, and 1000 ppm did not affect body weight and weights of liver, spleen, kidney or heart. Average carcass weights of broilers were greater when supplementing BHA at the rate of 12.5 mg/d/bird from 3- to 7-wk of age and BHT at the same rate for the last 5 d of the trial compared with those only fed unoxidized sunflower oil at 55 g/kg of dietary DM (Lin et al., 1989). Rainbow trout were utilized in a 2 x 2 x 2 factorial design with 2 feeding rates of oxidized fish oil (peroxide value of 5 and 120 meq/kg of oil), 2 feeding rates of α-tocopheryl acetate (0 and 33 mg/kg of food), and 2 feeding rates of ethoxyquin supplementation (0 and 125 mg/kg of food). Live weight gains and carcass composition were not different among treatments (Hung et al., 1981). In summary feeding ethoxyquin did not affect performance of chicks and fish.

Effect of Feeding Antioxidants on Oxidative Status and Stability

Dunkley et al. (1967) conducted a trial to evaluate the effects of supplementation of either tocopherol at 0.0025% of dietary DM or ethoxyquin at 0.0125% of dietary DM on oxidative stability of milk from dairy cows. Oxidative stability of the milk fat was increased by the supplementation of tocopherol but not by ethoxyquin. A second experiment was conducted to study the effect of feeding increasing dietary concentrations of ethoxyquin (0.015 and 0.15% of dietary DM) on milk quality. They reported an increase in endogenous tocopherol concentration and oxidative stability of the milk when feeding either concentration of ethoxyquin but the improvement was greater when more ethoxyquin was fed. The same research group also reported that supplemental ethoxyquin in the diet was transferred to milk and accompanied by appearance of an unidentified compound using a fluorimetric method (Dunkley et al., 1968).

Oxidative status of mammals can be monitored by several markers such as SOD, GPx, and TBARS. The activity of erythrocyte SOD was decreased compared with the value before treatment after 3 injections of Vitamin E (i.m., 500 IU/injection) and Se (i.m., 15 mg/injection) on alternate days up to the 5th day as a therapy for subclinical ketosis. Comparing the activities of erythrocyte SOD after treatments, cows receiving additional vitamin E and Se had the lowest SOD activity followed by those receiving 5 injections of 25% dextrose daily at 540 mL plus 1 injection of 4 mg/mL of dexamethasone at 2.5mL, whereas cows receiving no treatment had the highest SOD activity (Sahoo et al., 2009). Vázquez-Añón et al. (2008) reported that the activity of plasma SOD was decreased (22.02 vs. 19.34 U/g of protein) for cows with vs. without supplementation of 200 mg of Agrado Plus /kg of dietary DM when an unoxidized blend of unsaturated oil was fed, whereas SOD activity was increased (23.74 vs. 26.35 U/g of protein) by feeding Agrado Plus with oxidized oil compared to cows without Agrado Plus. The varied responses of activity of SOD may due to differences in degrees of oxidative stress among animals used in the studies.

Usually supplementation of Se is correlated positively with GPx activity in RBC or plasma. Hafeman et al. (1974) conducted a study of increasing concentrations of Se supplementation (0, 0.005, 0.1, 0.5, or 1.0 ppm) on erythrocyte GPx of the rat. Activity of GPx was increased markedly with increased concentration of Se in the diet. The difference among these groups became greater as the diets were fed over a longer period of time. Activity of plasma GPx was not different between cows fed an inorganic vs. organic source of Se (0.3 mg/kg of dietary DM) from 25 d before calving to 70 DIM (Cerri et al., 2009). The GPx activity in RBC of horses was increased with increasing the

distance of endurance race up to 80 km for horses under vitamin E (5000 IU/d of α-tocopheryl acetate) supplementation and horses supplemented with 5000 IU/d of vitamin E plus 7 g/d of ascorbic acid (Williams et al., 2004).

The supplementation of vitamin A (5000 IU/d), vitamin E (100 IU/d), and vitamin C (50 mg/d) resulted in decreased concentration of plasma TBARS in HIV-infected patients compared to the patients who received a placebo (Jaruga et al., 2002), indicating the reduced production of lipid peroxides in liver. In another human study involving 24 participants who were nonsmokers and not supplemented with vitamin (Jialal and Grundy, 1993), concentrations of plasma TBARS did not differ between volunteers who received 1.0 g/d of vitamin C, 800 IU/d of vitamin E, and 30 mg/d of vitamin A and those receiving placebo capsules. Two amounts of vitamin E (0 or 1000 IU/d) and of Se (0 or 3 mg/kg of dietary DM) were studied for 6 wk using multiparous dairy cows. Concentration of TBARS in erythrocytes was decreased by supplementation of vitamin E regardless of Se supplementation (Brzezinska-Slebodzinska et al., 1994). Holstein steers were fed supplemental vitamin E at 4 concentrations (0, 250, 500, or 2000 mg/d) for either 42 or 126 d. Concentrations of TBARS in meat increased with increased length of display of retail cuts but accumulation of TBARS was less in beef from vitamin E – supplemented steers than from controls (Liu et al., 1996). Descalzo and Sancho (2008) reported that meat from pasture-fed steers had lower TBARS concentration than that from grain-fed steers. They suggested that cattle grazed on good quality pasture incorporated enough vitamin E in their tissues to prevent lipid peroxidation in meat. A 2 x 2 factorial design was arraged with two types of corn oil (oxidized vs. unoxidized) and with or without Agrado Plus to test the effect of synthetic

antioxidants on shelf-life of pork after pigs were or were not given antioxidants. After 21 d in display case, the concentrations of TBARS in loin chop were lowest from pigs fed fresh oil with Agrado, whereas TBARS were greatest in loin chop from pigs fed oxidized oil without Agrado (D. M. Fernández-Dueñas, 2009). Supplementation of lipid-soluble antioxidant effectively reduced the lipid peroxidation by scavenging peroxyl radicals generated through the chain-reaction.

Limited studies have been published on the effects of synthetic antioxidants on oxidative stability. Bartov et al. (1991) reported that initial oxidation of uterine tissue from laying hens after 30 d of frozen storage as measured by thiobarbituric acid (**TBA**) was reduced by supplementing ethoxyquin at 250 mg/kg of food compared with those given 5 and 125 mg/kg of vitamin E and ethoxyquin, respectively. Lin et al. (1989) reported that adding BHA at the rate of 12.5 mg/d/bird for 7 wk plus BHT at the same rate for the last 5 d before slaughter with unoxidized sunflower oil increased the oxidative stability of dark and while meat from boilers after 9 d of refrigerated storage in contrast with meat from boilers given only unoxidized sunflower oil. In a study by Bailey et al. (1996), 4 feeding rates of ethoxyquin (0, 125, 500, and 1000 ppm) were given to chicks from 30-d to 6-wk of age. Both 500 and 1000 ppm ethoxyquin rates resulted in lower TBARS concentration in liver and spleen tissues but not in kidney compared to 0 ppm, indicating that ethoxyquin is effective to reduce tissue peroxidation.

Murai and Andrews (1974) reported that channel catfish fed oxidized menhaden oil had reduced lipid content of liver and lipid peroxidation of pelleted diets when supplemented with 125 mg/kg of ethoxyquin.

Effect of Prepartum Heat Stress on Performance and Metabolites

Commonly, temperature-humidity index (**THI**) is used to indicate the degree of heat stress on cattle (Armstrong, 1994; West, 2003). A THI above 72 indicates heat stress such that performance and physiological status will be altered. Typically, major responses to heat stress by dairy cattle include the following: reduced DMI and activity, increased sweating, water intake, respiratory rate, body temperature and maintenance requirement, and changes in metabolic and hormonal status (Fuquay, 1981; Armstrong, 1994; West, 2003). Ultimately, heat stress results in loss of milk production (Collier et al., 1982, Rhoads et al., 2009).

Avendaño-Reyes et al. (2006) reported that non-cool cows which were only under shades tended to have a greater respiratory rate (95.7 vs. 89.5 breaths/min) than cooled cows which were cooled by soaking the entire body with a hose that delivered approximately 25 L of 27°C water/cow/cooling event for every 2 min each day from 1130 to 1430 h for a 60-d nonlactating period, but rectal temperatures and BW were not different between treatment groups. After calving, cows were moved to a common pen with only shade. Milk production was numerically greater for cows exposed to the cooled environment relative to cows exposed to heat stress (22.3 vs. 20.2 kg/d, but proportion of milk fat and milk fat yield were not affected by treatment. Amaral et al. (2009) reported that rectal temperatures in the afternoon were greater for cows offered shade alone from dry off (60 d) until calving than cows offered fans and sprinklers. Milk yield, milk fat content, and milk fat yield were greater for cows under prepartum evaporative cooling even though all cows were cooled after calving. The DMI (as % of BW) of cooled vs. noncooled cows were not different during the nonlactating period, but was greater for cows under prepartum heat stress compared to those given evaporative

cooling from calving to 14 DIM. Pregnant dairy cows were assigned randomly to either a shaded or a non-shaded group during the last 60 d of pregnancy (Collier et al., 1982). After calving, all cows were cooled with fans and sprinklers. The shaded cows yielded 13.6% more milk for a 305-d lactation. Wolfenson et al. (1988) reported that prepartum cows under shade alone had greater rectal temperature in the afternoon period than cows under shade with fans and sprinklers (39.2 vs. 38.7°C) but BCS did not differ between treatments. Milk yield corrected for fat was increased by prepartum cooling compared with noncooled cows during 150 d of lactation. A 2 x 2 factorial trial was conducted using 112 growing replacement heifers in which animals were assigned to one of 4 treatments: no shade or misting, only misting, only shade, or shade and misting. Misted heifers had lower rectal temperatures and respiratory rates than unmisted heifers. After 131 d on treatment, BW was greater (520 vs. 547 kg) for shaded than for the unshaded heifers (Mitlohner et al., 2001).

Amaral et al. (2009) reported that prepartum cows under shade alone had lower NEFA in plasma at parturition and for the following 28 DIM compared with cows exposed to shade plus fans and sprinklers. This result was due to lower DMI and greater BCS for the cooled cows. They also detected lower BHBA in plasma from 14 to 28 DIM for prepartum cows only under shade than cows under shade with fans and sprinklers. Pregnant multiparous Holstein cows were assigned randomly to 2 study pens where cows were provided with either sprinklers over the feed bunk or sprinklers, fans, and shade over the feed bunk around 30 d before calving during summer. After parturition, cows were housed under identical conditions. Body condition scores and serum NEFA were not different between treatment groups (Urdaz et al., 2006).

The somatic cell count (**SCC**) response of cows responding to heat stress is consistent. Wegner et al. (1976) reported that cows under mild to severe heat stress from June to November had increased SCC during the hot summer season (August to October) than the rest of the study period. Mohammed and Johnson (1985) also reported that the number of somatic cells increased 56% when cows were exposed to heat stress (28.9°C and 55% RH).

Effect of Prepartum Heat Stress on the Immune System

Nardone et al. (1997) conducted a trial in which primiparous Holsteins were housed either in a cool (THI = 65) or hot environment (THI = 82 from 0900 to 2000 h, THI = 76 from 2100 to 0800 h) from 3 wk before calving to 36 h after calving. Concentration of IgG in colostrum was lower for cows housed in the hot vs. cool prepartum environment. Secretion of IgM by PBMC isolated from cows calving in summer (THI = 79) was greater than those from cows calving in spring (Lacetera et al., 2005).

Several studies have been carried out to evaluate the effect of heat stress on immune cell function in the bovine. However, the results of these studies are inconsistent regarding lymphocyte function in cows exposed to a hot environment. Soper et al. (1978) tested PBMC immunostimulation on lactating dairy cows every 2 wk for 1 yr. The greatest PBMC response to mitogens was shown for 4- to 6- yr old cows in August when heat stress occured, whereas the least response was shown for 7- to 9- yr old cows in Feburary. Contrary to this result, after *in vivo* heat stress of lactating Holstein cows, the responses of polymorphonuclear leukocytes *in vivo* was not influenced by heat stress, but the decrease in proliferation of lymphocytes isolated from cows exposed to heat stress was less at 42°C (Elvinger et al., 1991). The same lab

(Kamwanja et al., 1994) also reported that proliferation of phytohemagglutinin-stimulated lymphocytes isolated from 3 Holstein cows was decreased when cells were exposed to 42°C compared with 38.5°C. Lymphocytes were isolated from Holstein cows and stimulated by Con A. Cows provided only shade prepartum had less proliferation compared with those provided shade plus fans and sprinklers (Amaral et al., 2009). Yet Lacetera et al. (2002) reported that the response of Con A-treated PBMC isolated from calves exposed to a constantly hot environment (35°C) was not different from those of calves exposed to thermoneutral conditions. These variations may be due to the duration of the exposure, intensity of heat stress, and immune function variables measured (Kelley et al., 1982).

The Effect of Cooling Systems for Dairy Cows in Hot Environments

The characteristic climate in the southeastern United States is the high ambient temperature and relative humidity. Hot and humid conditions are associated with numerous physiological changes that occur in the digestive system, acid-base chemistry, and blood hormones (West, 2003). In order to improve cow performance in such conditions, alterations of the cow's environment have been developed.

Shading.

Firstly, shade structures should be used to reduce the direct and indirect solar radiation reaching the animals during the day (Ryan and Boland, 1992; West, 2003). They have little effect on changing ambient temperature and humidity (Buffington et al., 1981).

Shading during the prepartum period. Reducing the negative effect of heat stress by shading for nonlactating and pregnant cows has shown benefits. Two studies evaluated the effect of heat stress relief by shading during the last 80 d of pregnancy on

prepartum and postpartum responses of Holstein cows (Collier et al., 1982; Lewis et al., 1984). Upon calving, all cows were managed uniformly. Cows with shade during the last 80 d of gestation had numerically greater milk yields in the subsequent lactation, lower rectal temperatures and respiratory rates, greater thryoxine and lower NEFA concentration in plasma, and gave birth to heavier calves (the difference about 3 kg) compared with cows offered no shade (Collier et al., 1982). Prepartum shading reduced postpartum plasma concentrations of prostaglandin $F_{2\alpha}$, but increased rectal temperatures compared with non-shaded cows (Lewis et al., 1984).

Shading during the postpartum period. Providing shade to cows in midlactation for 11 wk during summer in Florida resulted in lower rectal temperatures (38.9 vs. 39.4°C), reduced respiratory rates (54 vs. 82 breaths/min), and about 10% more milk yield (Roman-Ponce et al., 1977). Similarly, mid-lactating cows with shade for 102d had lower rectal temperatures (38.7 vs. 39.6°C) and respiratory rates (79 vs. 115 breaths/min), increased ruminal contractions (2.3 vs. 1.6 times/min), and greater milk yield corrected for stage of lactation (15.1 vs. 12.7 kg/d) compared with cows with no shade indicating that environmental modification altered physiological responses of the cows (Collier et al., 1981).

Mechanical Cooling.

Air movement (fans), wetting the cow (sprinklers and sprayers), evaporation to cool the air (misting), and their combinations are effective to enhance heat dissipation. Mist precools the air by evaporation before it reaches the hair coat and respiratory system of the cows, whereas sprinklers and sprayers dampen the hair and skin of the cows, increase the rate of evaporation and subsequent heat removed from the skin The main disadvantage of sprinklers is that they create an environment saturated with water

which markedly reduces the capability of animals to dissipate heat by evaporation (Flamenbaum et al., 1986).

Cooling during the prepartum period. Cooling with sprinklers and fans during the last 60 d of gestation reduced rectal temperatures (38.7 vs. 39.0°C), increased 150-d lactation mean milk yield by 3.5 kg/d, and increased calf birth weights by 3.3 kg compared to cows managed under shade only. Prepartum cooling may alleviate any detrimental effects of heat stress on the development of mammary parenchyma or its lactogenic capacity. The effect of adding shade and fans to a sprinkler system for periparturient cows was evaluated in California (Urdaz et al., 2006). Shade with sprinklers and fans during the last 3 wk of gestation did not affect either the incidence of postparturient disorders/diseases or serum concentration of NEFA in the prepartum period compared with cows managed with sprinklers only but milk yield was increased by 1.4 kg/d.

Cooling during the postpartum period. Two evaporative cooling systems (Korral Kool® vs. fans and sprinklers) were evaluated for lactating dairy cows for a 142-d period from the end of May through the middle of October (Ryan and Boland, 1992). The mean rectal temperature (39.0 vs. 38.8°C) and milk production (27.7 vs. 26.8 L/d) of cows managed with Korral Kool compared with cows managed with fans and sprinklers. Correa-Calderon et al. (2004) also reported that using a Korral Kool cooling system for lactating Holstein cows for 18 wk reduced rectal temperature by 0.9°C compared with cows under shade only. Flamenbaum et al. (1986) reported that mean rectal temperature of Israeli-Holstein lactating cows was reduced by 0.6°C by cooling (sprinklers and forced ventilation) for 5 cooling periods of 30 min each during the day

compared with cows under no cooling system. Shading and cooling cows during late gestation and postpartum will improve subsequent lactation performance and may result in heavier calves at birth.

CHAPTER 3
EFFECT OF FEEDING ANTIOXIDANTS AND PREPARTUM EVAPORATIVE COOLING ON PERFORMANCE OF TRANSITION HOLSTEIN COWS DURING SUMMER IN FLORIDA

Introduction

Dairy cattle undergo tremendous physiological and nutritional changes during the periparturient period. As a result they might experience a variety of metabolic disorders (ketosis, hepatic lipidosis, displaced abomasum, and hypocalcemia) and infectious diseases (retained fetal membranes, mastitis, endometritis/metritis) (Goff and Horst, 1997; Bernabucci et al., 2005; Sordillo et al., 2007; Sordillo and Aitken, 2009). Many disorders and diseases during this special period are associated with suppressed host defense mechanisms (Sordillo, 2005; Sordillo et al., 2009) and oxidative stress. Oxidative stress results from excess production of reactive oxygen species (**ROS**) and insufficient antioxidant production to remove these ROS (Miller et al., 1993; Bernabucci et al., 2002, 2005; Castillo et al., 2005; Sordillo et al., 2007).

Recently, clinical medicine has given increased attention to the detection and protection of ROS (Castillo et al., 2003). Oxidative status can be monitored by several biomarkers, such as superoxide dismutase (**SOD**) which is the enzyme that catalyzes the dismutation of superoxide radicals to hydrogen peroxide and oxygen, glutathione peroxidase (**GPx**) which is a Se-dependent enzyme that decomposes hydrogen peroxide, thiobarbituric acid reactive substances (**TBARS**) which present a composite number of lipid oxidative end products including malondialdehyde and indicates the level of lipid peroxidation (Trevisan et al., 2001; Bernabucci et al., 2005), and total antioxidant status (**TAS**) which provides more overall evaluation of oxidative status.

The relationship between heat stress and oxidative status in dairy cattle has been examined only minimally. Activities of SOD, GPx and concentration of TBARS in erythrocytes as indicators of increased oxidative challenge were increased during heat stress (Bernabucci et al., 2002; Saker et al., 2004), whereas a decrease in GPx activity by peripheral blood mononuclear cells (**PBMC**) was detected in spite of heat stress (Burke et al., 2007).

Supplementation with 200 mg/kg of dietary synthetic antioxidant (Agrado Plus, Novus International, St. Charles, MO) increased plasma SOD activity when cows were fed oxidized rather than fresh oil and increased plasma GPx activity across both types of oil fed (Vázquez-Añón et al., 2008). The effect of supplementation of synthetic antioxidants on oxidative status and performance of dairy cattle during the transition period is limited.

The aim of this study was to evaluate whether the supplementation with a blend of synthetic antioxidants (Agrado Plus®) would ameliorate the expected negative effect of heat stress on performance and oxidative status of Holstein cows.

Material and Methods

Animals, Treatments, and Management

The experiment was conducted at the University of Florida Dairy Research Unit (Hague, FL) during the months of July through December 2008. All experimental animals were managed according to the guidelines approved by the University of Florida Animal Research Committee. Periparturient Holstein primiparous (n = 22) and multiparous (n = 13) cows were blocked by parity and were assigned to treatments at 29 ± 7 days prior to their calving date. Four treatments were arranged in a 2 x 2 factorial design including 2 dietary concentrations of antioxidants (0 vs. 250 mg/kg of dietary DM,

AO) and 2 environmental housing conditions for pregnant cows. The AO is a liquid mixture of ethoxyquin and tertiary-butyl-hydroquinone which was added to corn oil by the manufacturer and shipped to the research site. The corn oil was mixed with ground corn just prior to preparing the concentrate portion of the diet in 909 kg batches. The corn oil was devoid of commercial antioxidants except AO. Pregnant cows were housed in an open-sided free-stall barn with sand bedding equipped with or without fans (J & D Manufacturing Eau Claire, WI) and sprinklers (Rainbird Manufacturing, Glendale, CA) in cooled vs. noncooled treatments, respectively. Sprinklers were intermittently operated every 6 min for 1.5 min. Lights were operational from 0600 h to 2000 h. Calan gates (American Calan Inc., Northwood, NH) were used to measure DMI of individual cows. Pregnant cows were fed twice daily ad libitum amounts of a bermudagrass silage-corn silage-based TMR (Table 3-1). Refusals of TMR were measured daily. Rectal body temperatures were recorded daily between 1430 and 1530 h using a GLA M700 digital thermometer (GLA Agriculture Electronics, San Luis Obispo, CA). After calving, all animals were moved to a sand-bedded, open-sided, free-stall barn equipped with fans and sprinklers and Calan gates. Cows were milked twice daily at 0700 and 2000 h and fed in ad libitum amounts a corn silage-alfalfa hay-based TMR twice daily at 0800 and 1300 h for 7 weeks. Dry matter intake was recorded daily. Prepartum and postpartum cows were weighed on the same day each week before the morning feeding. Rectal body temperature was measured on 4, 7, and 12 DIM using a GLA M700 digital thermometer.

Sample Collection and Analysis

Representative samples of corn silage, bermudagrass silage, alfalfa hay and grain mixes were collected weekly, composited monthly and ground through a 1-mm Wiley

mill screen (A. H. Thomas, Philadelphia, PA). Silage and hay samples were ground before compositing whereas grain mix samples were composited before grinding. Composited feed samples were analyzed for CP using a macro elemental analyzer vario MAX CN (Elementar Analysensystene GmbH, Hanau, Germany), NDF (Mertens, 2002), ADF (AOAC, 1995), ether extract (AOAC, 2003), and minerals (Dairy One, Ithaca, NY). The chemical composition of diets are shown in Table 3-2.

Milk samples were collected from two consecutive milkings weekly using bronopol-B-14 as a preservative, and analyzed for true protein, fat, and SCC by Southeast Milk laboratory (Belleview, FL) using a Bently 2000 NIR analyzer. Final concentrations of fat and protein were calculated after adjusting for milk production during those 2 milk collections.

Blood samples were collected at 0900 h on Monday, Wednesday and Friday weekly from the coccygeal vessels into sodium heparinized tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) from calving until 49 DIM. Blood samples were placed on ice immediately after collection until centrifuged at 1200 × *g* at 4°C for 15 min (Allegra® X-15R Centrifuge, Beckman Coulter). Plasma was separated after centrifugation and stored at -20°C for subsequent metabolite and hormone analyses.

Plasma concentrations of NEFA (NEFA-C kit; Wako Diagnostics, Inc., Richmond, VA; as modified by Johnson, 1993) and BHBA (Wako Autokit 3-HB; Wako Diagnostics, Inc., Richmond, VA) were determined weekly for 7 wk. A Technicon Autoanalyzer (Technicon Instruments Corp., Chauncey, NY) was used to determine weekly concentrations of plasma BUN (a modification of Coulombe and Favreau, 1963 and Marsh et al., 1965) and plasma glucose (a modification of Gochman and Schmitz,

1972). Concentrations of progesterone were determined on all plasma samples collected using Coat-A-Count Kit (DPC® Diagnostic Products Inc., Los Angeles, CA) solid phase 125I RIA. The sensitivity of the assay was 0.02 ng/mL and the intra-assay CV was 3.8%.

Processing of Red Blood Cell (RBC)

Approximately 7 mL of blood was collected on -15, 1, 8, 15, and 29 DIM from the coccygeal vessels into evacuated tubes containing 17.55 mg of K_2 EDTA (Vacutainer®, Becton Dickinson, Franklin Lakes, NJ, USA). The blood samples were placed on ice immediately following collection and transported to the laboratory within 3 h. To measure haematocrit, blood was drawn into a haematocrit capillary tube (Fisher Scientific, Cat. No. 22-362-566) and centrifuged for 2.5 min. Plasma and RBC were separated using a refrigerated centrifuge operating at 1200 × *g* for 10 mins at 4 °C (Allegra® X-15R Centrifuge, Beckman Coulter). Plasma was stored at -80°C until analyzed for TBARS. The RBCs were processed as follows. The buffy coat (leukocytes) was discarded via pipette. The RBC (2 mL) were transferred using a positive displacement pipette to a 13 x 100 mm polypropylene tube. Cold (4°C) physiological saline (4 mL) was added to the RBC, capped, and gently mixed. The mixture was centrifuged at 1200 × *g* for 10 min, and saline pippetted off. This washing process was repeated 2 more times for a total of 3 saline washes. After the third saline wash, the washed RBC were lysed by adding 2 mL of cold (4°C) UltraPure water (Cayman Chemical, Catalog No. 400000, Ann Arbor, MI) at which time a dark maroon color was achieved upon vortexing. Samples (400 uL) were stored in triplicate vials at -80°C for analysis for superoxide dismutase (**SOD**) and glutathione peroxidase (**GPx**), respectively.

Thiobarbituric Acid Reactive Substances (TBARS) Assay

Plasma concentrations of TBARS were determined by the method modified by Armstrong et al. (1998). Briefly, fresh thiobarbituric acid (TBA) buffer was made (200 mL) by adding 40 mL of glacial acetic acid and 1.060 g of TBA to 160 mL distilled water. Sodium dodecyl sulfate (SDS) reagent (8.1%) was prepared by mixing 8.1 g of SDS with 100 mL of distilled water. Malondialdehyde (MDA) stock solution (100 nmol/mL) was made by mixing 82 uL of MDA (Sigma-Aldrich Inc., cat no. 108383, St. Louis, MO) and 1 mL of concentrated HCl with 100 mL of distilled water. One portion of stock MDA and 9 portions of distilled water were combined to make the MDA working solution (10 nmol/mL). Working MDA standards of 0, 1, 2, 3 and 4 nmol/mL were prepared by adding 0, 10, 20, 30, and 40 uL of MDA working solution respectively to 100, 90, 80, 70, and 60 uL of distilled water. In each labeled test tube, 100 uL of diluted sample (1:100) or working MDA standards, 100 uL of SDS reagent, and 2.5 mL of TBA buffer were added by force in order to mix them well. Tubes were covered with a marble and incubated at 95°C for 1 h. After incubation, tubes were placed in an ice water bath for 10 min. Lastly, fluorescent readings were obtained using a florescence spectrophotometer (RF-1501, Shimadzu International, Columbia).

Superoxide Dismutase (SOD) Assay

The SOD activities of RBC were measured using the Superoxide Dismutase Assay Kit (Cayman Chemical, Catalog No. 706002, Ann Arbor, MI). This assay utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. All 3 types of SOD (Cu/ZnSOD, MnSOD and FeSOD) are measured in this 96-well microplate assay. Briefly, 2.5 mL of concentrated assay buffer was diluted with 22.5 mL of HPLC-grade water. Concentrated sample buffer (2.5 mL) was diluted

with 22.5 mL of HPLC-grade water. Diluted assay buffer and sample buffer was kept at room temperature while performing the assay. Erythrocyte lysate samples (10 μL) from 15 d before expected calving date, 1, 8, 15, and 29 DIM were thawed and diluted 5000 times with diluted sample buffer. The SOD Stock standard was prepared by diluting 20 μL of concentrated SOD standard with 1.98 mL of diluted sample buffer. Working standards of 0, 0.025, 0.05, 0.1, 0.15, 0.2, and 0.25 U/mL were made by adding 0, 20, 40, 80, 120, 160, and 200 μL respectively of SOD standard stock to 1000, 980, 960, 920, 880, 840, and 800 μL of diluted sample buffer. Diluted samples and working standards were kept on ice. Diluted radical detector was obtained by diluting 50 μL of concentrated radical detector with 19.95 mL of diluted assay buffer. The wells on a 96-well plate were categorized as SOD standard wells and sample wells. Each standard/sample was measured in duplicate. For the SOD standard wells, 200 uL of the diluted radical detector and 10 uL of working SOD standard (7 standards) were added to each corresponding well. For sample wells, 200 μL of the diluted radical detector and 10 μL of the diluted sample were added to each corresponding well. After this step, xanthine oxidase was prepared by adding 50 μL of the supplied enzyme to 1.95 mL of sample buffer. As quickly as possible 20 μL of xanthine oxidase was added to each well to initiate the reaction. The plate was covered, incubated on a shaker for 1 h, and read at 450 nm using a plate reader (SpectraMax 340PC^{384}, Molecular Devices, CA). Superoxide dismutase activity was calculated based on the standard curve. Enzyme activity was expressed as units per milliliter of packed cell volume (PCV). Plates contained samples from each treatment and all the samples from the same cow were

analyzed in the same plate. This method was used for all variables measured using the microplate reader.

Glutathione peroxidase (GPx) Assay

The GPx activities of erythrocytes were measured using the assay kit supplied by Cayman Chemical Company (catalog no. 703102, Ann Arbor, MI). This assay measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR).

$$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

The procedure of this assay is as below. The assay buffer and sample buffer were diluted 10 fold and kept at room temperature. Erythrocyte lysate samples from 15 d before expected calving date, 1, 8, 15, and 29 DIM were thawed and diluted 500 times with diluted sample buffer. Ten μl of supplied glutathione peroxidase was diluted with 490 uL of diluted sample buffer. Cayman sells a 96-well kit and a 480-well kit. The 96-well kit contains 3 vials of co-substrate mixture. Each vial was reconstituted by adding 2 mL of HPLC-grade water. The 480-well kit contains 5 vials of co-substrate mixture. Each vial was reconstituted by adding 6 mL of HPLC-grade water. The diluted samples, enzyme, and co-substrate mixture were kept on ice at all times. The wells in a 96-well plate were categorized as background wells, positive control wells, and sample wells. For background wells, 50 μL of co-substrate mixture and 120 μL of assay buffer were added to each well. For positive control wells, 20 μL of diluted enzyme, 50 μL of co-substrate mixture and 100 uL of assay buffer were added to each well. For sample wells, 20 μL of diluted RBC sample, 50 μL of co-substrate mixture, and 100 uL of assay

buffer were added to each well. The reaction was initiated by adding 20 µL of cumene hydroperoxide to all wells as quickly as possible. The plate was covered and shaken for a few seconds. Lastly, plate was read at 340 nm once every minute for at least 6 min. GPx activity was calculated using the formula below:

$$\text{GPx activity} = \frac{\Delta A340/\text{min}}{0.00373} \times \frac{0.19\ \text{ml}}{0.02\ \text{ml}} \times \text{sample dilution} = \text{nmol/min/ml}$$

ΔA340/min was calculated by subtracting the change in absorbance per minute for the background from the change in absorbance per minute for the sample. Enzyme activity was expressed as nmol/min/mL, which is the amount of enzyme used to oxidize 1.0 nmol of NADPH to $NADP^+$ per minute at 25 °C. Enzyme activity was expressed as nmol per min per mL of PCV.

Acute Phase Protein Assays

Plasma samples collected on Monday-Wednesday-Friday were used to determine the concentrations of acid soluble protein (**ASP**) and haptoglobin (**Hp**). Acid soluble protein was extracted from 50 µL of plasma with 1 mL of 0.6 M perchloric acid after 20 min of incubation at room temperature in duplicate. Tubes were centrifuged at 1200 × *g* for 30 min at room temperature. Supernatant was analyzed with the bicinchoninic acid kit (Sigma-Aldrich, Saint Louis, MO; Cat No. 096k9802). Concentrations of unknowns were obtained from the standard curve. The inter-assay variation was 9.2%. If CV% between replicates was greater than 10%, samples were re-analyzed.

Concentrations of plasma Hp were determined by measuring the differences of hydrogen peroxide activity with haptoglobin-hemoglobin (Hb) complex (modified by Tarukoski 1966). Briefly, O-dianisidine solution (4 L) was prepared by adding 2.4 g of O-dianisidine, 2.0 g of Na_2EDTA, and 55.2 g of NaH_2PO_4 to 4 L of distilled water and

adjusted to pH of 4.1. Hemoglobin stock solution (25 uL) was added to each tube to form a Hb-Hp complex with Hp from 5 uL of plasma sample. O-dianisidine solution (7.5 mL) was added to each tube. Tubes were incubated in a water bath for 45 min at 37°C. Then 100 uL of hydrogen peroxide were added to react with Hb-Hp complex to liberate oxygen which oxidized O-dianisidine to a yellow color compound. Samples from the tubes were transferred to the 96-well plate and read at 450 nm using the microplate reader.

Neutrophil Function, WBC and Lymphocyte

Blood (6 mL) was collected from coccygeal vessels at – 21, 0, 7, and 14 DIM in vacutainer (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) tubes containing acid citrate dextrose. Tubes were not put on ice but were gently mixed by hand approximately every 15 min. Counts of WBC, lymphocytes, and neutrophils were done using a Bayer Advia 120 cell counter (Fisher Diagnostic, Middletown, VA) within 3 h of collection. Phagocytosis and oxidative burst by neutrophils were assessed within 3 h after blood collection. Neutrophil, WBC and lymphocyte concentrations were estimated by a demacytometer. Whole blood (100 uL) was pipeted into each of 3 tubes. Then 10 µL of 50 µM dihydrorhodamine 123 (DHR) (Sigma-Aldrich, Saint Louis, MO) was added to all tubes. Tubes were vortexed and incubated in an oven at 37°C for 10 min with constant rotation using the Clay Adams nutator (BD Bioscience, San Jose, CA). Ten µL of 20 µg/mL solution of phorbol 12-myristate, 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) were added to the second tube only. An *Escherichia coli* bacterial suspension (10^9 cells/µL) labeled with propidium iodide (Sigma-Aldrich) was added to the third tube to establish bacteria to neutrophil ratio of 40:1 (*Escherichia coli* strain were isolated from a dairy cow with mastitis and grown *in vitro* for labeling). Tubes were

vortexed and incubated in oven at 37^{o}C for 30 min with constant rotation using the Clay Adams nutator (BD Bioscience, San Jose, CA). Then all tubes were removed and placed immediately on ice to stop phagocytosis and oxidative burst activity. Tubes were processed in a Q-Prep Epics immunology workstation on the 35 cycle. Cold distilled water (500 µL) and 0.4% tryphan blue (10 µL) were added to each tube. Then tubes were vortexed, kept on ice, and 10,000 neutrophil cells were read using the Facsort flow cytometer (BD Biosciences, San Jose, CA). The percentage of total neutrophils able to phagocytize *E. coli*, undergo oxidative burst, and efficiency of bacteria killing (mean fluorescence intensity, **MFI**) were measured using the flow cytometer.

Ovalbumin Challenge

All cows were injected i.m. with 1 mg of ovalbumin (Sigma-Aldrich, Saint Louis, MO) diluted in 1 mL of sterile Quil A adjuvant (0.5 mg of Quil A/ mL of PBS) (Accurate Chemical & Scientific Corp. Westbury, NY) at -4 and -2 wk relative to expected calving date and at calving. Blood samples (8 mL) for measurement of anti-ovabumin IgG were collected at -4, -2, 0, 1, 2, 3, 4, and 7 wk relative to calving. Samples were taken in vacutainer tubes containing no anticoagulant before the ovalbumin injection. Serum concentration of anti-ovalbumin IgG was measured by an Enzyme Linked ImmunoSorbent Assay (ELISA) as described by Mallard et al. (1997). Briefly, flat bottom 96-well polystyrene plates (Immulon 2, Dynex Tech., Chantilly, VA) were coated with a solution of ovalbumin dissolved in carbonate-bicarbonate coating buffer (1.4 mg OVA/ mL of carbonate-bicarbonate buffer). Plates were incubated at 4°C for 48 h, then washed with PBS and 0.05% Tween-20 solution (pH = 7.4). Plates were blocked with a PBS-3% Tween-20 and bovine serum albumin (Sigma Chemical, St. Louis, MO) solution and incubated at room temperature for 1 h. Plates were washed and serum

samples and control sera diluted at l/50 and l/200 were added in duplicate using a quadrant system (Wright, 1987). Positive and negative control sera to anti-ovalbumin IgG were obtained from a pool of sera of known high (21 d after the third injection of ovalbumin) and low (no ovalbumin injection) concentrations, respectively. All samples from the same cows were analyzed in the same plate and plates contained balanced number of animals from each diet group. Plates were incubated at 24°C for 2 h and washed with the previously described buffer solution. Subsequently, alkaline phosphatase conjugate rabbit antibovine IgG whole molecule (Sigma Chemical, St. Louis, MO) was dissolved in Tris/HCl Buffer, added to the plates and incubated for 1 h at room temperature. After incubation, plates were washed 4 times and substrate solution [P-nitrophenyl phosphate disodium (Sigma Chemical, St. Louis, MO)] was added and the plate was incubated at room temperature for 30 min. Plates were read on an automatic ELISA plate reader (MRX Revelation; Dynex Technologies Inc., Chantilly, VA) and the optical density was recorded at 405 nm and the reference at 650 nm.

Vaginoscopy

Cows were evaluated for cervical discharge on 7, 16, and 25 DIM using the metricheck® (Metricheck, Simcro, New Zealand) tool. The vulva was cleaned using a povidone-iodine scrub (0.75% titratable iodine and 1% povidone solution, Agripharm, Memphis, TN, USA) and dried with clean gauze. The metricheck was inserted in the vagina close to the cervix. The floor of the vagina was scraped, the discharge collected in a 50-ml conical tube (Fisher Diagnostics, Middletown, VA), and scored according to Sheldon et al. (2006). Scoring system was as follows: 0 = translucent or clear, 1 = flecks of white or off-white pus, 2 = discharge containing < 50% white or off-white

mucopurulent material, 3 = discharge containing > 50% white or yellow mucopurulent material, 4 = discharge containing > 50% sanguineous mucopurulent material.

Uterine Cytology

At 40 ± 2 days postpartum, one of the uterine horns was flushed with saline. After thorough sanitation of the vulva and entrance of the vagina using chlorhexidine diacetate (Nolvasan, Fort Dodge, Overland Park, KS), an 18 French, 30 mL, 56 cm, 2-way Foley catheter was placed randomly in 1 uterine horn at approximately 2 cm past the bifurcation of the uterus. The cuff of the catheter was inflated with 7 to 10 mL of air according to the diameter of the uterine horn, and 20 mL of sterile isotonic saline solution was infused into the uterine horn and then recovered using a 35 mL sterile syringe. The aspirated fluid was transferred to a 50 mL sterile conical tube, placed in ice and transported to the laboratory within 4 h of collection. In the laboratory, the aspirated fluid was centrifuged at 750 x *g* for 10 min and the supernatant discarded. The pellet was resuspended with 2 mL of saline, and an aliquot of 20 uL was pipetted onto glass slides and smeared in triplicate. Smears were air-dried and stained using a Diff-Quick (Fisher Diagnostics, Middletown, VA) stain. Slides were examined under a microscope and the number of total leukocytes, epithelial endometrial cells, and neutrophils were counted to complete a 100 cell-count per slide, and percentage neutrophiles was calculated. Subclinical endometritis was diagnosed when the proportion of neutrophils exceeded 5% after 30 DIM (Gilbert et al., 2005).

Statistical Analyses

Treatments were arranged in a 2 x 2 factorial completely randomized design. Repeated measurements were made on nearly all variables and were analyzed using the PROC MIXED procedure of SAS (Release 9.2) according to the following model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + C_{l(ijk)} + W_m + (\alpha W)_{im} + (\beta W)_{jm} + (\alpha\beta W)_{ijm} + (\gamma W)_{km} + (\alpha\gamma W)_{ikm} + (\beta\gamma W)_{jkm} + (\alpha\beta\gamma W)_{ijkm} + \varepsilon_{ijklm}$$

where Y_{ijkl} is the observation, μ is overall mean, α_i is the fixed effect of diet ($i = 1, 2$), β_j is the fixed effect of environment ($j = 1, 2$), $(\alpha\beta)_{ij}$ is the interaction of diet and environment, γ_k is the fixed effect of parity ($k = 1, 2$), $(\alpha\gamma)_{ik}$ is the interaction of diet and parity, $(\beta\gamma)_{jk}$ is the interaction of environment and parity, $(\alpha\beta\gamma)_{ijk}$ is the interaction of diet, environment, and parity, $C_{l(ijk)}$ is the random effect of cow within diet and environment and parity ($l = 1, 2, \ldots n$) + W_l is the fixed effect of week ($l = 1, 2, 3, 4, 5, 6, 7$), $(\alpha W)_{il}$ is the interaction of diet and week, $(\beta W)_{jl}$ is the interaction of environment and week, $(\alpha\beta W)_{ijl}$ is the interaction of diet, environment, and week, $(\gamma W)_{kl}$ is the interaction of parity and week, $(\alpha\gamma W)_{ikl}$ is the interaction of diet, parity, and week, $(\beta\gamma W)_{jkl}$ is the interaction of environment, parity, and week, $(\alpha\beta\gamma W)_{ijkl}$ is the interaction of diet, environment, parity, and week, and ε_{ijklm} is the residual error. Different temporal responses to treatments were further examined using the SLICE option of the MIXED procedure. Mean treatment, parity, and time (week or day relative to calving) effects are presented as least square means. A covariate representing the initial measurement for IgG against ovalbumin and BCS of individual cows was included in the model.

Data were tested to determine the structure of best fit, namely AR(1), ARH(1), CS, or CSH, as indicated by a lower Schwartz Baesian information criterion value (Littell et al., 1996). If repeated measures were taken on unequally spaced intervals, the sp(pow) covariance structure was used.

Measures of blood cell numbers, neutrophil function, acute-phase proteins, oxidative markers, and IgG against ovalbumin were tested for normality before and after

transformation (log or square root transformation) by using the PROC UNIVARIATE procedure of SAS (SAS Institute, 2007). Probability values > 0.05 using the Shapiro-Wilk test for variables were considered normal. After data were transformed, the PROC MIXED procedure with the same model described above was used. Progesterone data (DIM of first ovulation, number of ovulations, peak concentration of progesterone in the first ovulation, the length of the first cycle, and accumulated progesterone over 49 DIM) were analyzed using Proc MIXED of SAS with the accumulated progesterone analysis requiring the repeated measures function. Metricheck scores were analyzed using logistic regression with the Odds-Ratio option. If cervical discharge of cows was scored as 0 or 1, cows were classified as healthy whereas scores of 2, 3, or 4 resulted in a diagnosis of metritis/endometritis. These reclassified scores were analyzed as binomial data. Uterine cytology data were used to classify cows as either clean or with subclinical endometritis. This binary data also was analyzed using logistic regression techniques. Differences discussed in the text were significant at $P \leq 0.05$ and tended to be significant at $0.05 < P \leq 0.15$.

Results and Discussion

Body Temperature, BW, BCS, and DMI

In the prepartum period, cows stayed in the assigned freestall barn area for 29 ± 7 d before calving. However the data for body temperature, BW, and DMI are reported only for the last 21 d prepartum because most cows contributed data during this time period. Mean rectal temperature over the last 21 d prior to calving was less for cooled vs. noncooled cows (39.2 vs. 39.6°C, $P < 0.001$, Table 3-3) as expected. This difference was consistent across prepartum days as tests of all interactions of week with treatments were not significant. Rectal temperatures above 39.2°C may represent

reduced ability of lactating dairy cows to adapt to thermal stress resulting in reduced performance (Staples and Thatcher, 2003). Therefore providing fans and sprinklers to shaded freestalls helped reduce heat stress and established the planned differences of prepartum environments for cows assigned to the study.

Prepartum intake of DM did not differ among the treatment groups, averaging 10 kg/cow per day (Table 3-3). However effect of treatment on DM intake when expressed as a percentage of BW differed according to parity. Feed intake (% of BW) by primiparous cows was unaffected by treatments but DMI by multiparous cows fed the control diet was decreased by cooling whereas intake was increased by cooling when cows were AO (diet by cooling by parity interaction, $P = 0.03$, Figure 3-1). Typically cows under significant heat stress reduce DMI. Multiparous cows fed the AO diet demonstrated this response. The DMI of multiparous cows fed the control diet and evaporatively cooled responded unexpectedly as mean DMI was only 1.09% of BW. Multiparous cows on this treatment were much heavier than those in the other groups, averaging 843 kg, about 147 kg heavier than multiparous cows on the other treatments (Table 3-3). Mean prepartum BW of primiparous cows did not differ across treatments resulting in a diet by environment by parity interaction ($P = 0.001$, Figure 3-2). This disparity in BW across treatments likely occurred due to the removal of some lighter BW cows from this treatment (control diet-cooled environment) because of poor health postpartum. Overconditioned cows may consume less DM postpartum (Jones and Garnsworthy, 1989) and both multiparous cows on the control diet-cool treatment in this study were poor eaters and had a mean prepartum BCS of 3.75 whereas other treatment groups were ≤ 3.4 (Table 3-3). Cows maintained under evaporative cooling

had greater body condition throughout the prepartum period (3.48 vs. 3.18, Table 3-3, *P* = 0.05). This difference was a result of removing cows assigned to this treatment from the experiment due to health reasons. Evaporative cooling of prepartum cows did not affect BW change although BW gain between 3 and 1 wk before calving was numerically greater for cooled vs noncooled cows (1.17 vs. 0.25 kg/d, *P* = 0.24, data not shown).

As expected, mean DMI during the first 7 wk postpartum was greater (*P* < 0.001) for multiparous compared with primiparous cows (16.8 vs. 13.8 kg/d, Table 3-4). However no difference in DMI was detected between parities when DMI was expressed as a percentage of BW (2.67 vs. 2.69%) thus indicating that more DM can be consumed by larger animals (BW of 643 vs. 516 kg for multiparous and primiparous cows, respectively). Effect of parity by diet by environment interaction on DMI was the same whether expressed as amount (*P* < 0.01) or as a proportion of BW (*P* = 0.001, Table 3-4). Postpartum DMI was greater when primiparous cows were evaporatively cooled prepartum and fed the control diet compared to the uncooled cows fed the control diet (2.98 vs. 2.62% of BW) but prepartum cooling had no effect on postpartum DMI of primiparous cows fed AO (2.49 vs. 2.68% of BW). However prepartum evaporative cooling of multiparous cows fed the control diet resulted in less DMI compared to those not cooled (1.99 vs. 3.24% of BW) whereas prepartum cooling had no effect on postpartum DMI of multiparous cows fed AO (2.83 vs. 2.64% of BW, Figure 3-3, diet by environment by parity interaction, *P* = 0.001). The cooled multiparous cows fed the control diet were the heaviest (Figure 3-4) and the heaviest conditioned at calving which they maintained throughout the study (Figure 3-5). Cows on this treatment had a BCS

of 3.75 at calving whereas the BCS of the other treatment groups ranged from 3.06 to 3.37 at calving (data not shown). These cows were the poorest eaters (DMI at 1.99% of BW), continuing their prepartum pattern of poor DMI (Table 3-3). Therefore the interaction effect of treatment with parity on postpartum DMI was likely largely due to greater body condition for the one group of animals at calving. Amaral et al. (2009) reported lower DMI by multiparous cows during the first 14 DIM when they were evaporatively cooled prepartum compared to cows provided shade only. Similarly, cows fed the control diet in the current study consumed less DM postpartum when cooled prepartum compared to those not cooled (2.57 vs. 2.79% of BW). However postpartum DMI of cows fed AO was not affected by prepartum cooling (2.66 vs. 2.66% of BW, diet by environment interaction, $P = 0.05$, Table 3-4). Mean DMI postpartum was not affected by diet although DMI was lower during the first week postpartum for cows fed AO compared to control cows (1.36 vs. 1.72% of BW, diet by week interaction, $P = 0.10$, Figure 3-6). This difference in DMI at wk 1 postpartum may have been due to a greater incidence of serious health disorders (mastitis, metritis, retained fetal membranes, milk fever, or displaced abomasum) for cows fed AO (n = 11/20) vs. the control diet (n = 3/15) (Table 3-9). In contrast to the current study, Vázquez-Añón et al. (2008) reported that feeding AO to lactating dairy cows increased DMI.

Neither mean BCS (3.21) nor change in BCS over 7 wk postpartum were influenced by treatments (Table 3-4). This result is consistent with a study in which the feeding of AO did not influence BCS of lactating dairy cows (Vázquez-Añón et al., 2008).

Milk Production and Milk Composition

As expected multiparous cows produced more milk (Table 3-4) than primiparous cows (32.8 vs. 24.4 kg/d, $P = 0.001$) likely due to greater DMI by multiparous cows. Mean and pattern of milk yield measured over the first 7 wk were not different among treatments (Table 3-4 and Figure 3-7). This is consistent with 3 studies in which milk yield was not influenced by dietary supplementation with AO (Bowman et al., 2008; He et al., 2008; Preseault et al., 2008). However, lactating cows did respond to supplemental AO by increasing milk yield adjusted for fat (3.5% FCM) (Vázquez-Añón et al., 2008). Smith et al. (2002) is the only group to report improvements in uncorrected milk yield (38.5 vs. 32.3 kg/d, $P < 0.05$) despite a 2.6 kg/d decrease in DMI when adding synthetic antioxidants (50 ppm) in the form of ethoxyquin to the diet for 2 wk. Possibly differences in concentration of natural dietary antioxidants such as vitamins A and E and Se in the control diets may account for differences in milk yield responses to AO across these studies. In the current study, mean prepartum and postpartum intakes were 174,853 and 83,781 IU/d for vitamin A, 848 and 446 IU/d for vitamin E, and 4.6 and 4.7 mg/d for Se, respectively.

Mean concentration (3.25 vs. 3.61%, $P = 0.03$) and yield (0.87 vs. 1.04 kg/d, $P = 0.04$) of milk fat were less for cows fed AO compared to control cows (Table 3-4). Smith et al. (2002) reported numerical decreases in milk fat concentration when ethoxyquin was fed at 0, 50, 100, or 150 ppm (3.6, 3.2, 3.5, and 3.4%, respectively) although milk fat production was unchanged. Milk fat depression is usually associated with changes in biohydrogenation of long chain polyunsaturated fatty acids to conjugated linoleic fatty acids by ruminal bacteria in cows fed high concentrate/low forage diets and/or diets rich in plant-oil supplementation (Bauman and Griinari, 2001; Zebeli and Aetaj, 2009).

Vázquez-Añón et al. (2007) reported a trend for reduced escape of linolenic acid from continuous cultures fed AO indicating that AO may stimulate some of the steps in the biohydrogenation process, although the outflow of *cis*-9, *trans*-11 CLA was not changed by adding AO. Likewise concentration of *trans*-10 C18:1 and the 3 CLA isomers (*cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, and *trans*-9, *trans*-11 CLA) in milk fat were not changed by feeding AO (Vázquez-Añón et al.,2008). Indeed milk fat yield was increased (0.95 vs. 1.00 kg/d) in dairy cows fed AO in that study. Their reported improvements in milk fat yield might be associated with positive effects of AO on efficiency of ruminal microbes (Vázquez-Añón et al., 2007). The AO appears to act as a microbial modifier in the rumen because feeding AO improved fiber digestibility and conversion of dietary N to microbial N in continuous culture systems (Vazquez-Anon et al., 2007), Cows in the current study were fed diets of low NDF and ADF concentrations (Table 3-2; NRC, 2001) and supplemented with polyunsaturated fat in the form of corn oil. A more acidic ruminal environment in the presence of additional polyunsaturated fatty acids may be a situation in which a microbial modifier such as AO can shift fatty acid metabolism toward CLA and result in reduce milk fat production.

Although the effect of prepartum cooling did not affect mean concentration or yield of milk fat, the pattern of milk fat concentration and yield over weeks postpartum differed; cows not evaporatively cooled prepartum experienced decreased milk fat % at wk 3 and 4 postpartum compared to cows evaporatively cooled prepartum (environment by week interaction, $P = 0.01$, Figure 3-8). Production of milk fat over time responded in a similar fashion (environment by week interaction, $P = 0.02$, Figure 3-9). Production of 3.5% FCM was reduced at wk 3 and 4 postpartum due to lack of prepartum cooling

(environment by week interaction, $P = 0.07$, Figure 3-10). Dairy cows under similar management conditions (Amaral et al., 2009) also tended to produce milk of less fat concentration due to lack of prepartum cooling (3.5 vs. 3.9% milk fat) and yielded less milk fat (0.9 vs. 1.3 kg/d). Avendaño-Reyes et al. (2006) reported that cows cooled with water spray and fans during pregnancy had an increase in milk fat yield in the subsequent lactation compared with cows managed under heat stress. It is unknown why lack of evaporative cooling prepartum would depress milk fat postpartum. Concentration of milk fat often is reduced when lactating cows undergo moderate serious heat stress, thought to be due to respiratory alkalosis. Collier et al. (1982) indicated that the effects of heat stress during the last trimester of pregnancy reduced placental and maternal hormone concentration, which in turn reduced mammary gland growth and function that may have led to the reduction in milk fat concentration.

Mean concentration of milk true protein tended to be greater for cows fed AO (3.07 vs. 2.94%, $P = 0.06$, Table 3-4). Much of this increase was due to a difference detected at wk 1 postpartum (diet by week interaction, $P = 0.04$, Figure 3-11) therefore AO did not improve milk protein long-termWhen feeding AO to lactating cows, no dietary effect on milk protein content and yield were reported (Vázquez-Añón et al., 2008). Prepartum cooling did not affect concentration or yield of milk protein. Milk protein concentration dropped acutely when cows were housed under heat stress for 5 d compared with the same cows housed under thermoneutral conditions for 5 d (Ominski et al., 2002).

Somatic cell count was greater in milk produced by cows without prepartum evaporative cooling at wk 5 and 6 after calving (environment by week interaction, $P =$

0.03, Figure 3-12). This was largely driven by a cow diagnosed with mastitis during this time postpartum although 5 additional animals had elevated SCC during this same time as well. The SCC was numerically greater at 5 of the 7 wk postpartum for cows not cooled prepartum. Partially relieving heat stress during the immediate prepartum period may reduce the susceptibility of cows to mammary gland infections postpartum. Wegner et al. (1976) reported that 64 cows housed in mild to severe heat stress starting in June, through the hot summer months of July and August, and into the cooler month of November had increased SCC from August to October. The number of somatic cells in milk from cows exposed to heat stress for 5 d was elevated compared with that from cows exposed to thermo-neutral conditions for the same period (Mohammed and Johnson, 1985).

Plasma Metabolites

The mean concentration of plasma NEFA tended to be greater for cows fed the control diet compared to that of cows fed AO (325 vs. 246 μEq/L, $P = 0.07$). However this dietary difference in plasma NEFA concentration was for multiparous cows only. Mean plasma concentrations of NEFA tended to be greater for multiparous cows fed the control diet (469 vs. 305 μEq/L) but the mean plasma concentrations of NEFA for primiparous cows were not affected by diet (182 vs. 187 μEq/L, diet by parity interaction, $P = 0.06$). These greater concentrations of plasma NEFA for multiparous cows in control – cool treatment group occurred only during the first 3 wk postpartum (diet by environment by parity by week interaction, $P = 0.03$; Figure 3-13). Therefore feeding AO to multiparous cows reduced plasma concentrations of NEFA during the early postpartum period. Elevated concentrations of plasma NEFA during the first 3 wk

postpartum were likely due to greater mobilization of adipose tissue in support of milk and maintenance requirements. Loss of BW and feed efficiency as well as energy balance (figure 3-14, diet by environment by parity by week interaction, $P = 0.03$) during the first 3 wk postpartum for these 4 treatment groups followed rather closely the same ranking as the plasma concentrations of NEFA; namely 2.05, 1.95, 1.69, 0.86 kg/d for BW loss; 2.55, 2.12, 1.90 1.73 kg of 3.5% FCM/kg of DMI, and 802, 492, 259, and 270 μEq/L of NEFA for multiparous-control diet, multiparous-AO diet, primiparous-control diet, and primiparous-AO diet groups, respectively.

The weekly postpartum pattern of concentration of plasma BHBA was similar to that of plasma NEFA; that is, plasma concentrations of BHBA were greater in multiparous cows fed the control diet during the first 3 wk postpartum (diet by parity by week interaction, $P < 0.01$, Figure 3-15). Greater loss of BW during this time for this group of cows likely accounted for the elevated BHBA concentrations. Thus feeding AO to multiparous cows reduced plasma concentrations of BHBA but not those of primiparous cows. Urine of all cows were checked for ketosis using a ketostik at 4, 7, and 12 DIM. Ketosis was diagnosed for 22 of the 35 cows at least once during the 3 d. A greater proportion of multiparous cows were diagnosed with ketonemia than primiparous cows (10/13 vs. 12/22) and mean concentration of BHBA was greater for multiparous cows (8.9 vs. 6.1 mg/dL, $P < 0.01$, Table 3-5). Incidence of ketosis was not different between the 4 treatment groups; namely 4/7 for control-cool, 5/8 for control-noncool, 6/10 for AO-cool, and 7/10 for AO-noncool.

Mean plasma concentration of glucose was greater for cows fed AO compared with that of control cows (65.7 vs. 62.7 mg/100 mL, $P = 0.03$, Table 3-5). This

increased glucose concentration may be due to greater glucose synthesis or less glucose utilization. Glucose production from propionate was likely similar between the 2 dietary groups because DMI did not differ. However the inclusion of AO in the diet may have caused a shift towardsbacterial species that produce propionic acid, such that glucose synthesis was increased. The reduced production of milk fat by cows fed AO may suggest a bacterial shift in the rumen against fiber digesters. On the other hand, glucose utilization may have been less for cows fed AO. There was a weak tendency detected (P = 0.11) for cows fed AO to produce 3.3 kg/d less 3.5% FCM compared with controls (26.2 vs. 29.5 kg/d, Table 3-4). If glucose production was similar between the 2 groups but glucose utilization was less due to lowered milk production, concentrations of plasma glucose could be elevated. The cost of energy to maintain the immune system should be taken into account in energy expense equations (Lochmiller and Deerenberg, 2000). If feeding AO improved the energy efficiency of the cow's immune cells, glucose utilization would be less and concentrations of plasma glucose could rise.

Neither mean nor weekly pattern of concentration of plasma BUN differed among treatment groups (10.9 mg/dL, Table 3-5). The overall mean concentrations of BUN did change with week postpartum (P < 0.001), increasing from 9.9 mg/dL at wk 1 to 12.2 mg/100 mL at wk 7 postpartum. This probably reflected increasing intake of CP with increasing intake of DM. Blood urea nitrogen is a major end product of N metabolism by ruminal microorganisms and can be an indicator of efficiency of utilization of dietary N (Nousiainen et al., 2004).

Postpartum Body Temperature and Oxidative Markers in Blood

Mean rectal temperatures of postpartum cows (taken at 4, 7, and 12 DIM) were greater for primiparous than multiparous cows (39.0 vs. 38.7°C, P = 0.01, Table 3-6).

This elevated temperature may reflect greater stress typically experienced by primiparous cows from the new experiences of calving and lactating for the first time. Cows without evaporative cooling prepartum had reduced mean body temperature postpartum compared with cows provided evaporative cooling (38.6 vs. 39.0°C, $P < 0.01$). This may result from cow's adaption to heat stress before calving. McDowell et al. (1969) reported that cows exposed to heat stress for 2 wk experienced improved surface evaporation compared to cows exposed for only 1 wk. After calving, all cows were under the same environmental conditions with fans and sprinklers. Assuming that heat production by the 2 groups of cows were similar postpartum, those that lacked cooling prepartum had greater evaporative heat loss than cooled cows which resulted in lower body temperature postpartum.

Plasma TBARS represented a composite number of lipid peroxidative end products (Bernabucci et al., 2005), but it should perhaps be considered as an index of oxidative stress (Armstrong and Browne, 1994). Plasma concentration of TBARS was greatest at -15 d prepartum and at 1 DIM and then decreased through 29 DIM (effect of week, $P < 0.001$, Figure 3-16). This pattern was followed by both parities and all treatment groups (tests of interactions of week with other independent variables were not significant). Data suggest that these postpartum cows were under less oxidative stress than prepartum cows. Bernabucci et al. (2005) collected 5 prepartum blood samples (30 to 4 d prepartum) and 5 postpartum blood samples (4 to 30 DIM) from 24 Holstein cows for analysis of oxidative markers. Plasma concentrations of TBARS were not different between prepartum and postpartum cows with the exception that cows exceeding a BCS of 3.0 had greater plasma concentrations of TBARS post vs.

prepartum (2.1 vs.1.5 nmol/mL). Cows calving in the summer in Italy had numerically greater plasma concentrations of TBARS in the prepartum (21 and 3 d prepartum) compared to the postpartum period (1, 3, and 35 DIM, Bernabucci et al., 2002). Cows in the current study may have been consuming more antioxidants than those in Italy and thus responded differently.

Prepartum cooling did not reduce oxidative stress as measured with TBARS (2.05 vs. 1.79 nmol/mL for cooled and hot respectively, Table 3-6). Likewise, Bernabucci et al. (2002) reported that moderate heat stress (summer vs. spring calving cows) had no effect on concentration of plasma TBARS in cows during the transition period.

Feeding AO tended to reduce mean plasma concentration of TBARS when cows were evaporatively cooled prepartum (2.33 vs. 1.78 nmol/mL) but diet had no effect when prepartum cows were offered shade alone (1.74 vs. 1.83 nmol/mL, diet by environment interaction, $P = 0.07$, Figure 3-17). Bernabucci et al. (2005) suggested that plasma concentrations of TBARS were positively correlated with NEFA and BHBA values. In the current study, when the NEFA values for the first 4 wk postpartum were examined (same time period of measurement for TBARS), the mean concentration for the cooled cows fed the control diet were only numerically greater compared with that of the other 3 treatments (503 vs. 321, 390, and 325 nmol/mL for control-cool, AO-cool, control-hot, and AO-hot, respectively, $P = 0.26$ for diet by environment interaction). The same treatment group (control-cool) had the greatest 4-wk mean concentration of plasma BHBA (8.4 vs. 6.8, 7.2, and 5.7 mg/100 mL) but the test of diet by environment interaction was $P = 0.95$). Therefore newly calved cows that are mobilizing more

adipose tissue may be under greater oxidative stress but plasma concentrations of NEFA and BHBA are imperfect indicators of that stress.

Activity of GPx per mL of erythrocyte decreased from 15 d prepartum to 8 DIM by 7% and then plateaued (effect of week, $P = 0.02$; Figure 3-18). Bernabucci et al. (2005) also reported GSH-Px activity of erythrocytes to drop between -4 d and +11 d of calving. This periparturient pattern followed that of TBARS.

The effect of prepartum cooling on mean activity of erythrocyte GPx uncorrected and corrected for PCV was influenced by the feeding of AO. Heat stress of multiparous cows fed the control diet elevated GPx corrected for PCV (8,854 vs.12,247 nmol/min/mL). Including AO in the diet of multiparous cows reversed this effect of heat stress by reducing GPx activity (10,720 vs. 8,697 nmol/min/mL, Table 3-6) whereas the activity of erythrocyte GPx corrected for PCV of primiparous cows was not affected by diet or environment (diet by environment by parity interaction, $P = 0.01$, Figure 3-19). The same 3-way interaction for GPx activity per mL of erythrocyte was detected. Lack of evaporative cooling of multiparous cows fed the control diet elevated GPx (34,960 vs.40,505 nmol/min/mL of RBC). Including AO in the diet of multiparous cows reversed this effect of heat stress by reducing GPx activity (35,716 vs. 30,203 nmol/min/mL of RBC) whereas the activity of erythrocyte GPx per mL of erthrocyte of primiparous cows was not affected by diet or environment (diet by environment by parity interaction, $P = 0.05$, Figure 3-20). The mechanism by which AO alleviated the negative effect of heat stress by reducing the GPx activity for multiparous cows is unclear. It is possible that AO scavenged ROS molecules and reduced the load of peroxides generated from increased panting under lack of evaporative cooling, as a result, GPx activity was

suppressed. Vazquez-Anon et al. (2008) reported that feeding AO at 200 mg/kg increased the activity of plasma GPx (0.39 vs. 0.55 U/mg of protein for the absence and presence of AO, respectively) for mid- to late-lactation dairy cows regardless of the type of soybean oil (oxidized vs. unoxidized) fed. Hafeman et al. (1974) reported that activity of erythrocyte GPx was increased when increasing Se supplementation, indicating that there is a correlation between the concentration of Se in diet and the activity of GPx. In current study, the concentration of Se that multiparous cows consumed postpartum followed the same pattern as the activity of erythrocyte GPx corrected for PCV (4.3, 5.8, 5.4, and 5.1 mg/d for control - cool, control – hot, AO – cool, and AO – hot, respectively).

Concentration of erythrocyte SOD corrected for PCV increased 22%, from 2,412 U/mL on d 15 prepartum to 2937 U/mL on 8 DIM (effect of week, $P = 0.02$, Figure 3-21). Bernabucci et al. (2005) reported this same dependent variable to increase about the same percentage as the current study from -17 d to -4 d prepartum but then decreased thereafter until 30 DIM. The periparturient pattern of SOD followed the opposite pattern of TBARS and GPx.

Mean concentration of SOD activity corrected for packed cell volume was 2955 and 2495 U/mL for multiparous and primiparous cows, respectively ($P = 0.05$). High producing dairy cows were more likely to suffer from oxidative stress (Castillo et al., 2005; Lohrke et al., 2005) which can be monitored by elevated activities of antioxidant enzymes. Parity affected SOD activity in the opposite way compared to that of plasma TBARS.

Feeding AO resulted in an increase in SOD per mL of erythrocytes at 8 DIM, reversing the trend for cows not fed AO (diet by week interaction, $P = 0.03$, Figure 3-22). Contrary to current study, Sahoo et al. (2009) reported that the activity of erythrocyte SOD was decreased compared with the value before treatment after 3 injections of Vitamin E (i.m., 500 IU/injection) and Se (i.m., 15 mg/injection) on alternate days up to the 5th day as a therapy for subclinical ketosis. Vázquez-Añón et al. (2008) reported that the activity of plasma SOD was decreased (22.02 vs. 19.34 U/g of protein) for cows with vs. without supplementation of 200 mg of Agrado Plus per kg of dietary DM when an unoxidized blend of unsaturated oil was fed, whereas SOD activity was increased (23.74 vs. 26.35 U/g of protein) by feeding Agrado Plus with oxidized oil compared to cows without Agrado Plus.

The different responses of SOD and GPx to feeding of AO may be because AO might do a better job of scavenging H_2O_2 than O_2^- radicals along the sequence of reactions of reduction of oxygen in the electron transport chain.

White Blood Cells

The predominant cells comprising white blood cells (WBC) are lymphocytes and neutrophils, making up from 86 to 91% of the WBC in the current study (Table 3-7). The lymphocytes are mononuclear cells that are part of the adaptive immune system in that they produce antibodies against antigens to help the host organism resist infection by foreign pathogens. The neutrophils are polynuclear cells that are part of the innate immune system that are the first cells to arrive at sites of infection. The concentration of WBC decreased from 10,887 per µL of blood at 15 d prepartum to 7711 per µL of blood at 7 DIM (effect of DIM, $P < 0.001$, Figure 3-23). The type of WBC responsible for this decrease was the neutrophil. Neutroophils migrate to the mammary gland and the

uterus in response to the presence of pathogenic microorganisms after calving. The concentration of blood neutrophils was greatest at 15 d prior to calving (4617/µL) and decreased to 2272/µL by 7 DIM (effect of DIM, $P < 0.001$, Figure 3-22). Concentration of lymphocytes did not decrease over this same time period (Figure 3-22).

Prepartum cooling of cows tended to reduce mean concentration of WBC for the periparturient cow (7900 vs. 10,176 per µL of whole blood, $P = 0.09$, Table 3-7). The reduction was detected only at 7 and 14 DIM (environment by DIM interaction, $P = 0.05$, Figure 3-24) in which the concentration dropped 32% from 9577 to 6524 per µL of whole blood as cows transitioned from the prepartum to postpartum period. The WBC concentration of noncooled cows remained the same throughout the periparturient period (mean of 10,220 per µL of whole blood. The blood cell type that was responsible for this postpartum drop in WBC of cooled cows was the neutrophil. Concentration of blood neutrophils decreased from 0 to 7 DIM by 57% when cows were cooled prepartum but only by 24% when cows were not evaporatively cooled (environment by DIM interaction, $P = 0.11$, Figure 3-25). On the other hand, the concentration of lymphocytes did not change (mean of 3455 per µL of whole blood) during the transition period for cows cooled prepartum (Figure 3-26). Therefore the innate immune system of bovine appeared to be more sensitive than the adaptive system to parturition. Lack of prepartum cooling appeared to minimize the typical decrease in immuno-suppression reported for cows in the first 2 wk postpartum. Possibly the prepartum exposure of cows to hotter environmental conditions prepared their immune system for the stress of parturition. The cows with the elevated WBC count postpartum had a lower mean rectal temperature at 4, 7, and 12 DIM (Table 3-6) suggesting that elevated body temperature

postpartum may reflect a degree of suppressed immune response. Cows not offered evaporative cooling prepartum had a 53% greater mean concentration of lymphocytes throughout the study (5411 vs. 3455 per µL of whole blood, *P* = 0.03, Table 3-7). The concentration of blood lymphocytes tended to decrease on the day of calving and then rebound for noncooled cows whereas that of the cooled cows did not change over time (environment by DIM interaction, *P* = 0.09, Figure 3-25). Again, hotter environmental conditions resulted in elevated blood concentration of immune cells.

Function of Blood Neutrophils

Effect of calving. Measures of neutrophil numbers and activities were lowest at 7 or 14 DIM compared with -15 d or day of calving indicating a partially suppressed immune system within a week or 2 after calving (effect of day, P < 0.001, Figure 3-22). The proportion of neutrophils carrying out phagocytosis against labeled E. coli was less at 7 compared to 0 DIM (72.6 vs. 83.3%, effect of day, P < 0.001) and the MFI for phagocytosis also decreased from 60.4 to 33.5 on these same days (effect of day, P < 0.001, Figure 3-27). Proportion of neutrophils undergoing oxidative burst was less at 7 compared to 0 DIM (84 vs. 78%, effect of day, P < 0.01, Figure 3-28) and the MFI for oxidative burst also decreased progressively over time from 1687 at -15 DIM to 868 at 14 DIM (effect of day, P < 0.01, Figure 3-27). This decrease after calving has been reported by other investigators as indicators of suppressed immune response immediately postpartum.

Effect of parity. In general, the immune system of multiparous cows appeared to be more suppressed than that of primiparous cows. The concentration of neutrophils (2894 vs. 3545/µL of blood, P = 0.11), the MFI of phagocytosis (36 vs. 62, P < 0.001), the percentage of neutrophils conducting oxidative burst (76 vs. 82, P = 0.03), and the

MFI of neutrophils conducting oxidative burst (1041 vs. 1431, P = 0.03) were lower for multiparous cows (Table 3-7). This effect of parity for these dependant variables was consistent across all days of measure with the exception of percentage of neutrophils conducting oxidative burst. Multiparous cows had a reduced percentage only at 7 DIM compared with primiparous cows (71 vs. 86%, parity by week interaction, P = 0.05, Table 3-7).

Effect of treatments. The concentration of blood neutrophils (number per µL) throughout the study was unaffected by diet or prepartum evaporative cooling.

Prepartum cooling increased phagocytosis of *E. coli* by blood neutrophils of multiparous cows (77.1 vs. 71.6%) but the reverse occurred for primiparous cows (77.1 vs. 71.6%, cooling by parity interaction, *P* = 0.03, Figure 3-29). This decrease in % neutrophils undergoing phagocytosis due to the cooling of primiparous cows was matched with a decrease in MFI for phagocytosis (69 vs. 55) but MFI for phagocytosis by neutrophils of multiparous cows was not affected by prepartum cooling (37 vs. 34, cooling by parity interaction, *P* = 0.05, Figure 3-30). Feeding AO did not influence the proportion of neutrophils that phagocytized *E. coli*. However the MFI for phagocytosis was affected by diet. The MFI for phagocytosis was reduced by feeding AO to primiparous cows (69 vs. 55) but was unchanged by feeding AO to multiparous cows (33 vs. 39, diet by parity interaction, *P* = 0.02, Figure 3-31). This reduction by AO on MFI of phagocytosis as cows transitioned from prepartum to 7 and 14 DIM was greater for primiparous than for multiparous cows (diet by parity by time interaction, *P* = 0.04, Figure 3-32).

The consumption of oxygen during the generation of ROS is a critical process termed oxidative burst to kill bacteria after phagocytosis. Killing ability by neutrophils (oxidative burst) tended to be compromised by feeding AO when prepartum cows were cooled (83 vs. 77%) vs. noncooled (77 vs. 80%, diet by environment interaction, P = 0.10, Figure 3-33). This effect tended to occur at 7 and 14 DIM (diet by environment by day interaction, P = 0.08, Figure 3-34). Including AO in the diet of hotter cows prepartum resulted in a similar pattern of oxidative burst by neutrophils as cows fed the control diet and kept with evaporative cooling. Feeding ethoxyquin at 150 ppm inhibited phagocytosis of leucocytes of tilapia (Yamashita et al., 2009) indicating that to some extent, the synthetic antioxidants can suppress partially neutrophil activity.

Several studies have examined the impact of feeding vitamin antioxidants on neutrophil function. When cows were injected with 3000 IU of vitamin E at 10 and 5 d before calving, intracellular kill of bacteria by neutrophils was increased at calving (Hogan et al., 1990). The same research group (1992) also reported that supplementation of vitamin E (400 to 600 mg/d) or Se (0.3 mg/kg of dietary DM) increased the proportion of bacteria killed by neutrophils. Weiss and Hogan (2005) also reported that bacterial killing ability by neutrophils tended to be increased for cows fed selenite at 0.3 mg/kg of dietary DM with 500 IU/d of vitamin E compared with cows fed an organic source of Se. Boyne and Arthur (1979) concluded that the percentage of neutrophils that phagocytized and killed bacteria was greater for cows that received sufficient Se (0.1 ppm of dietary DM) than cows given a Se-deficient diet. Grasso et al. (1990) reported that cows supplemented with 2 mg/d of sodium selenite during the transition period had greater bacterial killing ability by neutrophils in milk compared with

cows not supplemented with Se. However function of neutrophils (the proportion of neutrophils that phagocytized bacteria and number of intracellular bacteria per neutrophil) isolated from whole blood were not influenced by supplementation with either 0 or 30 g/d of vitamin C starting from 2 wk before calving through 7 DIM (Weiss and Hogan, 2007). No published study has reported on the effect of feeding synthetic antioxidants on neutrophil function.

Ovalbumin Challenge and Acute-phase Proteins

Cows were given 3 separate injections of ovalbumin at approximately 4 and 2 wk before calving and on the day of calving. Plasma concentrations of IgG for ovalbumin increased after each injection as expected (Figure 3-35). The IgG concentration at wk 4 before calving was used as a covariate in the statistical analysis because analysis of wk 4 alone in a reduced model resulted in a $P = 0.12$ for the test of diet by environment interaction. The mean circulating concentrations of IgG against injected ovalbumin was less for multiparous cows fed AO vs. the control diet (0.80 vs. 0.63 optical density) whereas the response to diet was unchanged for primiparous cows (0.68 vs. 0.73 OD, diet by parity interaction, $P = 0.05$). This difference between multiparous cows occurred primarily from calving to 7 wk postpartum (Figure 3-36). From my knowledge, this is the first study to report the effect of feeding synthetic antioxidants on IgG responses to ovalbumin injections of bovine. Under the current conditions of this trial, supplementing synthetic antioxidants appeared to suppress adaptive immunity responses of multiparous cows. A linear increase in IgG concentration with increased dietary supplementation of vitamin E (285, 570, and 1140 IU/d, respectively) for bull calves was detected at 21 d after ovalbumin ingection (Rivera et al., 2002). Similar to this finding,

calves injected with 125 IU of vitamin E at 7 wk of age had greater IgG values compared with those receiving no vitamin E (Reddy et al., 1987).

Primiparous cows had greater mean plasma concentration of ASP than multiparous cows (49.5 vs. 39.8 µg/mL, $P = 0.02$, Table 3-7) with the greater values occurring during the first 2 wk after calving (parity by week interaction, $P < 0.001$, Figure 3-37). This result is in agreement with our previous studies (Amaral et al., 2008).

Primiparous cows had a greater mean rectal temperature than multiparous cows on 4, 7, and 12 DIM (Table 3-6) suggesting that primiparous cows were experiencing more stress and responding with greater circulation of acid soluble protein. When plasma ASP values were plotted for cows diagnosed as healthy (n = 18) vs. unhealthy (mastitis, metritis, or retained fetal membranes in the first 14 DIM, n = 17), unhealthy cows had greater mean concentrations of plasma ASP (51.2 vs. 40.1 µg/mL, $P < 0.01$) and had greater peak concentrations of plasma ASP between 6 to 22 DIM (78.0 vs. 50.9 µg/mL, healthy vs. unhealthy by time interaction, $P = 0.001$, Figure 3-38). Lastly, cows supplemented with AO had greater plasma concentrations of ASP from 6 to 15 DIM compared with the control group (diet by DIM interaction, $P = 0.03$, Figure 3-39). This dietary effect on plasma ASP during the first 2 wk postpartum may have resulted from a greater incidence of endometritis (10/20 vs. 3/15) and retained fetal membranes (2/10 vs. 0/15) for cows fed AO vs. control, respectively (Table 3-9).

Similarly to ASP, plasma concentrations of Hp in early in lactation tended to be greater for primiparous compared to multiparous cows (parity by day interaction, $P = 0.09$, Figure 3-40). Also in agreement with ASP responses to disease was the effect of mastitis and metritis on plasma Hp. Plasma concentrations of Hp were greater between

6 and 15 DIM in unhealthy vs. healthy cows (healthy vs. unhealthy by time interaction, $P < 0.01$, Figure 3-41). Plasma concentrations of Hp may not be as sensitive an acute phase protein to reflect disease as plasma concentrations of ASP (6 vs. 4 significant time points). Diet did not affect mean or weekly pattern of plasma Hp. Although prepartum cooling did influence plasma concentrations of Hp over DIM, the influence was mixed (environment by DIM interaction, $P = 0.03$, Figure 3-42); namely that cooled cows had greater plasma Hp at 6 DIM but less at 13 DIM.

Progesterone

The reproductive system of multiparous cows appeared to respond better than primiparous cows in the first 7 wk postpartum. Multiparous cows had a longer first estrous cycle (19.3 vs. 15.1 d, $P = 0.06$) and a greater peak concentration of plasma progesterone in the first cycle (8.2 vs. 5.8 ng/mL, $P = 0.04$) although the number of ovulations were fewer (1.3 vs. 1.9, $P = 0.04$, Table 3-8). Thirty-two of the 35 cows ovulated within the first 49 DIM. When considering only the 32 cows, treatment did not influence the day of first ovulation which averaged 19.7 ± 2.6 DIM (Table 3-8). However when the 3 cows that did not ovulate were assigned 49 DIM as their day of first ovulation, the day of first ovulation was affected by treatment. Providing evaporative cooling to cows fed the control diet tended to increase the number of days to first ovulation compared to noncooled cows (26.0 vs. 19.8 DIM) whereas cooling of cows fed AO tended to decrease time to first ovulation (20.5 vs. 28.9 DIM, diet by environment interaction, $P = 0.08$). This pattern was repeated for length of first estrous cycle in that providing evaporative cooling to cows fed the control diet resulted in a reduced length of cycle compared to noncooled cows (14.6 vs. 19.1 DIM) whereas cooling of cows fed AO increased length of first cycle (20.1 vs. 15.0 DIM, diet by environment interaction, $P =$

0.04). Likewise the plasma progesterone values accumulated over all 21 d of sampling times tended to be greater for these same 2 groups of cows (P = 0.10). Therefore noncooled cows fed the control diet and cooled cows fed AO had an earlier ovulation, a longer first estrous cycle, and produced more progesterone over 7 wk than the other 2 treatment groups. This effect can not be explained adequately by treatment differences in plasma concentrations of NEFA or in feed efficiency, nor by lower incidence of health disorders (Table 3-10). Multiparous cows fed the control vs. the AO diet had greater peak concentrations of plasma progesterone in the first estrous cycle (10.6 vs. 5.8 ng/mL) whereas that of primiparous cows did not differ between diets (6.0 vs. 5.7 ng/mL, parity by diet interaction, P = 0.05, Figure 3-43).

Vaginoscopy and Uterine cytology

Cervical discharge was scored based upon extent of presence of pus on 7, 16, and 25 DIM. Cows having clear discharge or only specks of pus were classified as healthy whereas significant amounts of pus resulted in diagnosis of metritis (7 and 16 DIM) or endometritis (25 DIM) (Sheldon et al., 2006), creating a binomial set of data. Compared with multiparous cows, primiparous cows had a greater incidence of uterine disease at 7 DIM (96 vs. 62%), at 16 DIM (91 vs. 42%), and at 25 DIM (59 vs. 23%). Primiparous cows were 18.2 (P = 0.02), 12.8 (P = 0.01), and 11.5 (P = 0.01) times more likely to have a uterine infection on 7, 16, and 25 DIM than multiparous cows based on odds ratio analysis. At 25 DIM, cows fed AO had a greater incidence of endometritis than cows not fed AO (60 vs. 27%, odds ratio of 9.6, P = 0.02).

At 40 ± 2 DIM, one uterine horn was flushed and the proportion of neutrophils calculated after counting 100 stained cells identified using a microscope. Neutrophils comprised a greater proportion of cells when cows were fed AO (21.9 vs. 6.8%, P =

0.02). Cows were diagnosed as having subclinical endometritis when the proportion of neutrophils exceeded 5% (Gilbert et al., 2005) thus creating a binominal data set. The incidence of subclinical endometritis was greater in cows fed AO (80 vs. 33%, odds ratio of 3.4, $P < 0.01$). This negative effect of AO on increasing uterine subclincial endometritis at 40 DIM agrees with AO's negative effect on increasing endometritis at 25 DIM as reported in the previous paragraph. Primiparous cows tended to have more cases of endometritis than multiparous cows (68 vs. 46%, odds ratio = 9.3, $P = 0.06$), a pattern that agrees with the greater incidence of metritis for primiparous vs. multiparous cows. Lastly, cows lacking prepartum evaporative cooling tended to have a greater incidence of subclinical endometritis (72 vs. 47%, $P = 0.08$).

Summary

Parity. Primiparous cows appeared to be under greater stress postpartum than multiparous cows as evidenced by greater or tendency for greater mean body temperature at 4, 7, and 12 DIM (39.0 vs. 38.7°C), greater metricheck score which is greater than 1 at 7 DIM (95 vs. 62%), 16 DIM (91 vs. 42%), and 25 DIM (59 vs. 23%), greater incidence of subclinical endometritis around 40 DIM (68 vs. 46%), and increasing concentrations of BHBA with increasing DIM. As a consequence of a greater incidence of uterine diseases, the immune responses were stimulated; namely, an increase in the concentration of blood neutrophils (3545 vs. 2894 per µL), in MFI for phagocytosis (62 vs. 36) and for oxidative burst (38 vs.32) by neutrophils, in % of neutrophils undergoing oxidative burst (82 vs. 76%), and in plasma concentration of acute phase proteins (acid soluble protein and haptoglobin) in the first 2 wk postpartum. Also as a result of increased metritis and endometritis, ovarian activity was affected negatively; namely, a shorted length of the first estrous cycle (15.1 vs. 19.3 d) and a

lowerpeak concentration of progesterone in the first cycle (5.8 vs. 8.2 ng/mL). Lastly, oxidative markers in the blood (TBARS, SOD, and GPx) reacted differently. Mean plasma concentrations of TBARS were greater (2.12 vs. 1.71 nmol/mL) whereas mean RBC concentrations of SOD corrected for packed cell volume were lower (2495 vs. 2955 U/mL) for primiparous vs. multiparous cows. Activity of GPX per mL of RBC were lower at 15 d before parturition (34,601 vs. 40,096 nmol/min/mL) but greater at 29 DIM (36,629 vs. 32,440 nmol/min/mL) for primiparous vs. multiparous cows.

Cooling. Prepartum evaporative cooling during the 3 wk prior to calving had or tended to have several positive benefits. Prepartum cooling resulted in a lower mean rectal temperature during the prepartum period (39.2 vs. 39.6°C) but a greater mean rectal temperature at 4, 7, and 12 DIM (39.0 vs. 38.7°C). This slightly greater mean rectal temperature was not increased enough to negatively affect production or health. Indeed prepartum cooling resulted in greater mean concentration of milk fat during 7 wk (3.54 vs. 3.32%) and mean production of 3.5% FCM during the first 4 wk postpartum (26.5 vs. 23.0 kg/d). Additionally, incidences of mastitis (1/17 vs. 6/18), displaced abomasum (0/17 vs. 3/18), and subclinical endometritis (47 vs. 72%) were reduced due to prepartum cooling. Associated with the reduction in health disorders, the number and activity of select immunity cells were affected as evidenced by lower concentration of circulating WBC postpartum (2049 vs. 2804 per µL of blood) and of circulating lymphocytes (3455 vs. 5411 per µL of blood) and increased proportion of neutrophils undergoing oxidative burst of *E. coli* of cows fed the control diet (83 vs. 77%). Assuming that an elevated concentration of oxidative markers in blood is indicative of greater oxidative stress, prepartum cooling of multiparous cows resulted in less

oxidative stress as evidenced by lower activity of GPx in RBC (8,854 vs. 12,247 nmol/min/mL) and of SOD in RBC (2503 vs. 3111 U/mL). The only negative effects of prepartum cooling were a greater plasma concentration of TBARS for control-fed cows (2.33 vs. 1.74 nmol/mL), a decreased length of the first estrous cycle of control-fed cows (14.6 vs. 19.1 d), and a reduction in phagocytosis by neutrophils (74 vs. 81% and 55 vs. 67 MFI) in primiparous cows.

Synthetic antioxidants. Feeding AO did not influence DM intake or uncorrected milk production. However supplemental AO had mixed effects on milk composition. Concentration of milk true protein was increased (3.07 vs. 2.94%) but concentration of milk fat was decreased (3.25 vs. 3.61%) resulting in less production of milk fat (0.88 vs. 1.04 kg/d) and of 3.5% FCM (26.2 vs. 29.5 kg/d). Therefore AO seems to influence the microbial population in the rumen. In addition, cows fed AO had a greater incidence of endometritis (60 vs. 27%) at 25 DIM and of subclinical endometritis at 40 DIM (80 vs. 33%). These reproductive disorders may have contributed to a lower peak concentration of plasma progesterone in the first estrous cycle of multiparous cows (5.8 vs. 10.6 ng/mL).

A greater peak concentration of plasma ASP postpartum in cows fed AO vs. control diet may reflect greater stress.

The effect of AO on oxidative markers or immune cells was not consistent. Under 4 different scenarios of more stressful (primiparous or prepartum noncooling) or less stressful (multiparous or prepartum cooling) scenarios, cows fed AO responded differently. Results are the following:

Nonstressful situation improved. Feeding AO to cows managed under prepartum evaporative cooling conditions resulted in

1. a longer first estrous cycle (20.2 vs. 14.6 d).
2. A decrease in plasma concentration of TBARS (1.78 vs. 2.33 nmol/mL).

Nonstressful situation aggravated. Feeding AO to cows managed under prepartum evaporative cooling conditions resulted in

1. a decrease proportion of neutrophils undergoing oxidative burst (77 vs. 83%).

Stressful situation improved. Feeding AO to multiparous cows managed without prepartum evaporative cooling resulted ina reduced activity of GPX in RBC corrected for packed cell volume (8,697 vs. 10,720 nmol/min/mL).

Stressful situation aggravated: Feeding AO to primiparous cows

1. reduced MFI of phagocytosis by neutrophils (36 vs. 57%)
2. Feeding AO at 250 mg per kg of dietary DM to periparturient Holstein cows fed diets of minimal fiber density resulted in reduced production of fat-corrected milk, increased incidence of uterine infections, reduced neutrophil activity, and mixed effects on oxidative markers in blood.

Table 3-1. Ingredient composition of diets fed to nonlactating and lactating Holstein cows.

Ingredient, % of DM	Nonlactating	Lactating
Corn silage	30.0	39.5
Bermudagrass silage	35.0	. . .
Alfalfa hay	. . .	12.5
Ground corn	9.5	18.9
Soybean meal	9.1	7.8
Soy Plus[1]	1.2	7.3
Citrus pulp	6.7	4.0
Corn gluten feed	. . .	4.4
Corn oil	2.0	2.0
Mineral/vitamin mix	6.5[2]	3.7[3]

[1] West Central Soy, Ralston, IA.

[2] Contained 18.3% CP, 20.5% Ca, 0.3% P, 3.1% Mg, 9.6% Cl, 0.3% K, 1.6% Na, 2.4% S, 11 ppm Co, 176 ppm Cu, 8 ppm I, 158 ppm Fe, 142 ppm Mn, 7 ppm Se, 268,00 IU vitamin A, 40 IU vitamin D, and 1300 IU of vitamin E (DM basis).

[3] Contained 24% CP, 9% Ca, 1% P, 4% K, 3% Mg, 10% Na, 1.1% S, 1200 ppm Mn, 158 ppm Fe, 500 ppm Cu, 1500 ppm Zn, 8.25 ppm Se, 2% Cl, 20 ppm I, 147,756 IU/kg of vitamin A, 43,750 IU/kg of vitamin D, and 787 IU/kg of vitamin E (DM basis).

Table 3-2. Chemical composition of diets fed to nonlactating and lactating Holstein cows.

Chemical	% DM			
	Nonlactating cows		Lactating cows	
	Control	Antioxidant[1]	Control	Antioxidant
CP	15.1	15.3	17.9	17.7
NDF	37.6	37.7	26.0	25.8
ADF	23.5	23.6	15.8	16.1
Ether extract	3.9	4.0	4.5	4.7
NEL, Mcal/kg	1.31	1.34	1.75	1.75
Ca	1.86	1.85	0.83	0.95
P	0.32	0.33	0.40	0.39
Mg	0.43	0.44	0.33	0.35
K	1.25	1.25	1.46	1.45
Na	0.15	0.15	0.31	0.31
S	0.34	0.33	0.23	0.24
Cl	0.99	0.93	0.38	0.45
Cu	29	28	25	30
Fe	256	252	18	18
Mn	107	100	78	87
Zn	46	48	69	59

[1] Agrado Plus (Novus International Inc., St. Charles, MO).

Table 3-3. Effect of feeding synthetic antioxidants (AO) and prepartum cooling on body temperature, body weight, BCS, and DMI of nonlactating pregnant Holstein cows during summer in Florida.

Measure	Treatment							
	Control diet				AO diet[1]			
	Cooled		Non-cooled		Cooled		Non-cooled	
	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous
Number of animals	5	2	6	2	5	5	6	4
Body temperature, °C	39.2 ± 0.1	39.4 ± 0.2	39.5 ± 0.1	39.8 ± 0.2	39.2 ± 0.1	39.2 ± 0.1	39.6 ± 0.1	39.6 ± 0.1
DMI, kg/d	10.6 ± 0.6	9.2 ± 0.9	10.4 ± 0.5	9.4 ± 0.9	10.3 ± 0.7	11.7 ± 0.6	9.7 ± 0.5	9.0 ± 0.6
BW, kg	568 ± 18	843 ± 28	585 ± 16	680 ± 28	596 ± 18	691 ± 18	587 ± 16	720 ± 20
DMI, % of BW	1.86 ± 0.10	1.09 ± 0.16	1.77 ± 0.09	1.41 ± 0.16	1.69 ± 0.12	1.70 ± 0.10	1.62 ± 0.09	1.25 ± 0.11
BCS[2]	3.37 ± 0.17	3.75 ± 0.27	3.21 ± 0.16	3.06 ± 0.27	3.37 ± 0.16	3.41 ± 0.19	3.35 ± 0.16	3.09 ± 0.19

[1] Agrado Plus (Novus Internat. Inc., St. Charles, MO).
[2] Least squares mean of BCS at the time of enrollment in the study and at calving.
[3] N/A = Not available.

Table 3-3. Continued

Measure	*P* values														
	Diet, Control vs. AO	Cool vs. noncool	Diet by cooling	Parity (P)	Diet by P	Cooling by P	Diet by cooling by P	Week (W)	Diet by W	Cooling by W	Diet by cooling by W	P by W	Diet by P by W	Cooling by P by W	Diet by cooling by P by W
Number of animals	N/A[3]	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Body temperature, °C	0.38	<0.001	0.77	0.19	0.15	0.85	0.76	0.53	0.48	0.35	0.39	0.81	0.98	0.44	0.34
DMI, kg/d	0.56	0.09	0.10	0.33	0.12	0.39	0.18	<0.001	0.23	0.49	0.22	0.85	0.73	0.96	0.47
BW, kg	0.20	0.04	0.01	<0.001	0.02	0.02	0.001	0.21	0.14	0.40	0.43	0.47	0.36	0.95	0.97
DMI, % of BW	0.73	0.42	0.03	<0.001	0.03	0.91	0.03	<0.001	0.36	0.64	0.18	0.56	0.85	0.97	0.75
BCS	0.77	0.05	0.37	1.00	0.43	0.16	0.69	0.03	0.39	1.00	0.34	0.03	0.77	0.56	0.70

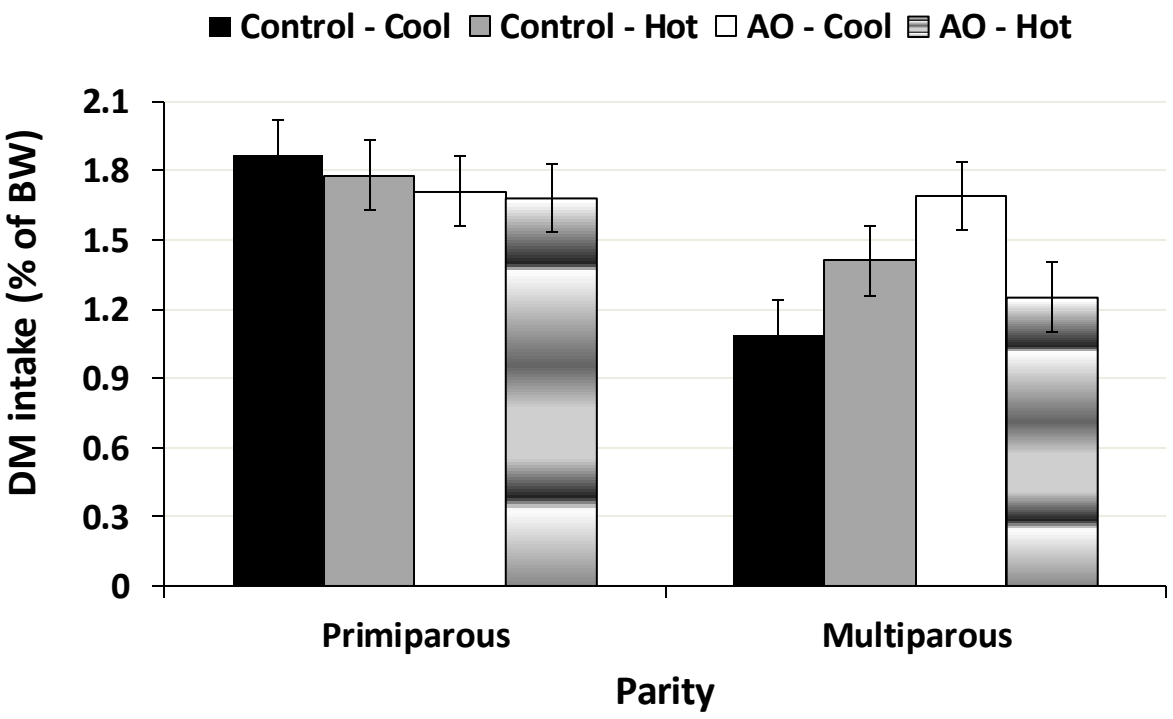

Figure 3-1. Least squares means for mean dry matter intake of prepartum primiparous (n = 22) and multiparous (n = 13) Holstein cows housed in cooled (Cool) or noncooled (Hot) freestalls and fed diets supplemented without (Control) or with dietary antioxidants (AO). Effect of diet by environment by parity interaction (P = 0.03).

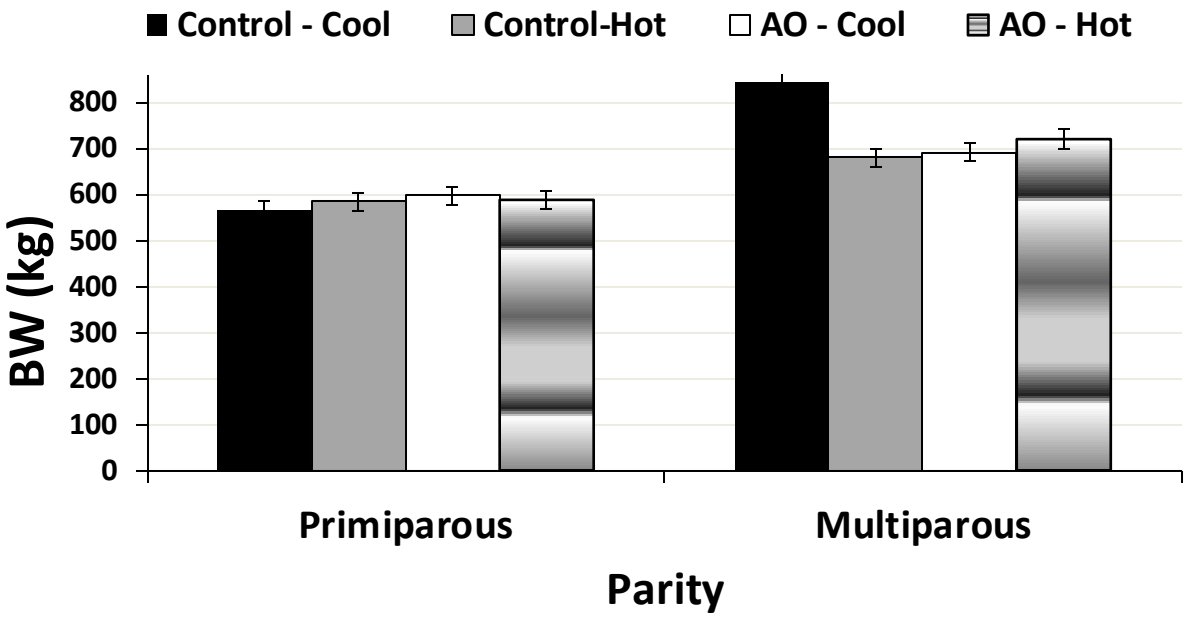

Figure 3-2. Least squares means for mean body weight of prepartum primiparous (n = 22) and multiparous (n = 13) Holstein cows housed in cooled (Cool) or noncooled (Hot) freestalls and fed diets supplemented without (Control) or with dietary antioxidants (AO). Effect of diet by environment by parity interaction (P = 0.001).

Table 3-4. Effect of feeding synthetic antioxidants (AO) and prepartum cooling on performance of lactating pregnant Holstein cows during summer in Florida.

Measure	Treatment							
	Control diet				AO diet[1]			
	Cooled		Non-cooled		Cooled		Non-cooled	
	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous
Number of animals	5	2	6	2	5	5	6	4
BW, kg	504 ± 16	724 ± 26	518 ± 15	590 ± 26	516 ± 16	625 ± 16	526 ± 15	631 ± 18
BCS	3.23 ± 0.13	3.46 ± 0.20	3.12 ± 0.12	3.08 ± 0.20	3.19 ± 0.12	3.33 ± 0.14	3.19 ± 0.12	3.08 ± 0.14
DMI, kg/d	15.0 ± 0.91	14.1 ± 1.4	13.5 ± 0.8	18.9 ± 1.4	12.7 ± 0.9	17.7 ± 0.8	14.1 ± 0.8	16.6 ± 1.0
DMI, % of BW	2.98 ± 0.14	1.99 ± 0.21	2.62 ± 0.12	3.24 ± 0.21	2.49 ± 0.14	2.83 ± 0.14	2.68 ± 0.12	2.64 ± 0.15
Milk, kg/d	27.7 ± 2.8	31.1 ± 2.5	24.5 ± 2.8	34.4 ± 4.4	23.6 ± 2.5	36.0 ± 4.4	22.0 ± 2.8	29.8 ± 3.1
Milk fat, %	3.35 ± 0.19	4.21 ± 0.31	3.21 ± 0.17	3.67 ± 0.30	3.22 ± 0.19	3.38 ± 0.19	3.12 ± 0.17	3.29 ± 0.21
Milk protein, %	2.97 ± 0.08	2.76 ± 0.14	3.02 ± 0.07	3.00 ± 0.14	2.98 ± 0.08	3.10 ± 0.08	3.09 ± 0.07	3.09 ± 0.09
Milk fat, kg/d	0.9 ± 0.1	1.3 ± 0.1	0.7 ± 0.1	1.2 ± 0.1	0.7 ± 0.1	1.2 ± 0.1	0.7 ± 0.1	1.0 ± 0.1
Milk protein, kg/d	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	0.9 ± 0.1
Milk SCC, x1000	174 ± 21	182 ± 28	89 ± 20	300 ± 28	105 ± 21	47 ± 21	190 ± 20	177 ± 22
3.5% FCM, kg/d	26.8 ± 2.4	33.8 ± 3.8	22.5 ± 2.2	34.8 ± 3.8	22.0 ± 2.4	34.3 ± 2.4	20.1 ± 2.2	28.4 ± 2.7
Feed efficiency	1.81 ± 0.16	2.48 ± 0.25	1.72 ± 0.15	2.00 ± 0.25	1.79 ± 0.16	1.97 ± 0.16	1.45 ± 0.14	1.79 ± 0.18

[1] Agrado-Plus (Novus Internat. Inc., St. Charles, MO).
[2] N/A = Not Available.

Table 3-4. Contiuned

Measure	*P* values														
	Diet, Control vs. AO	Cool vs. noncool	Diet by cooling	Parity (P)	Diet by P	Cooling by P	Diet by cooling by P	Week (W)	Diet by W	Cooling by W	Diet by cooling by W	P by W	Diet by P by W	Cooling by P byW	Diet by cooling by P by W
Number of animals	N/A[2]	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
BW, kg	0.48	0.06	0.02	< 0.001	0.16	< 0.01	0.01	< 0.001	0.12	0.56	0.29	0.52	0.80	0.16	0.41
BCS	0.83	0.10	0.59	0.62	0.72	0.23	0.97	0.001	0.59	0.25	0.18	0.58	0.32	0.82	0.53
DMI, kg/d	0.88	0.25	0.34	< 0.001	0.33	0.22	< 0.01	< 0.001	0.04	0.14	0.10	0.02	0.38	0.65	0.07
DMI, % of BW	0.69	0.06	0.05	0.87	0.14	0.01	0.001	< 0.001	0.10	0.21	0.26	0.28	0.60	0.72	0.30
Milk, kg/d	0.50	0.41	0.41	0.001	0.46	0.84	0.24	< 0.001	0.39	0.36	0.20	< 0.001	0.13	0.20	0.32
Milk fat, %	0.03	0.18	0.43	0.01	0.13	0.55	0.51	< 0.001	0.10	0.01	0.95	0.02	0.36	0.47	0.94
Milk protein, %	0.06	0.17	0.49	0.70	0.19	0.78	0.27	< 0.001	0.04	0.15	0.41	0.94	0.21	0.33	0.53
Milk fat, kg/d	0.04	0.11	0.86	< 0.001	0.72	0.96	0.40	< 0.001	0.17	0.02	0.83	< 0.01	0.48	0.33	0.55
Milk protein, kg/d	0.69	0.38	0.36	< 0.001	0.27	0.97	0.13	< 0.001	0.84	0.79	0.54	0.10	0.27	0.27	0.64
Milk SCC, x1000	0.35	0.31	0.23	0.82	0.22	0.27	0.80	< 0.001	0.15	0.03	0.68	0.27	0.26	0.68	0.40
3.5% FCM, kg/d	0.11	0.18	0.58	< 0.001	0.86	0.88	0.26	< 0.001	0.19	0.07	0.65	< 0.001	0.61	0.22	0.39
Feed efficiency	0.06	0.05	0.90	0.01	0.42	0.66	0.30	< 0.001	0.37	0.08	0.05	0.15	0.68	0.52	0.03

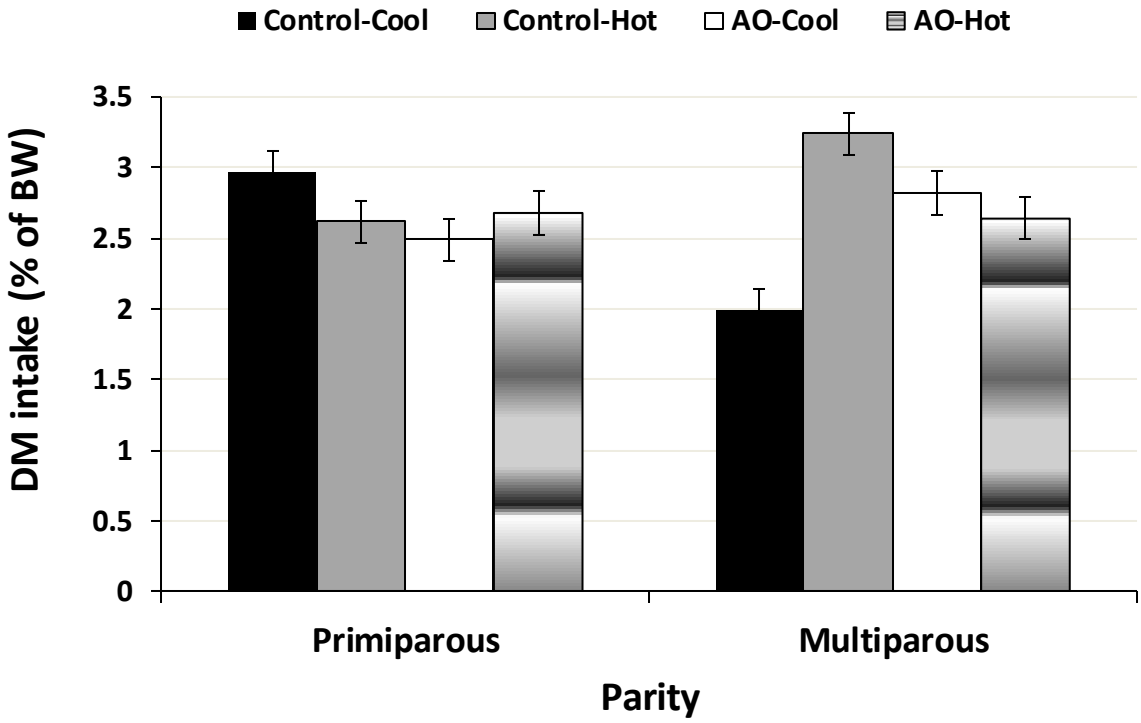

Figure 3-3. Least squares means for mean dry matter intake of postpartum primiparous (n = 22) and multiparous (n = 13) Holstein cows housed in cooled (Cool) or noncooled (Hot) freestalls and fed diets supplemented without (Control) or with dietary antioxidants (AO). Effect of diet by environment by parity interaction (*P* = 0.001).

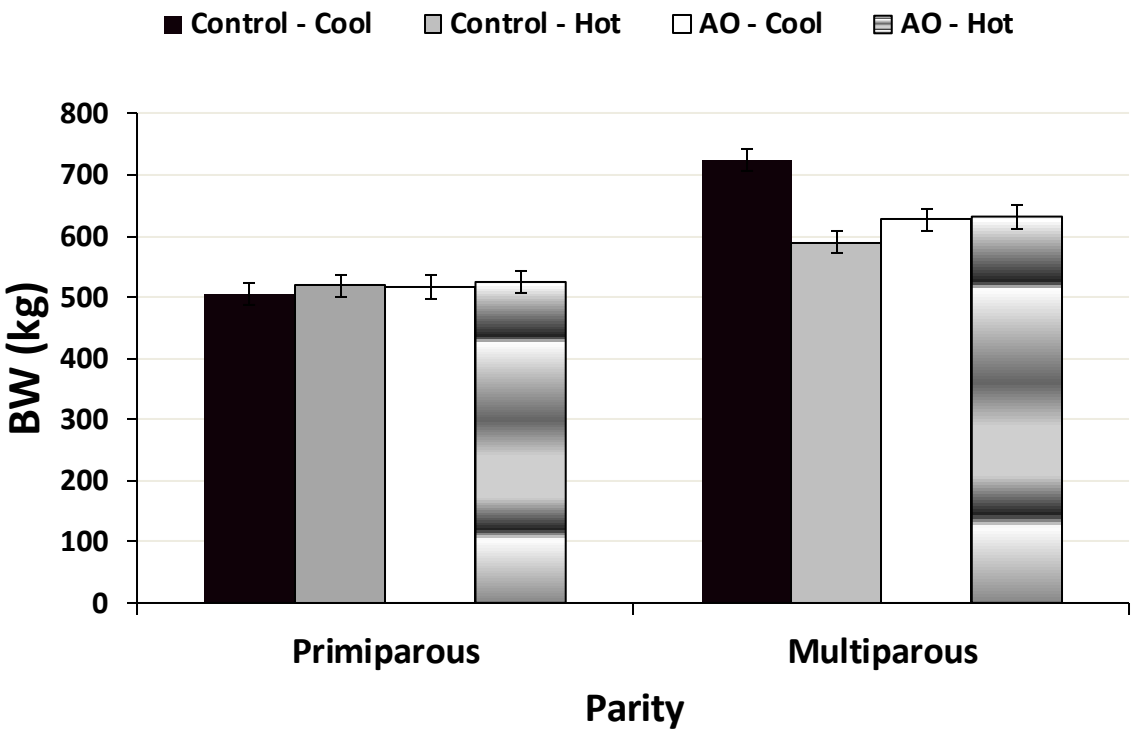

Figure 3-4. Least squares means for mean body weight (BW) of postpartum primiparous (n = 22) and multiparous (n = 13) Holstein cows housed in cooled (Cool) or noncooled (Hot) freestalls and fed diets supplemented without (Control) or with dietary antioxidants (AO). Effect of diet by environment by parity interaction (P = 0.01).

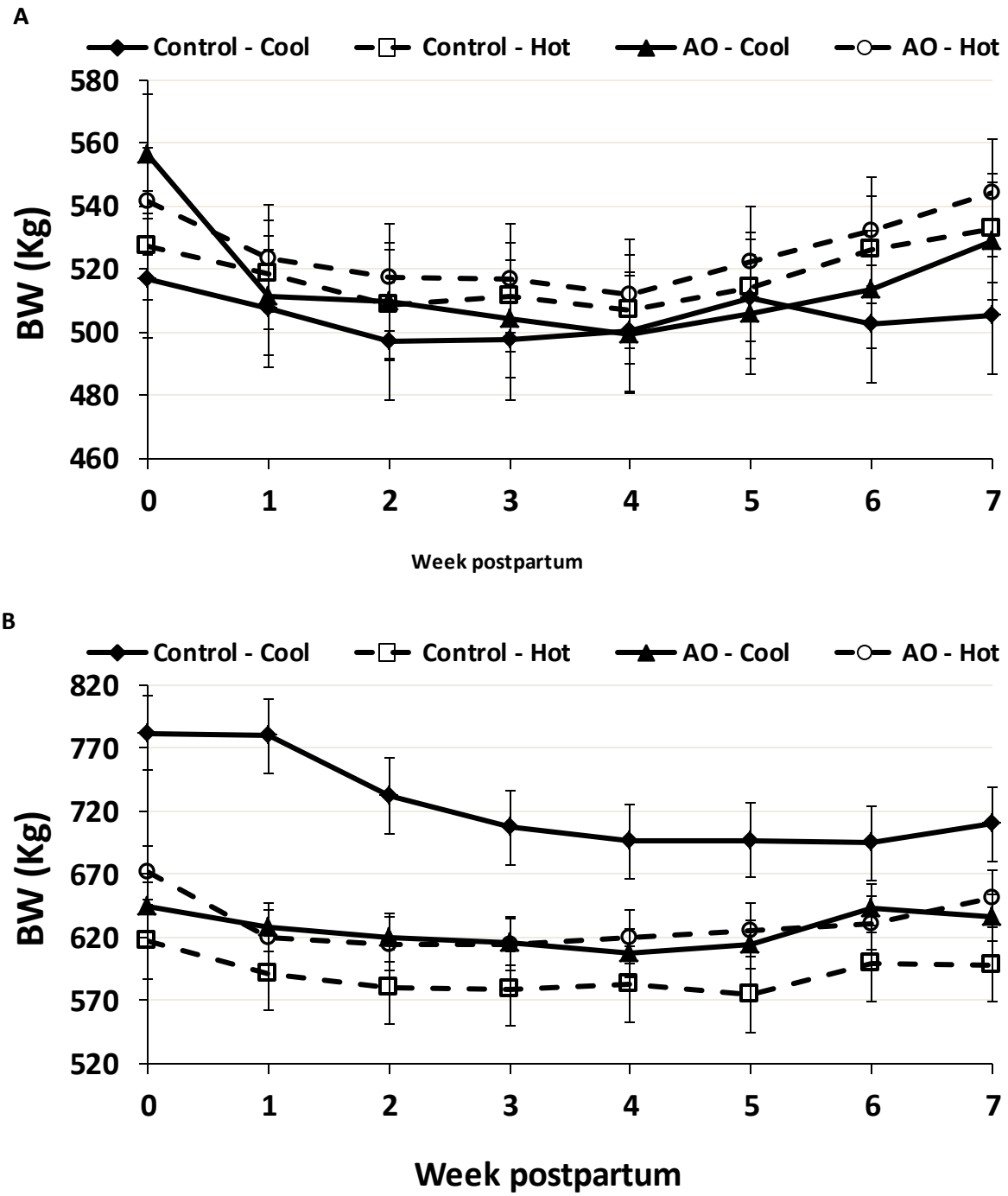

Figure 3-5. Least squares means for weekly body weight (BW) of postpartum primiparous (A, n = 22) and multiparous (B, n = 13) Holstein cows housed in cooled (Cool) or noncooled (Hot) freestalls and fed diets supplemented without (Control) or with dietary antioxidants (AO). Effect of parity by diet by environment by week (P = 0.41).

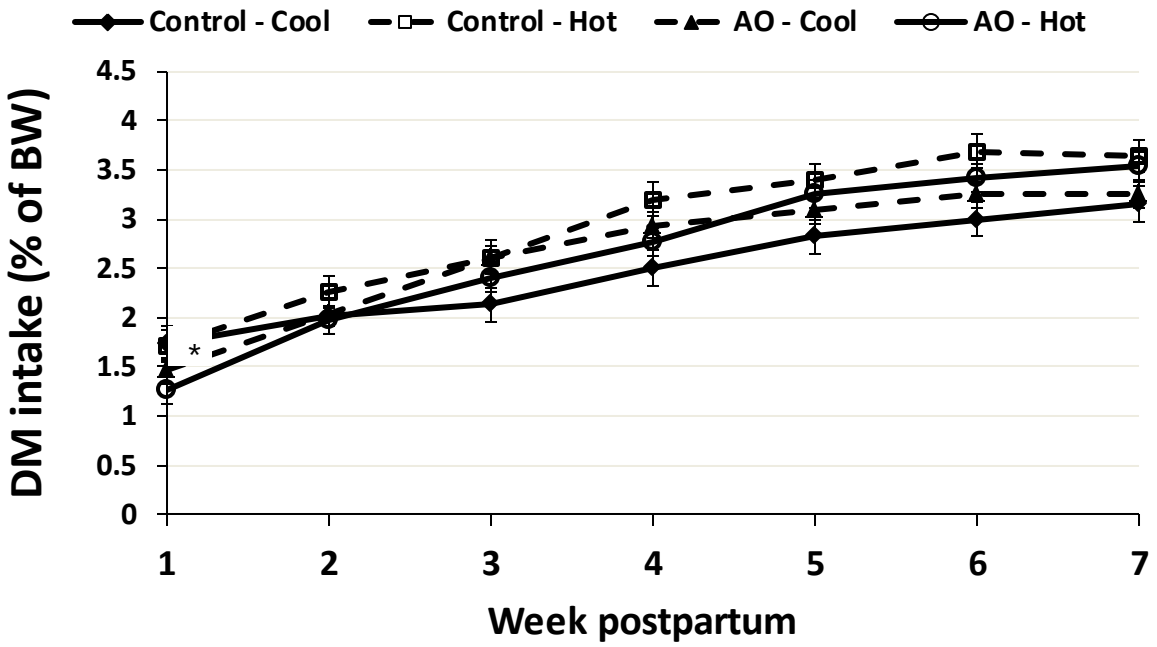

Figure 3-6. Least squares means for weekly dry matter intake of postpartum Holstein cows (n = 35) housed in cooled (Cool) or noncooled (Hot) freestalls and fed diets supplemented without (Control) or with dietary antioxidants (AO). Effect of diet by week interaction ($P = 0.10$). Week with asterisk indicates that diets differed ($P < 0.05$).

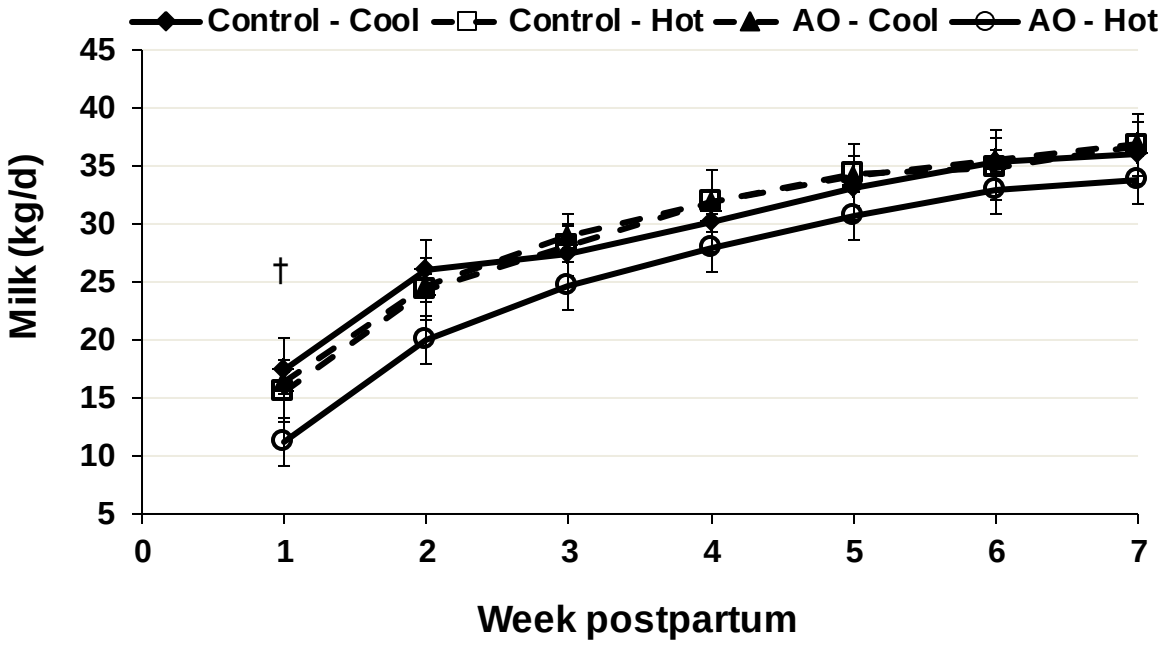

Figure 3-7. Least squares means for weekly milk production of Holstein cows (n = 35) housed in cooled (Cool) or noncooled (Hot) freestalls and fed diets supplemented without (Control) or with dietary antioxidants (AO). Effect of diet by environment by week ($P = 0.20$). Week with dagger indicates that means differed ($P < 0.10$).

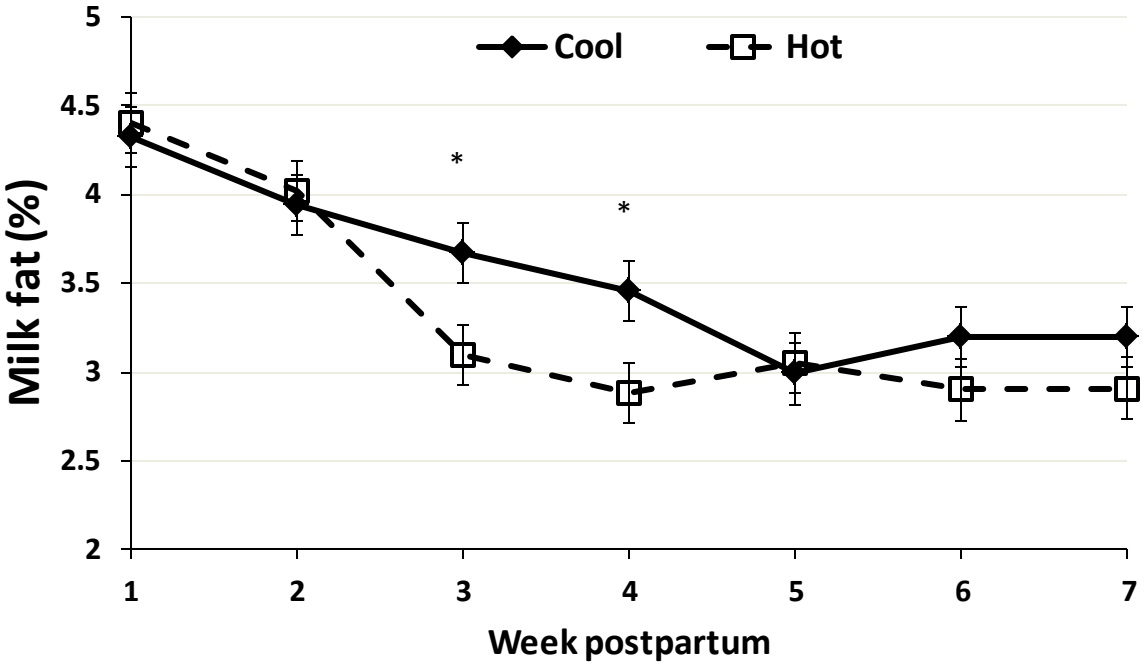

Figure 3-8. Least squares means for weekly concentration of milk fat of Holstein cows (n = 35) housed in cooled (Cool) or noncooled (Hot) freestalls. Effect of environment by week interaction ($P = 0.01$). Week with asterisk indicates that means differed using slice command ($P < 0.05$).

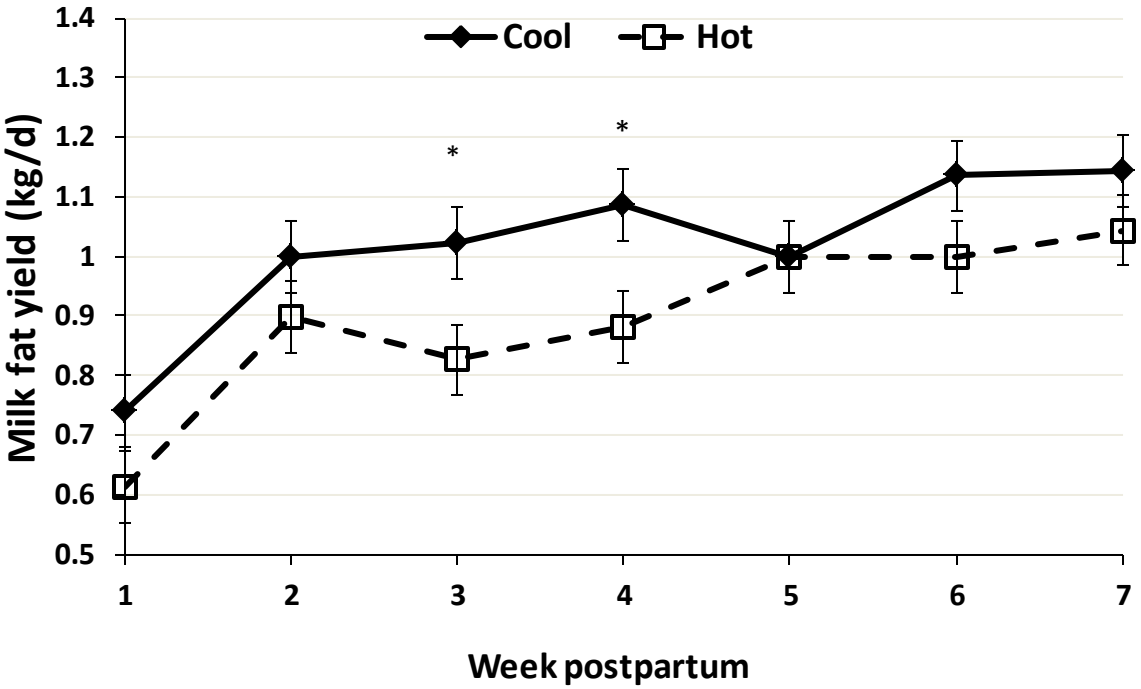

Figure 3-9. Least squares means for weekly milk fat production of Holstein cows (n = 35) housed in cooled (Cool) or noncooled (Hot) freestalls. Effect of environment by week interaction ($P = 0.02$). Week with asterisk indicates that means differed using slice command ($P < 0.05$).

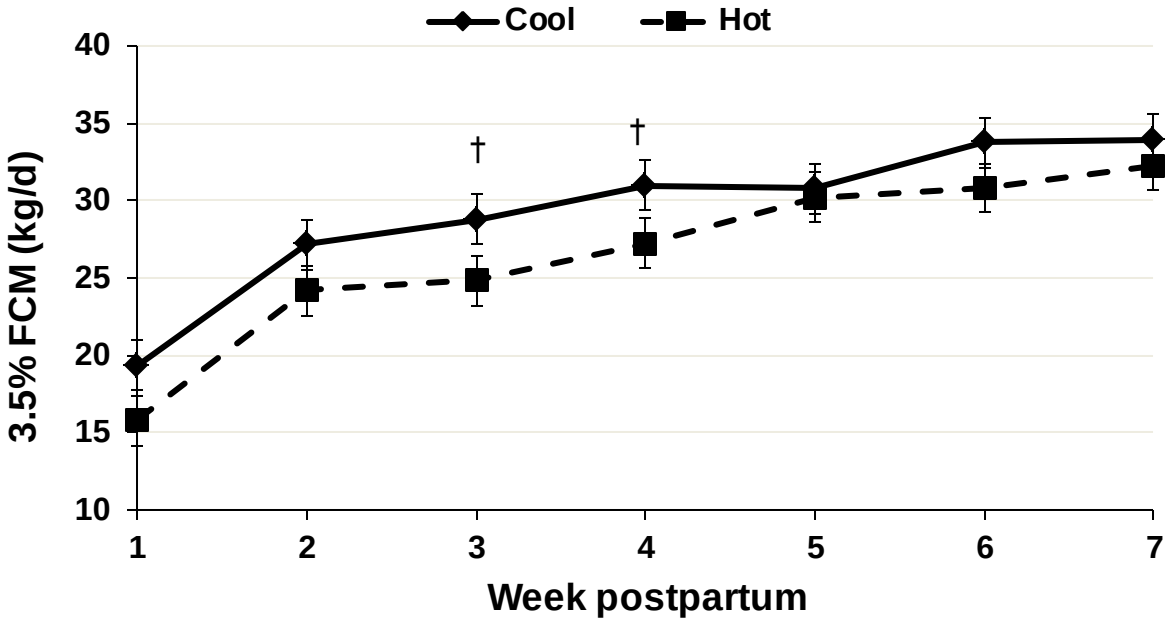

Figure 3-10. Least squares means for production of 3.5% fat-corrected milk by postpartum Holstein cows (n = 35) housed in shaded freestalls equipped with fans and sprinklers (Cool) or just shade (Hot). Effect of cooling by week interaction ($P = 0.07$). Week with dagger indicates that means differed using slice command ($P < 0.10$).

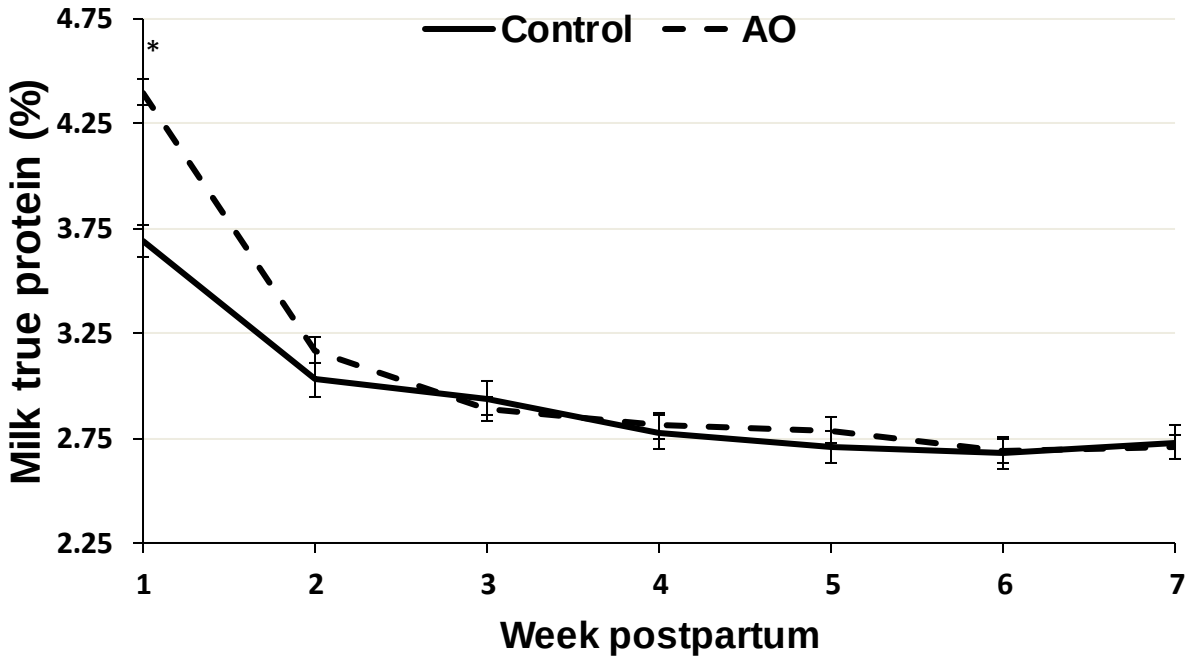

Figure 3-11. Least squares means for weekly concentration of milk protein of Holstein cows (n = 35) fed diets supplemented without (Control) or with dietary antioxidants (AO). Effect of diet by week interaction ($P = 0.04$). Week with asterisk indicates that means differed using slice command ($P < 0.05$).

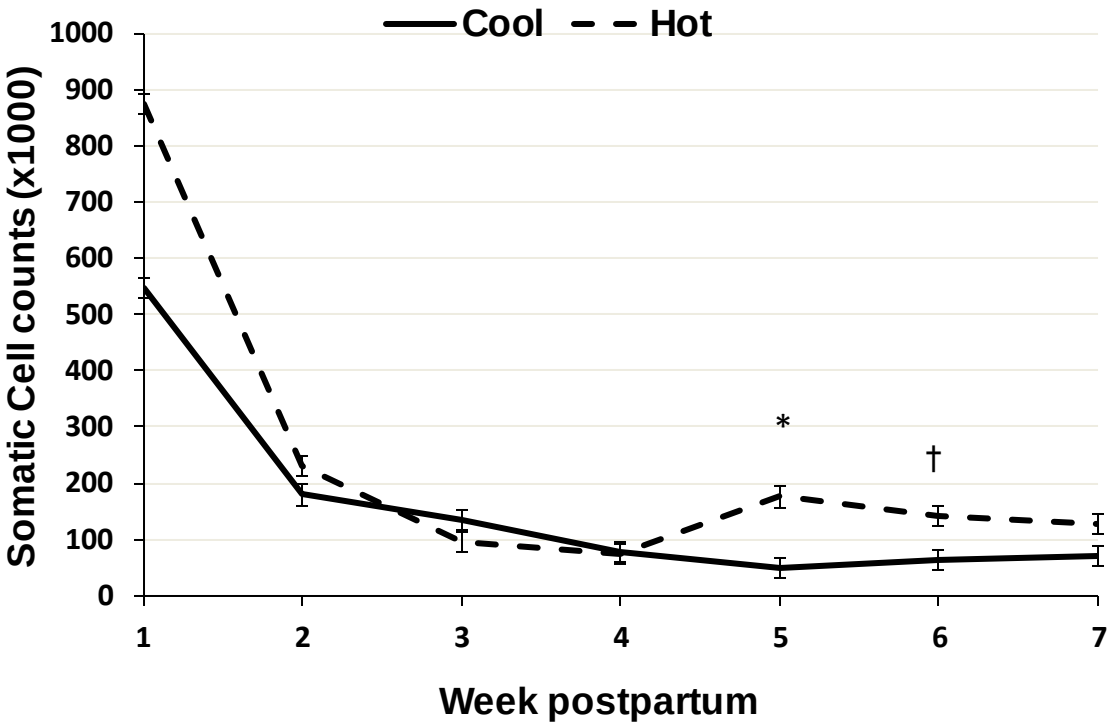

Figure 3-12. Least squares means for somatic cell counts of postpartum Holstein cows (n = 35) housed in shaded freestalls equipped with fans and sprinklers (Cool) or just shade (Hot). Effect of cooling by week interaction ($P = 0.03$). Week with asterisk indicates that means differed using slice command ($P < 0.05$), with dagger differed ($P < 0.10$).

Table 3-5. Effect of feeding synthetic antioxidants (AO) and prepartum cooling on postpartum plasma concentration of metabolites of lactating Holstein cows during summer in Florida.

Measure	Treatment							
	Control diet				AO diet[1]			
	Cooled		Non-cooled		Cooled		Non-cooled	
	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous
NEFA, μeq/L	195 ± 47	557 ± 74	168 ± 44	381 ± 74	222 ± 48	288 ± 47	152 ± 43	321 ± 52
BHBA, mg/dL	7.22 ± 0.97	10.46 ± 1.54	5.17 ± 0.94	9.28 ± 1.54	6.53 ± 1.00	8.62 ± 0.97	5.55 ± 0.91	7.40 ± 1.09
Glucose, mg/dL	64.2 ± 1.6	58.2 ± 2.6	68.5 ± 1.5	59.9 ± 2.6	69.9 ± 1.6	60.6 ± 1.6	70.8 ± 1.5	61.5 ± 1.8
BUN, mg/dL	11.8 ± 0.8	11.1 ± 1.2	10.4 ± 0.7	9.5 ± 1.2	11.5 ± 0.8	10.9 ± 0.8	10.7 ± 0.7	11.0 ± 0.9

[1] Agrado-Plus (Novus Internat. Inc., St. Charles, MO).

Table 3-5. Continued

Measure	*P* values														
	Diet, Control vs. AO	Cool vs. noncool	Diet by cooling	Parity (P)	Diet by P	Cooling by P	Diet by cooling by P	Week (W)	Diet by W	Cooling by W	Diet by cooling by W	P by W	Diet by P by W	Cooling by P by W	Diet by cooling by P by W
NEFA, µeq/L	0.07	0.16	0.31	< 0.001	0.06	0.78	0.14	<0.001	<0.01	<0.01	<0.01	<0.001	0.03	< 0.01	0.02
BHBA, mg/dL	0.22	0.11	0.75	< 0.01	0.30	0.85	0.73	<0.01	0.03	0.87	0.15	0.01	<0.01	0.61	0.22
Glucose, mg/dL	0.03	0.16	0.45	< 0.001	0.45	0.63	0.63	0.08	0.63	0.49	0.67	0.12	0.37	0.70	0.18
BUN, mg/dL	0.61	0.15	0.37	0.46	0.59	0.79	0.70	<0.001	0.38	0.52	0.08	0.59	0.94	0.98	0.19

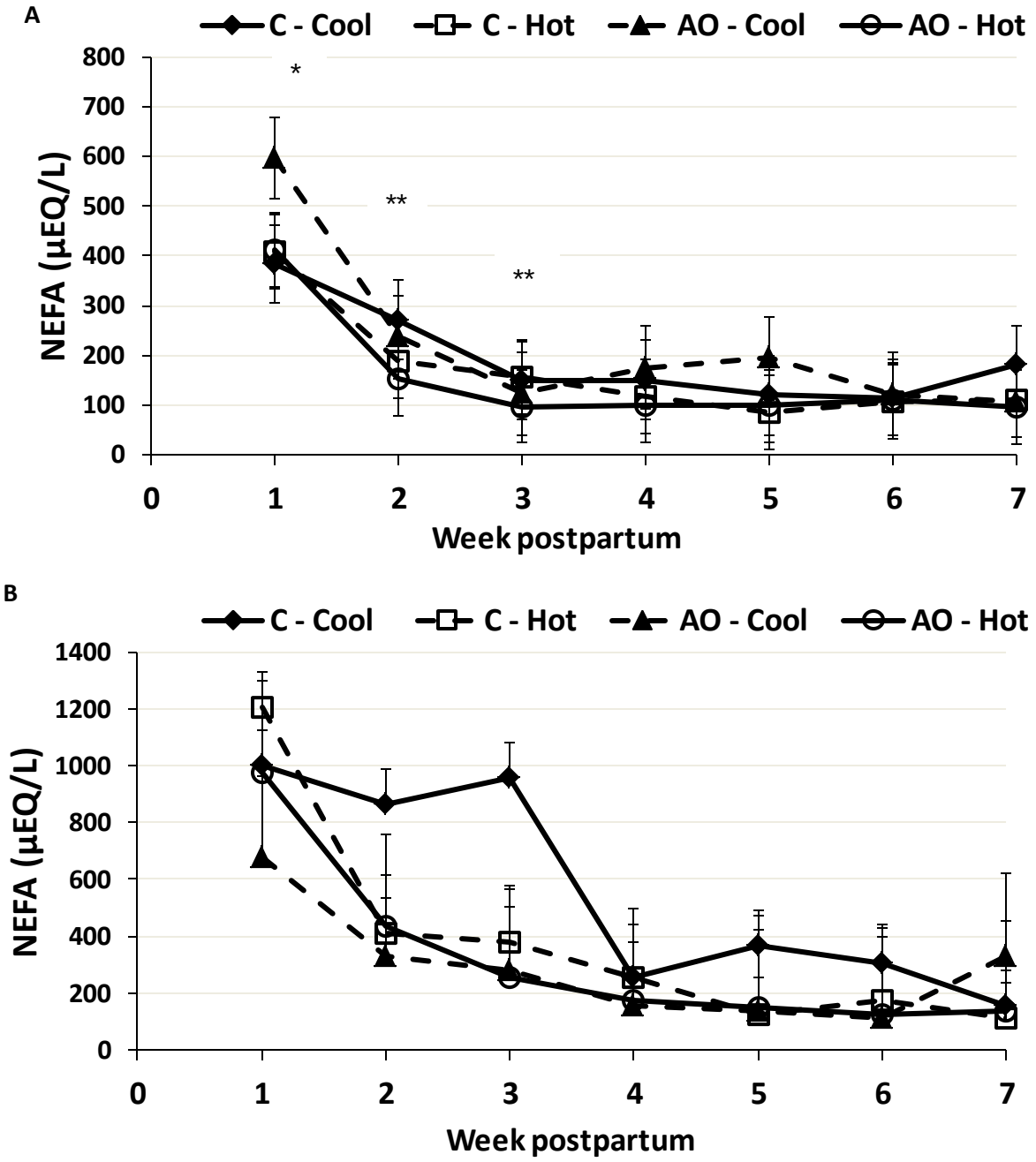

Figure 3-13. Least squares means for weekly plasma concentrations of NEFA of postpartum primiparous (A, n = 22) and multiparous (B, n = 13) Holstein cows fed diets supplemented without (Control, C) or with synthetic antioxidants (AO) and housed in shaded freestalls equipped with fans and sprinklers (Cool) or just shade (Hot). Effect of parity by diet by environment by week interaction ($P = 0.02$). Week with one asterisk indicates that means for the 8 treatments differed for that week using slice command ($P < 0.05$); with two asterisks differed at $P < 0.001$.

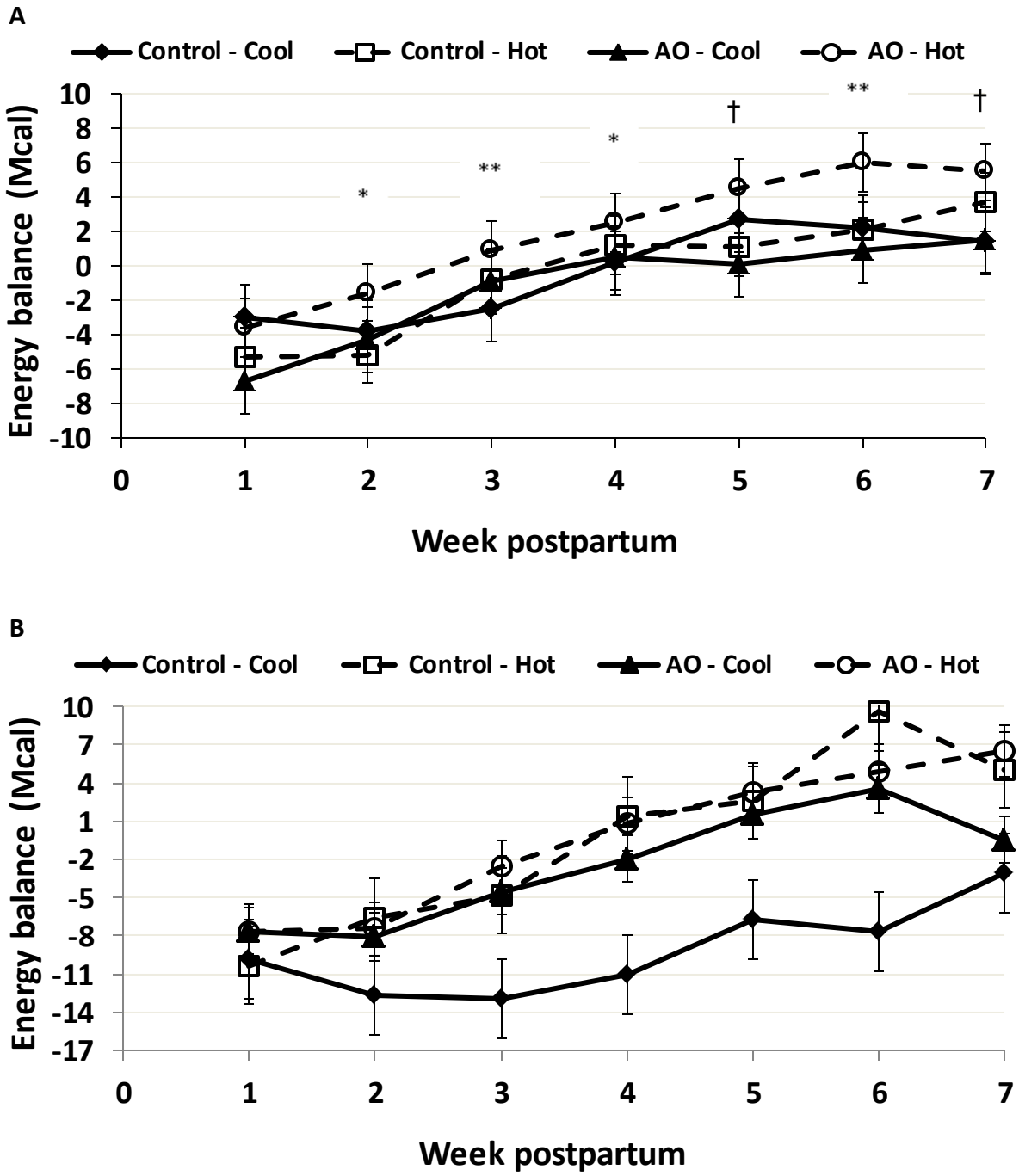

Figure 3-14. Least squares means for weekly energy balance of postpartum primiparous (A, n = 22) and multiparous (B, n = 13) Holstein cows fed diets supplemented without (Control) or with synthetic antioxidants (AO) and housed in shaded freestalls equipped with fans and sprinklers (Cool) or just shade (Hot). Effect of parity by diet by environment by week interaction (P = 0.03). Week with one asterisk indicates that 8 treatment means differed for that week using slice command ($P < 0.05$); with two asterisks differed at $P <$ 0.01; dagger differed at $P< 0.10$.

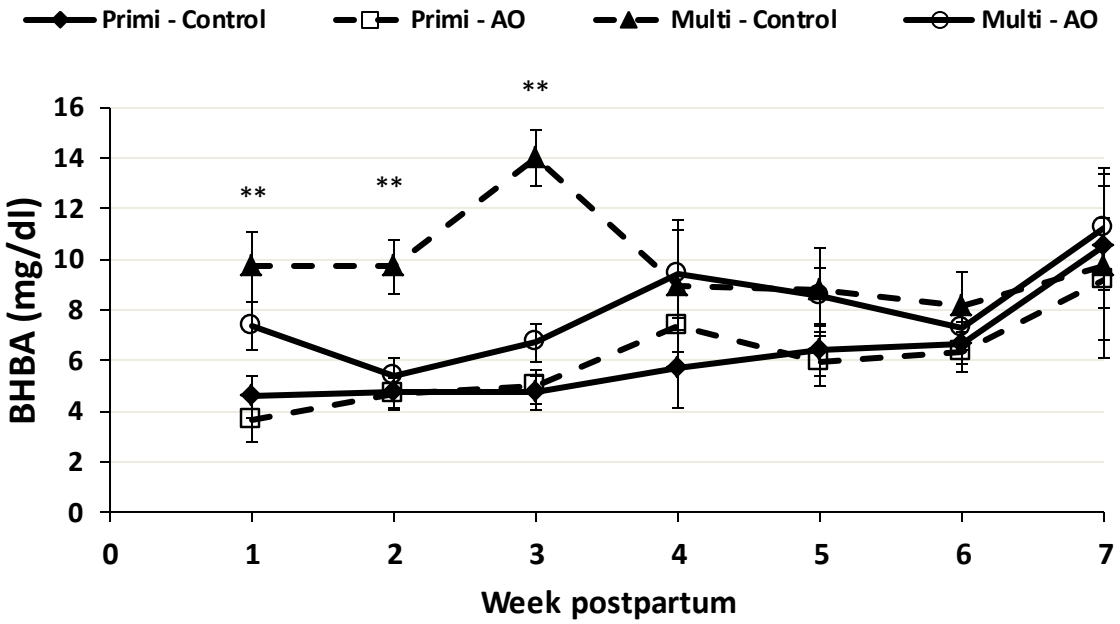

Figure 3-15. Least squares means for weekly plasma concentration of beta-hydroxyl butyric acid of primiparous (primi, n = 22) and multiparous (multi, n = 13) Holstein cows fed diets supplemented without (Control) or with synthetic antioxidants (AO). Effect of diet by parity by week interaction ($P < 0.01$). Week with two asterisks indicates that means differed for that week using slice command ($P < 0.01$).

Table 3-6. Effect of feeding synthetic antioxidants (AO) and prepartum cooling on postpartum body temperature and plasma concentration of oxidative markers of lactating Holstein cows during summer in Florida.

Measure	Treatment							
	Control diet				AO diet[1]			
	Cooled		Non-cooled		Cooled		Non-cooled	
	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous
Body temp, °C	39.0 ± 0.1	39.0 ± 0.2	38.7 ± 0.1	38.4 ± 0.2	39.0 ± 0.1	38.9 ± 0.1	39.0 ± 0.1	38.4 ± 0.2
TBARS[2], nmol/mL	2.32 ± 0.19	2.34 ± 0.29	2.08 ± 0.17	1.39 ± 0.31	1.95 ± 0.19	1.61 ± 0.19	2.15 ± 0.17	1.52 ± 0.21
GPx[3], nmol/min/mL	10,302 ± 720	8854 ± 1133	9384 ± 659	12,247 ± 1157	9305 ± 723	10,720 ± 726	9733 ± 659	8697 ± 801
GPx[4], nmol/min/mL	35,985 ± 2181	34,960 ± 3444	35,802 ± 1993	40,505 ± 3464	33,456 ± 2184	35,716 ± 2187	37,214 ± 1993	30,203 ± 2435
SOD[5], U/mL	2635 ± 272	2503 ± 427	2486 ± 248	3111 ± 439	2405 ± 271	2759 ± 275	2454 ± 248	3448 ± 302
SOD[6], U/mL	9247 ± 1078	9709 ± 1701	9635 ± 985	10,634 ± 1713	8806 ± 1079	9120 ± 1081	9484 ± 985	11,839 ± 1203
PCV[7], % of blood	28.6 ± 1.1	25.5 ± 1.8	26.0 ± 1.0	30.3 ± 1.8	27.4 ± 1.1	29.8 ± 1.1	26.2 ± 1.0	29.1 ± 1.2

[1] Agrado-Plus (Novus Internat. Inc., St. Charles, MO).
[2] TBARS = thiobarbituric acid reactive substances.
[3] GPx = glutathione peroxidase expressed as per ml of blood corrected for PCV.
[4] GPx = glutathione peroxidase expressed per ml of red blood cell.
[5] SOD = superoxide dismutase expressed as per ml of blood corrected for PCV.
[6] SOD = superoxide dismutase expressed per ml of red blood cell.
[7] Packed cell volume.

Table 3-6. Continuned

Measure	*P* values														
	Diet, Control vs. AO	Cool vs. noncool	Diet by cooling	Parity (P)	Diet by P	Cooling by P	Diet by cooling by P	Week (W)	Diet by W	Cooling by W	Diet by cooling by W	P by W	Diet by P by W	Cooling by P by W	Diet by cooling by P by W
Body temp, °C	0.70	< 0.01	0.51	0.01	0.26	0.12	0.52	0.56	0.79	0.87	0.55	0.88	0.68	0.95	0.15
TBARS	0.19	0.13	0.07	0.02	0.68	0.15	0.52	<0.001	0.51	0.54	0.70	0.71	0.77	0.25	0.62
GPx	0.34	0.72	0.10	0.46	0.67	0.44	0.01	0.45	0.83	0.46	0.91	0.24	0.24	0.83	0.76
GPx	0.15	0.62	0.33	0.88	0.25	0.63	0.05	0.02	0.72	0.37	0.69	<0.001	0.35	0.89	0.98
SOD	0.72	0.20	0.76	0.05	0.35	0.13	0.90	0.02	0.39	0.73	0.71	0.83	0.10	0.73	0.92
SOD	0.99	0.20	0.56	0.26	0.74	0.48	0.68	0.82	0.03	0.91	0.22	0.17	0.09	0.69	0.25
PCV	0.57	0.94	0.28	0.10	0.27	0.04	0.07	<0.01	0.91	0.43	0.82	0.78	0.22	0.42	0.67

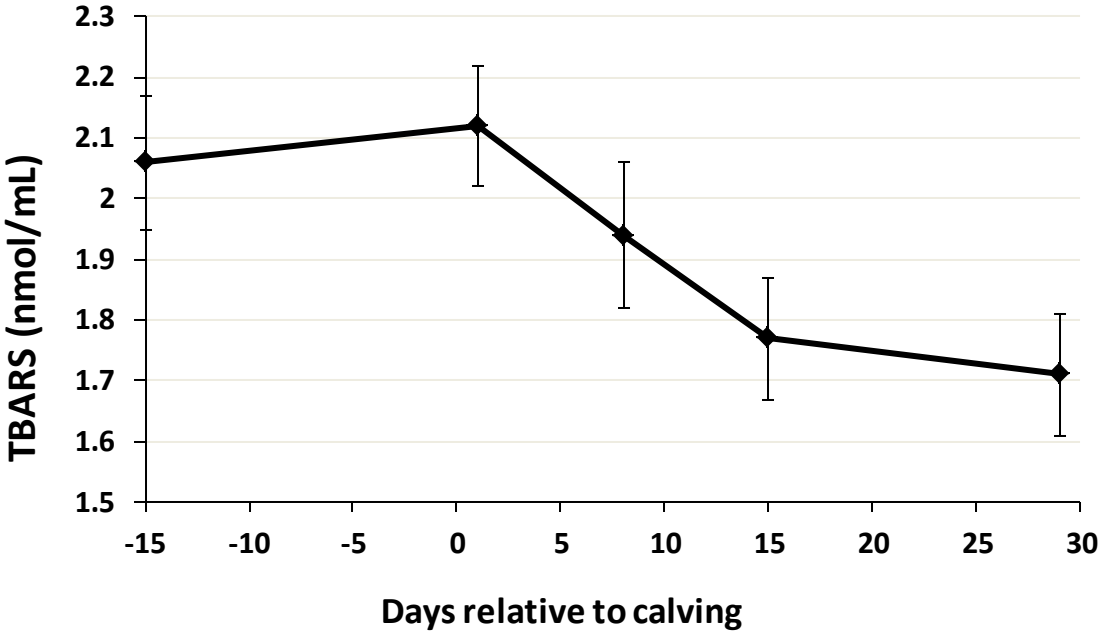

Figure 3-16. Least squares means for plasma thiobarbituric acid reactive substances (TBARS) on -15, 1, 8, 15, 29 d relative to calving. Effect of time ($P < 0.0001$).

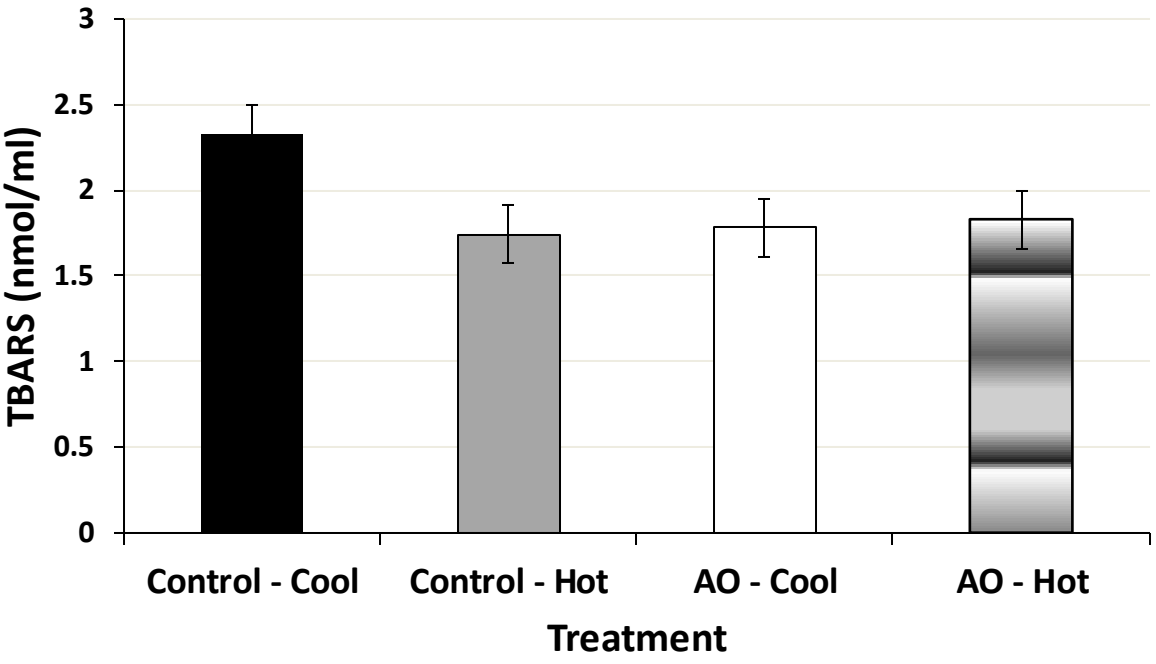

Figure 3-17. Least squares means for mean plasma concentration of thiobarbituric acid reactive substances (TBARS) of Holstein cows (n = 35) fed diets supplemented without (Control) or with dietary antioxidant (AO) and housed in shaded freestalls equipped with fans and sprinklers (Cool) or just shade (Hot). Diet by environment interaction (P = 0.07).

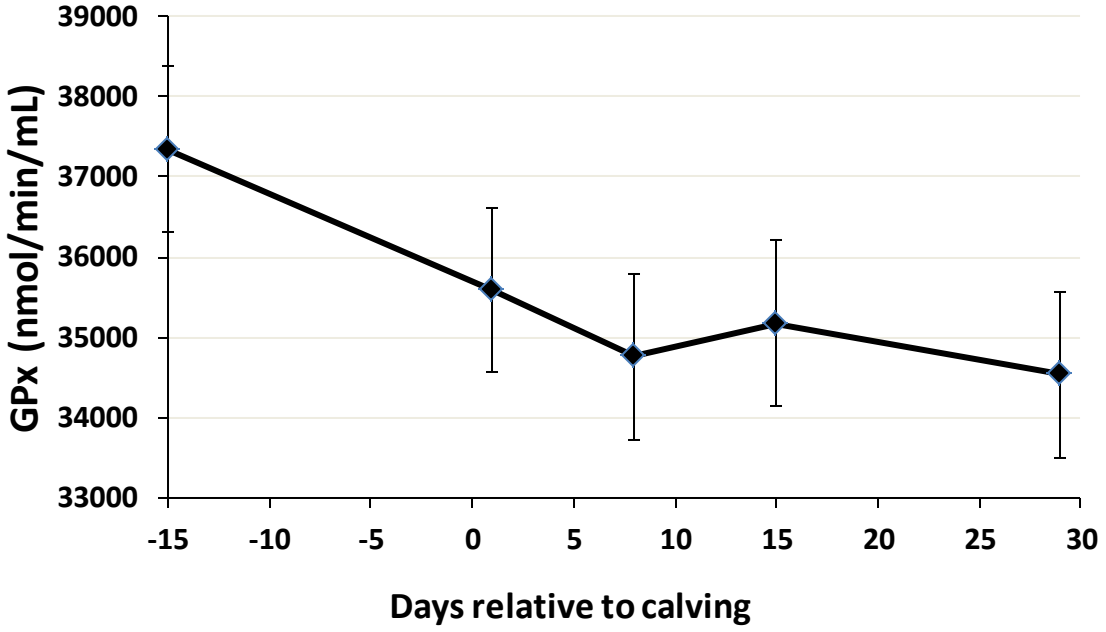

Figure 3-18. Least squares means for weekly activity of glutathione peroxidase (GPx) per mL of erythrocyte of Holstein cows (n = 35) fed diets supplemented without or with synthetic antioxidants and housed in shaded freestalls equipped with fans and sprinklers or just shade on -15, 1, 8, 15, and 29 d relative to calving. Effect of time (P = 0.02).

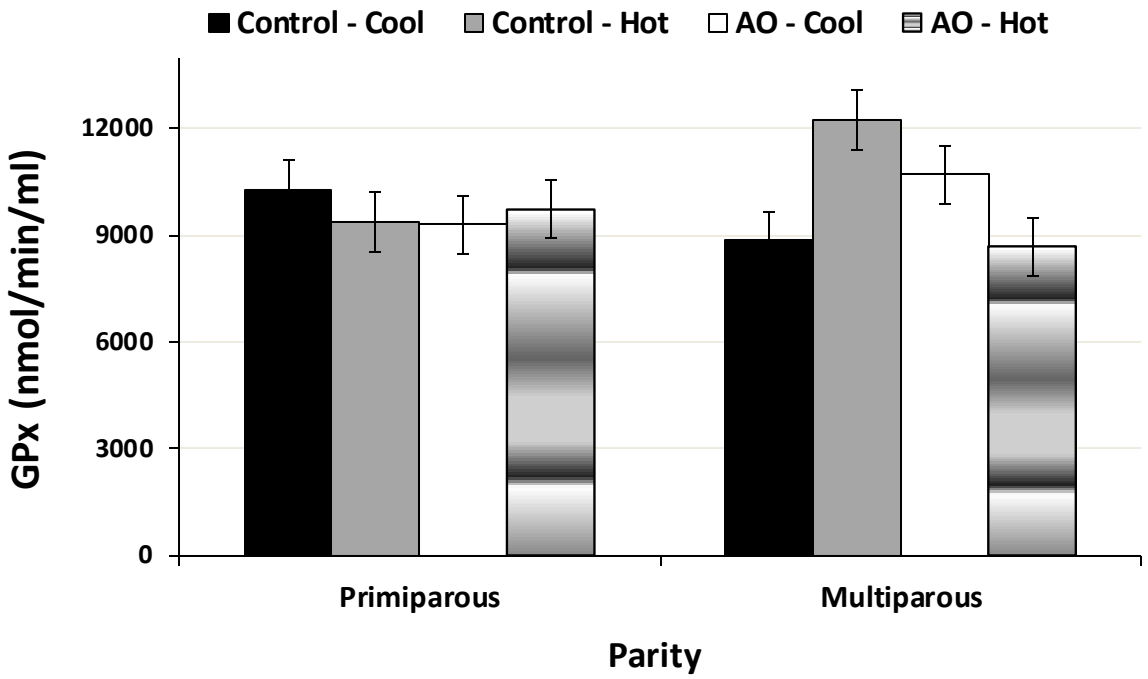

Figure 3-19. Least squares means for mean activity of glutathione peroxidase corrected for pack cell volume of primiparous (n = 22) and multiparous (n = 13) Holstein cows fed diets supplemented without (Control) or with synthetic antioxidants (AO) and housed in shaded freestalls equipped with fans and sprinklers (Cool) or just shade (Hot). Effect of diet by environment by parity interaction (P = 0.01).

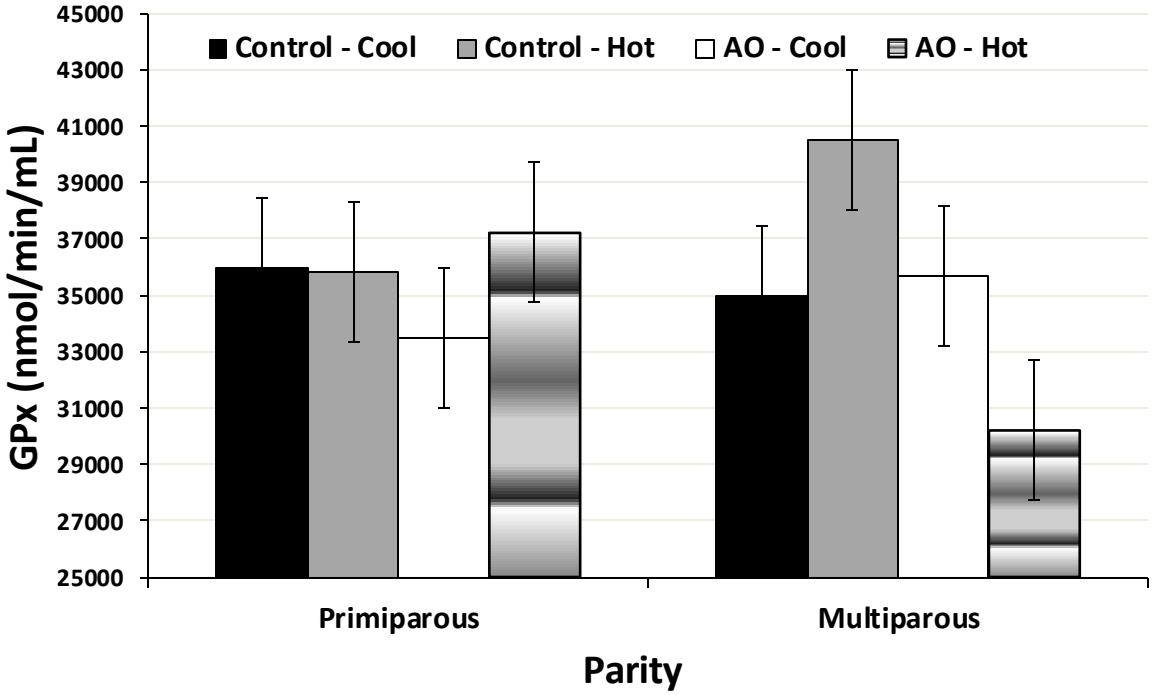

Figure 3-20. Least squares means for mean activity of glutathione peroxidase per mL of erythrocyte of primiparous (n = 22) and multiparous (n = 13) Holstein cows fed diets supplemented without (Control) or with synthetic antioxidants (AO) and housed in shaded freestalls equipped with fans and sprinklers (Cool) or just shade (Hot). Effect of diet by environment by parity interaction (P = 0.05).

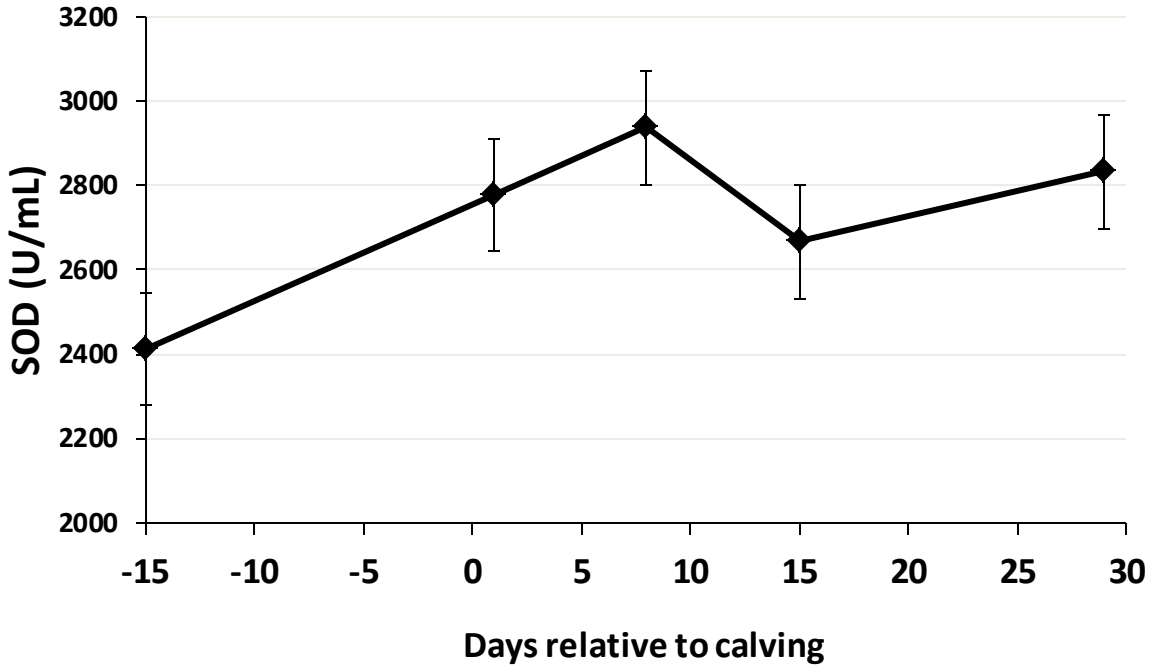

Figure 3-21. Least squares means for activity of superoxide dismutase (SOD) corrected for pack cell volume of Holstein cows (n = 35) fed diets supplemented without or with synthetic antioxidants and housed in shaded freestalls equipped with fans and sprinklers or just shade on -15, 1, 8, 15, and 29 d relative to calving. Effect of time (*P* = 0.02).

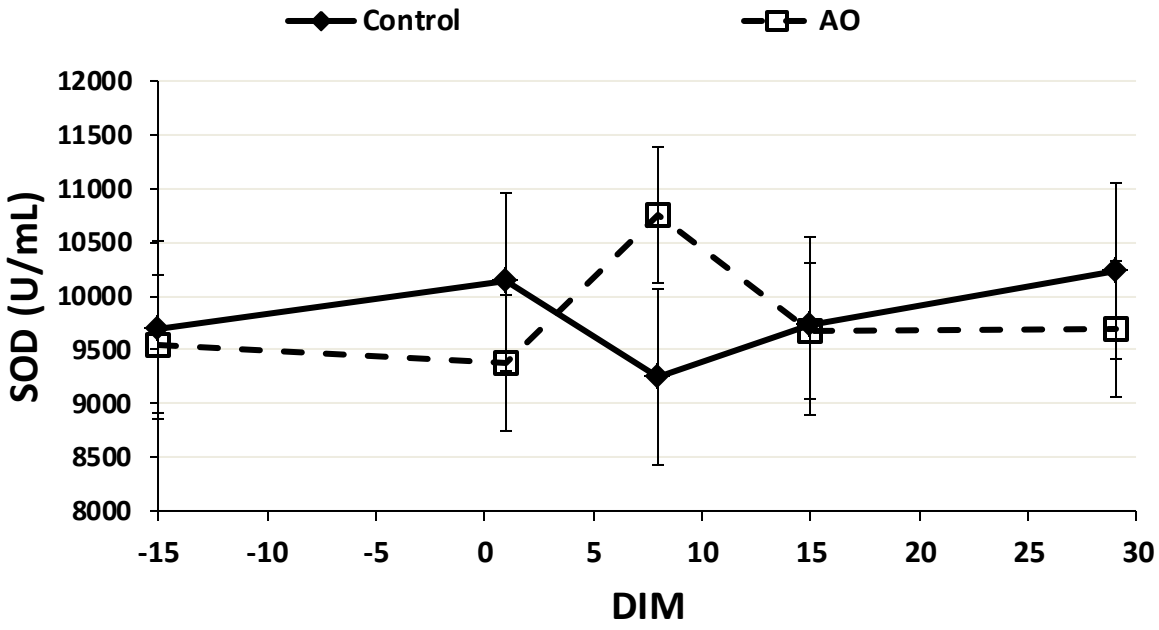

Figure 3-22. Least squares means for activity of superoxide dismutase (SOD) per mL of erythrocyte of Holstein cows (n = 35) fed diets supplemented without (Control) or with dietary antioxidants (AO) on -15, 1, 8, 15, and 29 d relative to calving. Effect of diet by time (P = 0.03).

Table 3-7. Effect of feeding synthetic antioxidants (AO) and prepartum cooling on concentration of white blood cells (WBC), lymphocytes, and neutrophils, function of blood neutrophils, and plasma concentration of acid soluble protein (ASP) and haptoglobin (Hp) of periparturient Holstein cows during summer in Florida.

Measure	Treatment							
	Control diet				AO diet[1]			
	Cooled		Non-cooled		Cooled		Non-cooled	
	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous
WBC, per µL	9,273 ± 1665	5,998 ± 1762	10,698 ± 1766	12,917 ± 3795	8,488 ± 1547	8,249 ± 1481	8,969 ± 1470	8,652 ± 1746
Lymphocytes, per µL	4272 ± 1081	2208 ± 944	4911 ± 1131	8145 ± 3484	3912 ± 996	3862 ± 978	4700 ± 1080	4559 ± 1304
Neutrophils, per µL	3,728 ± 563	2,983 ± 730	4,571 ± 661	3,010 ± 737	3,031 ± 489	2,819 ± 426	3,056 ± 429	2,772 ± 470
Phagocytosis, %	77.0 ± 3.3	80.4 ± 5.2	80.3 ± 3.2	70.4 ± 5.0	71.1 ± 3.0	73.8 ± 3.6	81.4 ± 3.3	72.8 ± 3.6
Phagocytosis, MFI[2]	63.3 ± 5.9	34.5 ± 5.1	75.3 ± 6.8	31.2 ± 4.7	48.5 ± 4.4	40.4 ± 4.2	62.7 ± 6.0	36.7 ± 4.0
Oxidative burst, %	84.1 ± 3.0	81.2 ± 4.6	84.7 ± 2.8	70.5 ± 4.3	77.1 ± 2.7	76.1 ± 3.2	81.8 ± 2.9	77.4 ± 3.3
Oxidative burst, MFI	1374 ± 211	1098 ± 321	1645 ± 219	1094 ± 297	1146 ± 183	950 ± 196	1589 ± 222	1025 ± 209
Acute phase proteins								
ASP, µg/mL	45.1 ± 4.6	39.9 ± 7.3	48.6 ± 4.2	35.3 ± 7.3	53.5 ± 4.6	41.5 ± 4.6	50.7 ± 4.2	42.6 ± 5.1
Hp, arbitrary units	0.015 ± 0.002	0.015 ± 0.002	0.013 ± 0.002	0.012 ± 0.002	0.012 ± 0.003	0.011 ± 0.003	0.011 ± 0.002	0.013 ± 0.002

[1] Agrado-Plus (Novus Internat. Inc., St. Charles, MO).
[2] MFI = Mean florescence intensity, to test the phagocytic ability of neutrophils.

Table 3-7. Continuned

Measure	*P* values														
	Diet, Control vs. AO	Cool vs. noncool	Diet by cooling	Parity (P)	Diet by P	Cooling by P	Diet by cooling by P	Week (W)	Diet by W	Cooling by W	Diet by cooling by W	P by W	Diet by P by W	Cooling by P by W	Diet by cooling by P by W
WBC, per µL	0.56	0.09	0.18	0.60	0.76	0.30	0.29	<0.001	0.67	0.05	0.41	0.67	.042	0.97	0.83
Lymphocytes per µL	0.85	0.03	0.18	0.81	0.89	0.16	0.15	0.07	0.85	0.09	0.73	0.14	0.96	0.94	0.57
Neutrophils, per uL	0.14	0.68	0.66	0.11	0.35	0.66	0.74	<0.001	0.47	0.11	0.24	0.50	0.54	0.98	0.97
Phagocytosis, %	0.42	0.83	0.15	0.27	0.93	0.03	0.86	0.001	0.41	0.97	0.34	0.29	0.60	0.65	0.15
Phagocytosis, MFI	0.68	0.16	0.79	<0.001	0.02	0.05	0.79	<0.001	0.93	0.93	0.61	0.40	0.04	0.11	0.17
Oxidative burst, %	0.46	0.64	0.10	0.03	0.24	0.13	0.40	<0.01	0.36	0.73	0.08	0.05	0.86	0.96	0.31
Oxidative burst, MFI	0.46	0.29	0.70	0.03	0.96	0.40	0.90	<0.01	0.81	0.47	0.77	0.22	0.23	0.96	0.99
Acute phase proteins															
ASP, µg/mL	0.22	0.85	0.97	0.02	0.92	0.78	0.43	<0.001	0.03	0.72	0.47	<0.001	0.74	0.89	0.86
Hp, arbitrary units	0.65	0.99	0.73	0.20	0.36	0.68	0.65	<0.001	0.24	0.03	0.72	0.09	0.97	0.60	0.35

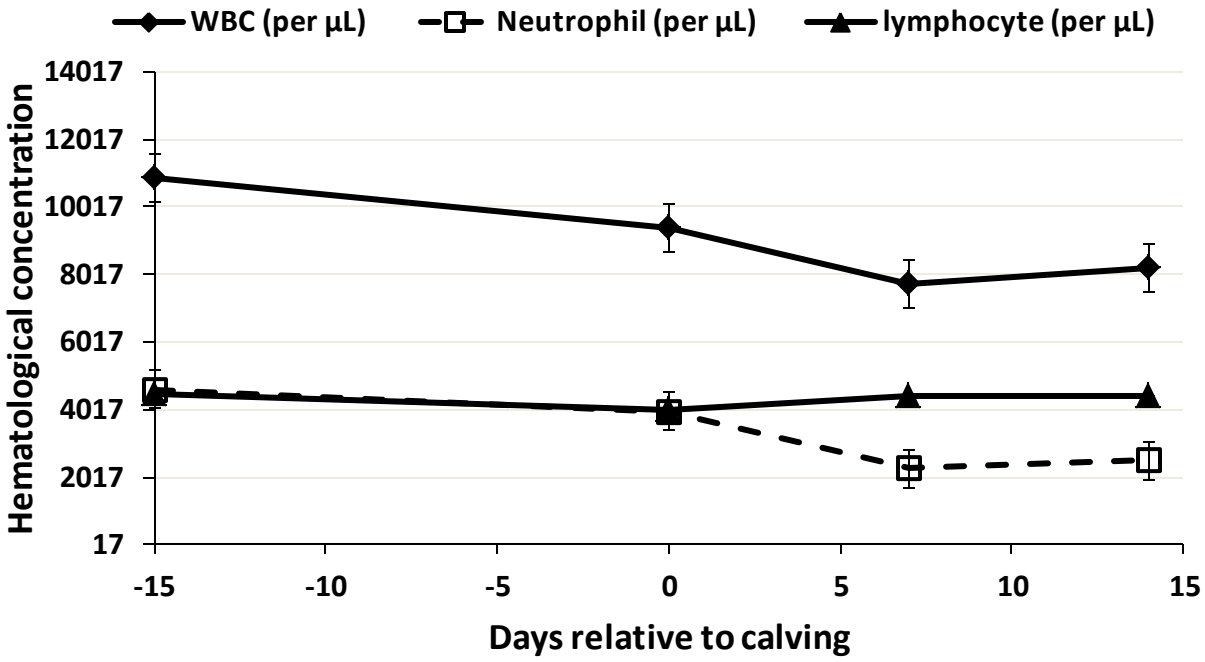

Figure 3-23. Least squares means for number of white blood cells (WBC), neutrophils, and lymphocytes per µL of whole blood on -15, 0, 7, 14 d relative to calving. Effect of time for WBC ($P < 0.001$), for neutrophil ($P < 0.001$), and for lymphocyte ($P = 0.07$).

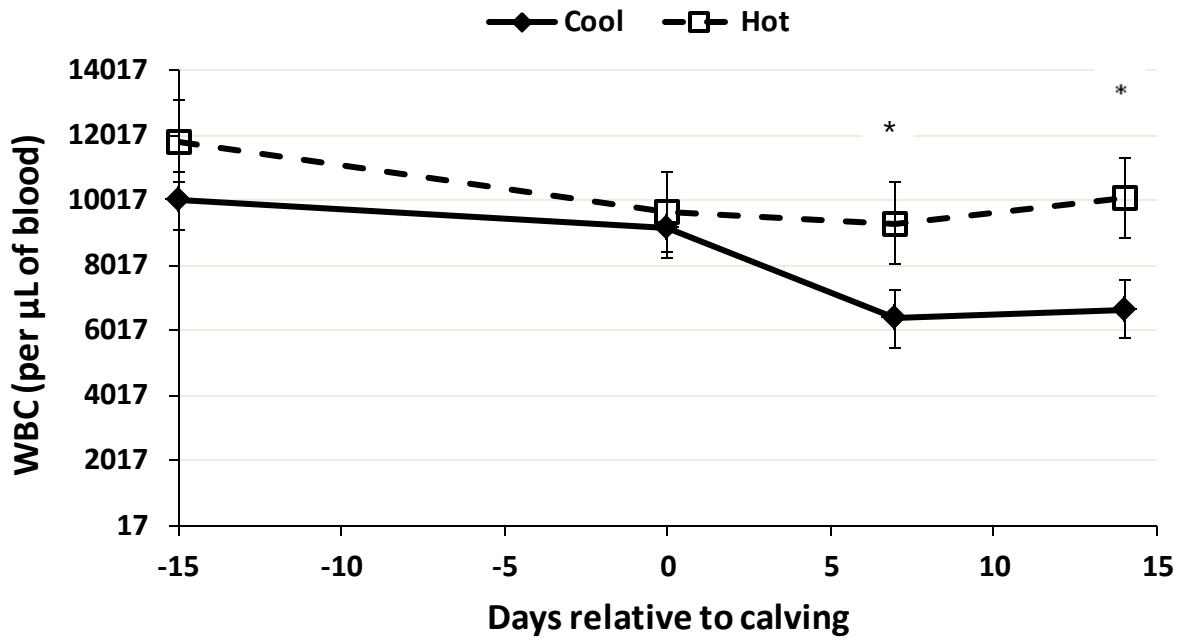

Figure 3-24. Least squares means for number of white blood cells (WBC) per µL of whole blood of Holstein cows (n = 35) housed in shaded freestalls equipped with fans and sprinklers (Cool) or just shade (Hot). Effect of environment by week interaction ($P = 0.05$). Days relative to calving with asterisk indicates that means differed ($P < 0.05$) using slice command.

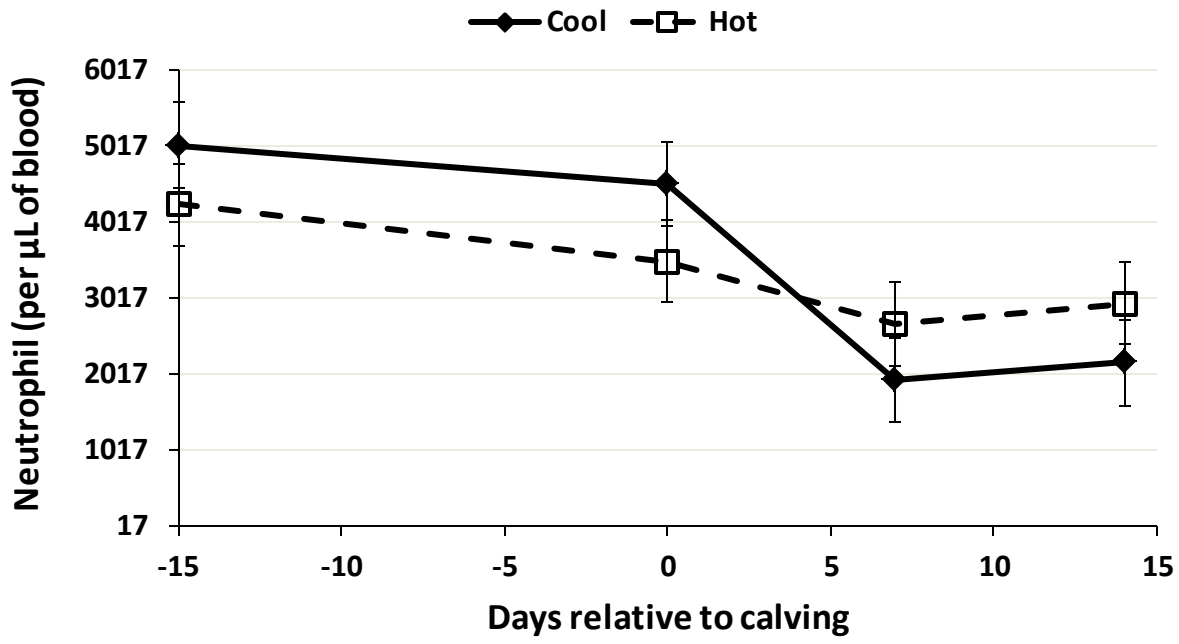

Figure 3-25. Least squares means for number of neutrophils per µL of whole blood of Holstein cows (n = 35) housed in shaded freestalls equipped with fans and sprinklers (Cool) or just shade (Hot). Effect of environment by week interaction (P = 0.11).

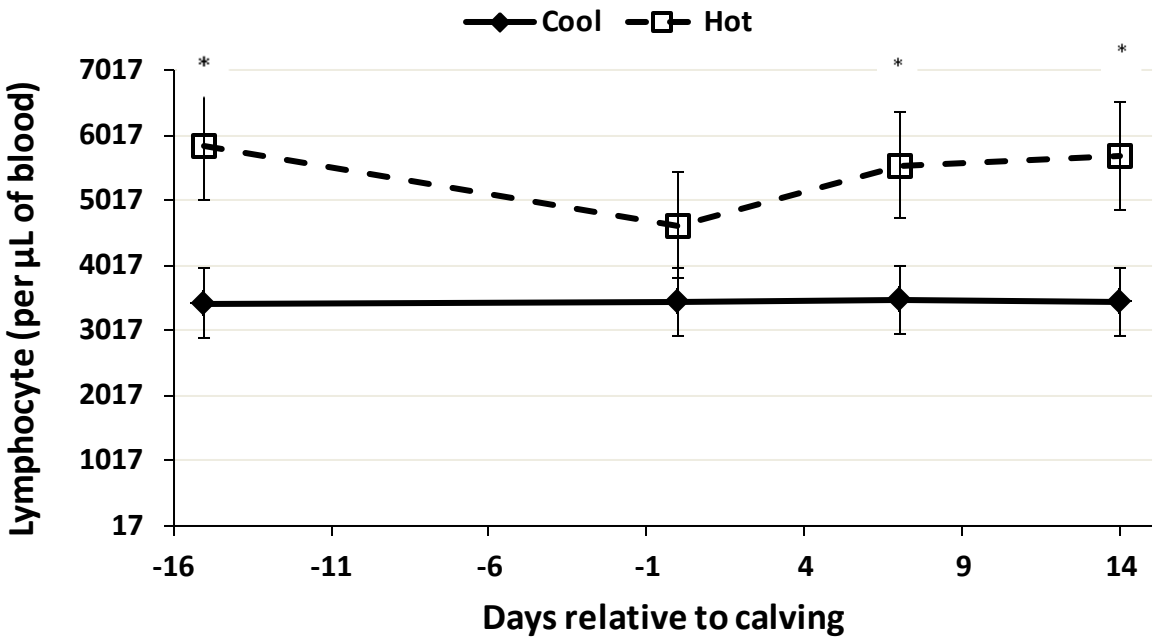

Figure 3-26. Least squares means for number of lymphocytes per µL of whole blood of Holstein cows (n = 35) housed in shaded freestalls equipped with fans and sprinklers (Cool) or just shade (Hot). Effect of environment by week interaction ($P = 0.09$). Days relative to calving with asterisk indicates that means differed ($P < 0.05$) using slice command.

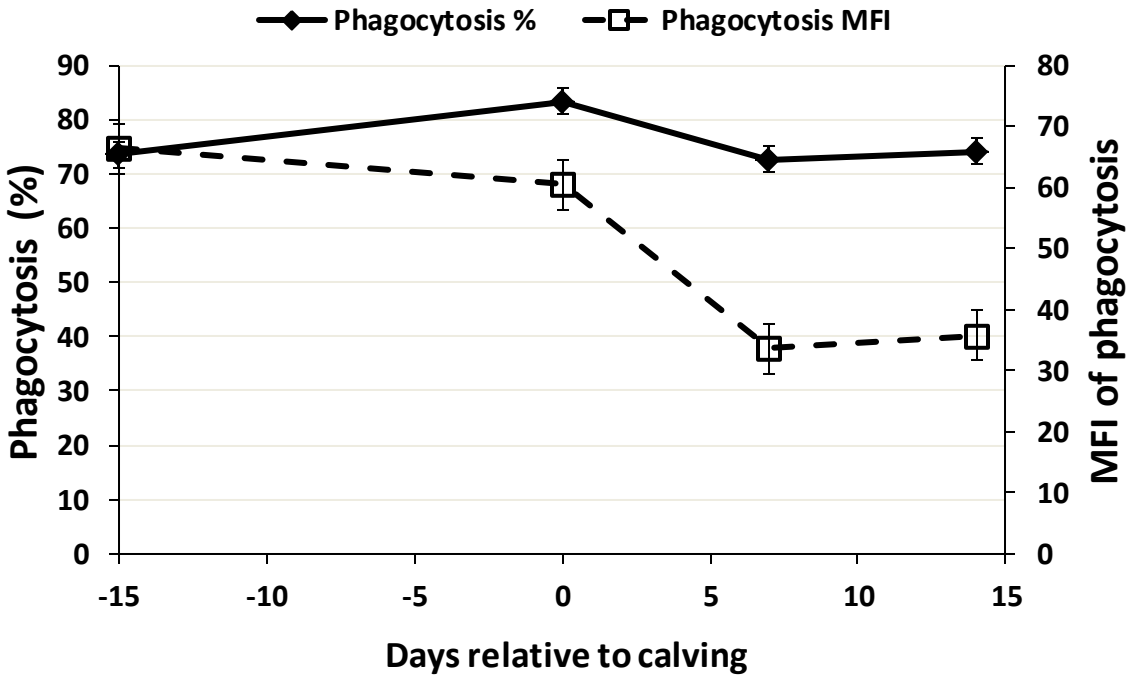

Figure 3-27. Least squares means for percentage of neutrophils with phagocytic activity (solid line) and neutrophil mean florescence intensity (MFI, indication of number of bacteria phagocytised per neutrophil, dash line) of Holstein cows (n = 35) on -15, 0, 7, 14 d relative to calving. Effect of time ($P = 0.001$) for percentage of neutrophils with phagocytic activity, effect of time ($P < 0.001$) for neutrophil MFI.

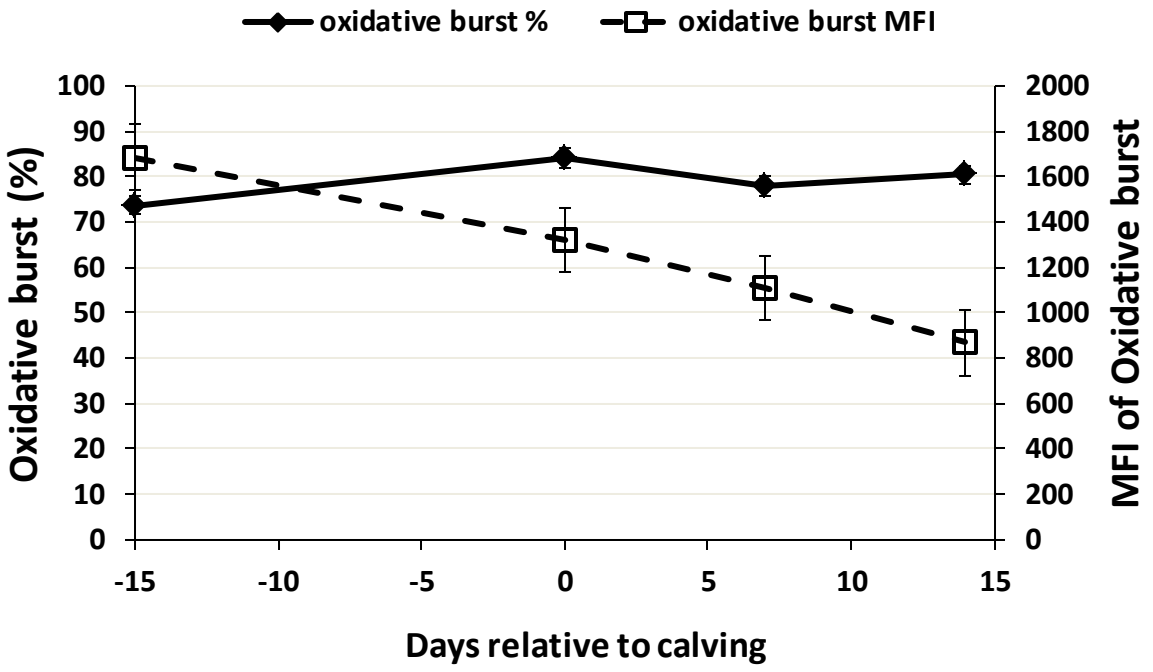

Figure 3-28. Least squares means for percentage of neutrophils with oxidative burst activity (solid line) and neutrophil mean florescence intensity (MFI, indication of intensity of reactive oxygen species produced per neutrophil, dash line) of Holstein cows (n = 35) on -15, 0, 7, 14 d relative to calving. Effect of time ($P <$ 0.01).

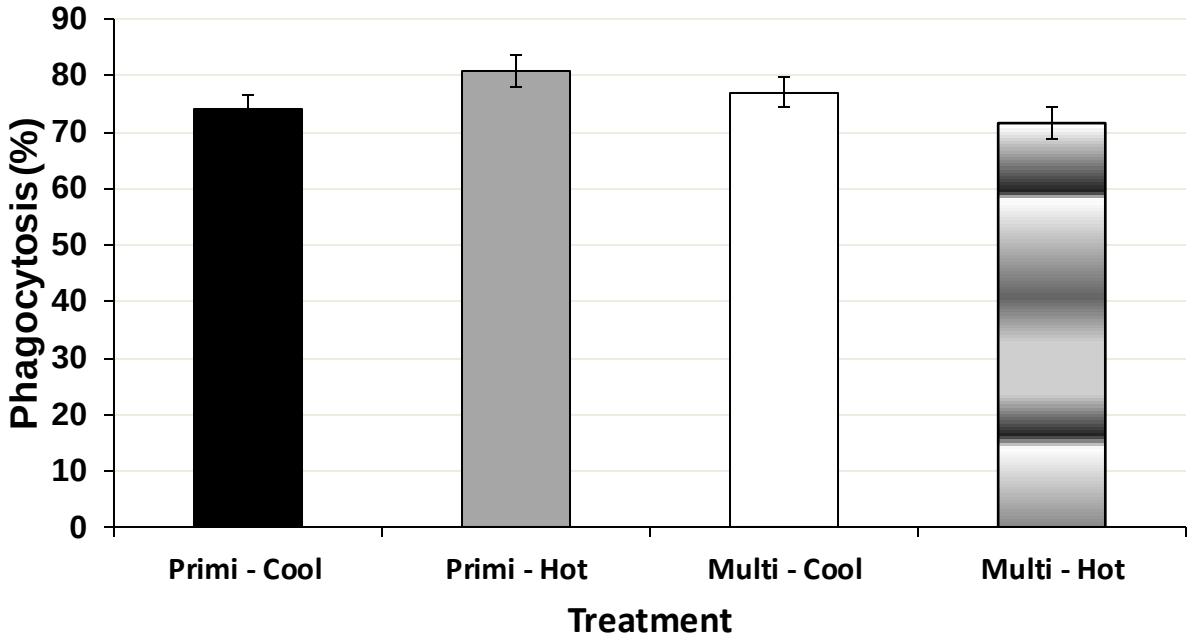

Figure 3-29. Least squares means for mean percentage of neutrophils with phagocytic activity of primiparous (primi, n = 22) and multiparous (multi, n = 13) Holstein cows housed in shaded freestalls equipped with fans and sprinklers (Cool) or just shade (Hot). Effect of environment by parity interaction (*P* = 0.03).

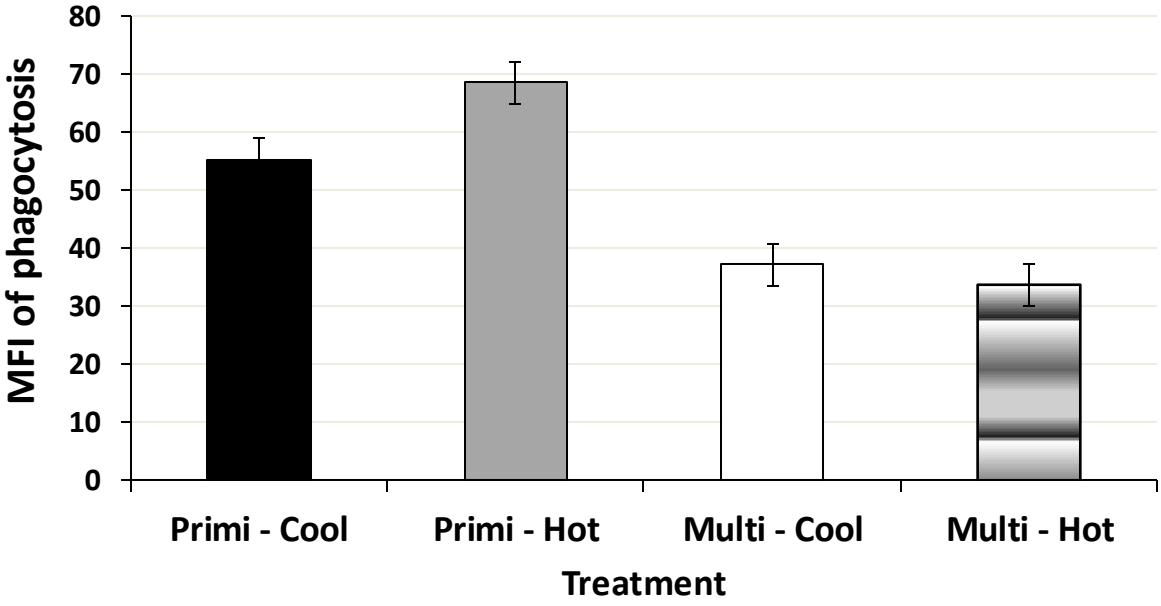

Figure 3-30. Least squares means for neutrophil mean fluorescence intensity (MFI, indication of number of bacteria phagocytised per neutrophil) of primiparous (primi, n = 22) and multiparous (multi, n = 13) Holstein cows housed in shaded freestalls equipped with fans and sprinklers (Cool) or just shade (Hot). Effect of environment by parity interaction (P = 0.05).

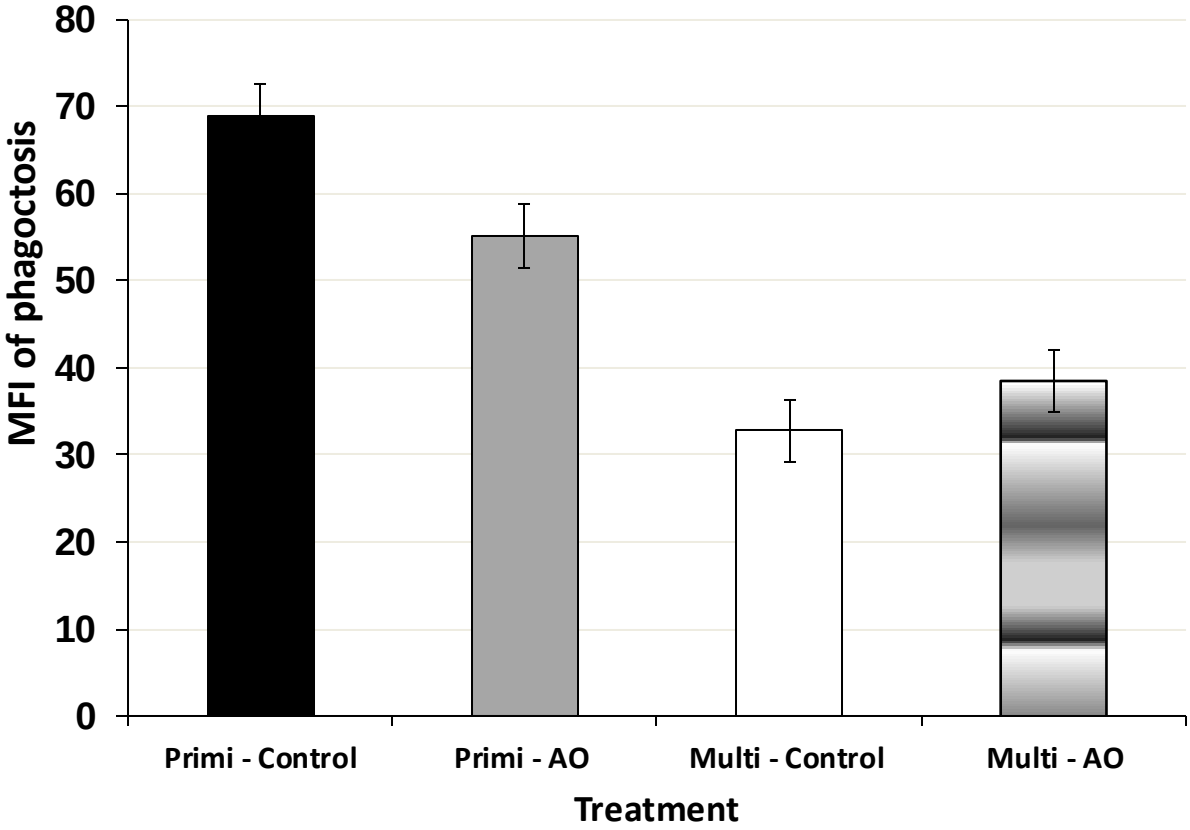

Figure 3-31. Least squares means for neutrophil mean fluorescence intensity (MFI, indication of number of bacteria phagocytised per neutrophil) of primiparous (primi, n = 22) and multiparous (multi, n = 13) Holstein cows fed diets supplemented without (Control) or with synthetic antioxidants (AO). Effect of diet by parity interaction (P = 0.02).

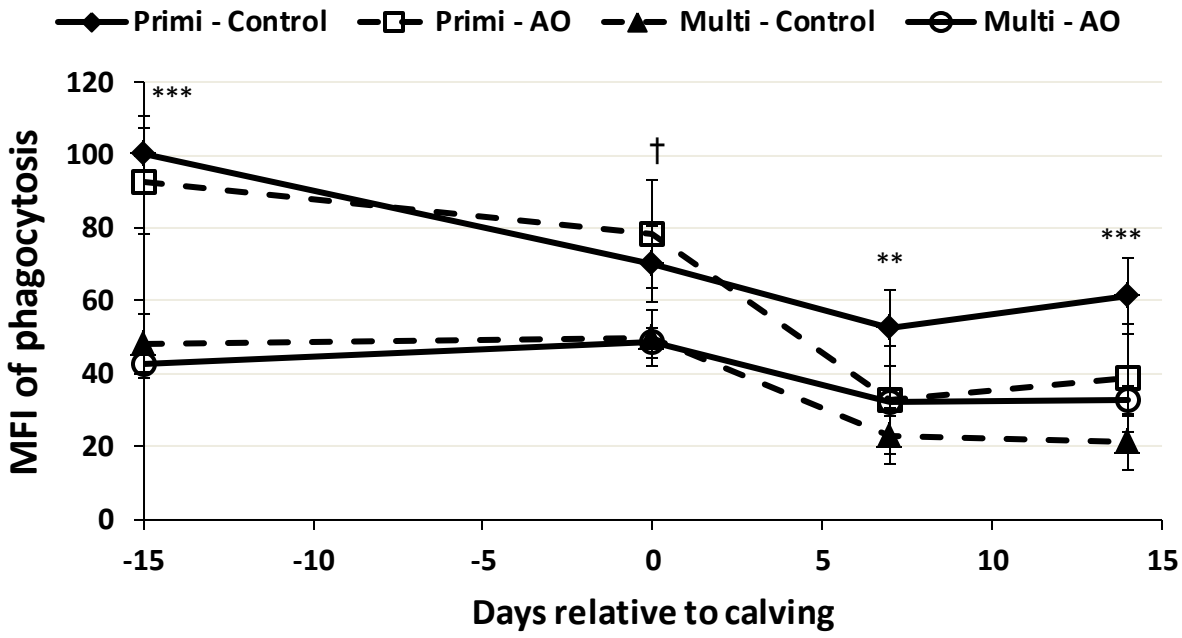

Figure 3-32. Least squares means for neutrophil mean fluorescence intensity (MFI, indication of number of bacteria phagocytised per neutrophil) of primiparous (primi, n = 22) and multiparous (multi, n = 13) Holstein cows fed diets supplemented without (Control) or with synthetic antioxidants (AO) on -15, 0, 7, 14 d relative to calving. Effect of diet by parity interaction ($P = 0.04$). Days relative to calving with two asterisks indicates that means differed for that day using slice command ($P < 0.01$), with three asterisks differed ($P < 0.001$), with dagger differed ($P < 0.10$).

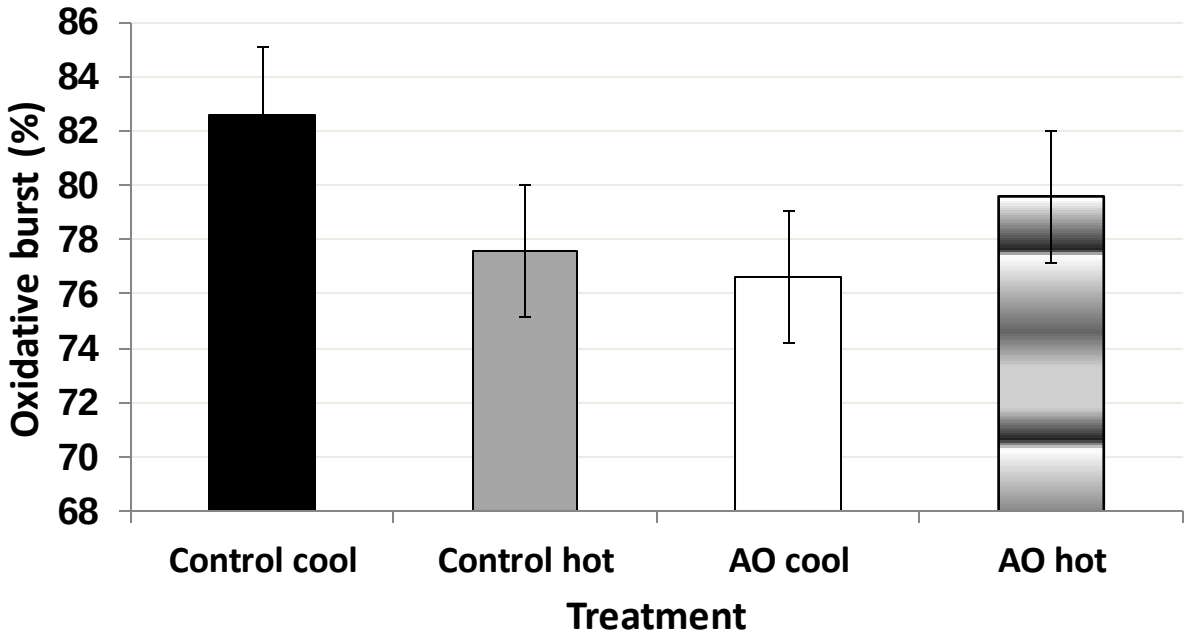

Figure 3-33. Least squares means for mean percentage of neutrophil with oxidative burst activity of primiparous (n = 22) and multiparous (n = 13) Holstein cows fed diets supplemented without (Control) or with synthetic antioxidants (AO) and housed in shaded freestalls equipped with fans and sprinklers (Cool) or just shade (Hot). Effect of diet by environment interaction (P = 0.10).

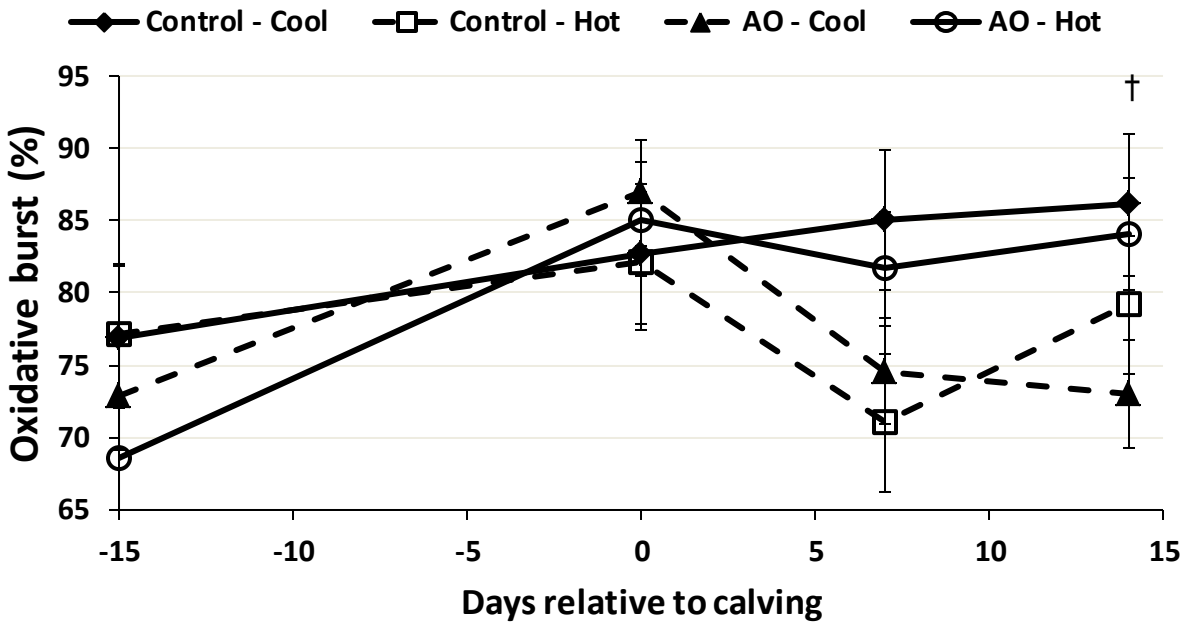

Figure 3-34. Least squares means for percentage of neutrophil with oxidative burst activity of Holstein cows (n = 35) fed diets supplemented without (Control) or with synthetic antioxidants (AO) and housed in shaded freestalls equipped with fans and sprinklers (Cool) or just shade (Hot) on -15, 0, 7, 14 d relative to calving. Effect of diet by environment by time interaction (P = 0.08). Days relative to calving with dagger indicates that means differed for that day using slice command (P < 0.10).

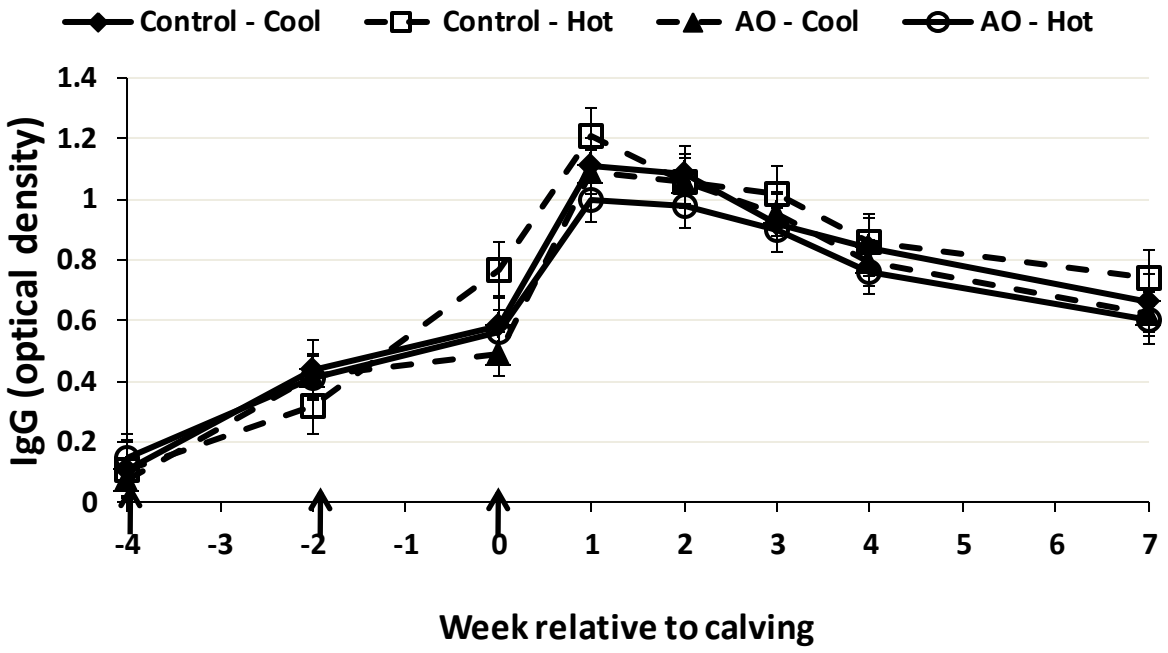

Figure 3-35. Least squares means for IgG response against ovalbumin of Holstein cows (n = 35) fed diets supplemented without (Control) or with synthetic antioxidants (AO) and housed in shaded freestalls equipped with fans and sprinklers (Cool) or just shade (Hot) on -4, -2, 0, 1, 2, 3, 4, and 7 wk relative to calving. Arrows on the top of week indicate the week of ovalbumin injection. Effect of diet by environment by week interaction (P = 0.69).

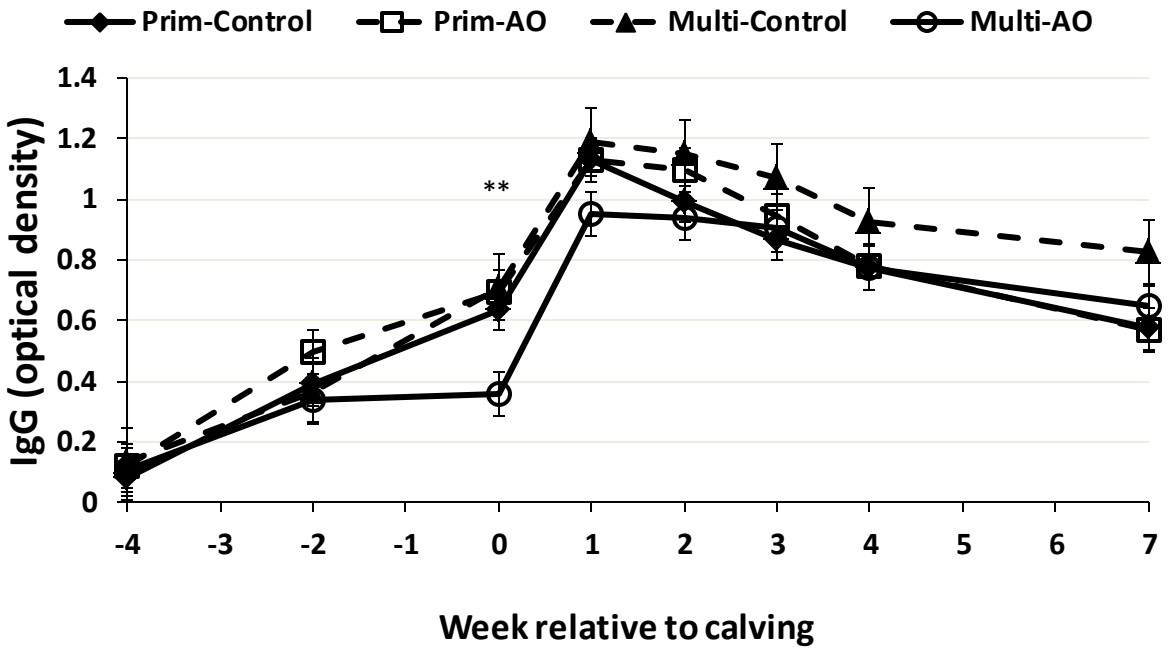

Figure 3-36. Least squares means for IgG response against ovalbumin of primiparous (primi, n = 22) and multiparous (multi, n = 13) Holstein cows fed diets supplemented without (Control) or with synthetic antioxidants (AO). Effect of diet by parity interaction ($P = 0.05$). Week with two asterisks indicates that means differed for that week ($P < 0.01$) using slice command.

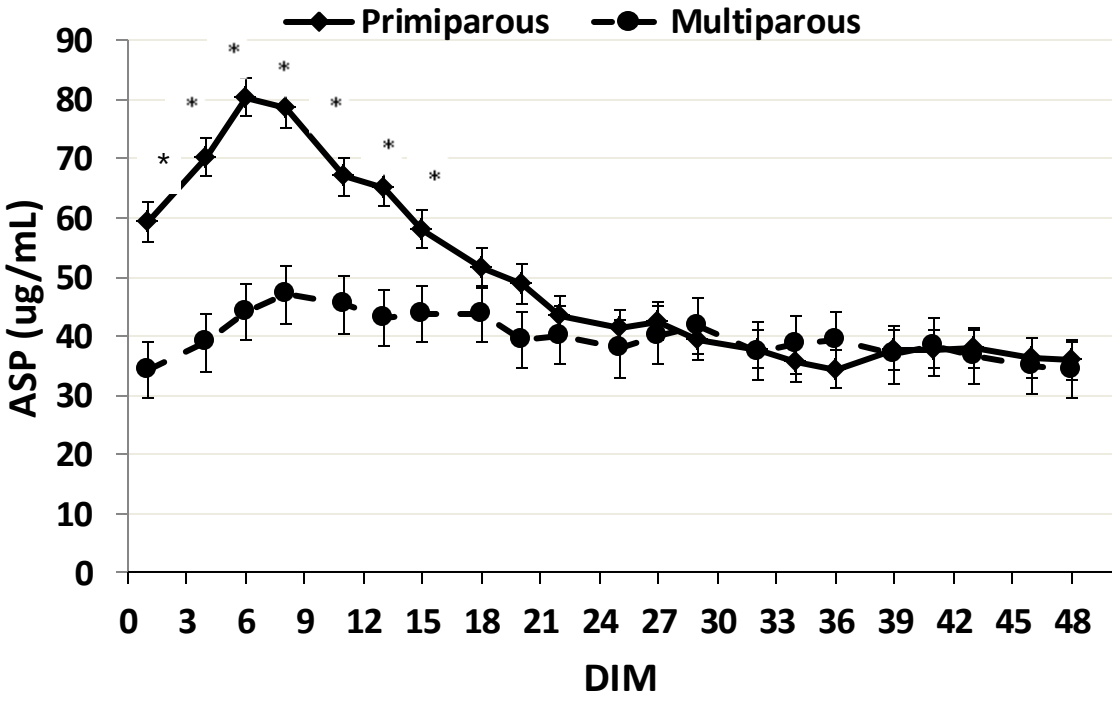

Figure 3-37. Least squares means for concentrations of acid soluble protein (ASP) of primiparous (n = 22) and multiparous (n = 13) Holstein cows fed diets supplemented with or without dietary antioxidant (Agrado Plus) and housed in shaded freestalls equipped with fans and sprinklers or just shade. Parity by DIM interaction ($P < 0.001$). Days in milk with asterisks indicates that means differed for that day using slice command ($P < 0.05$).

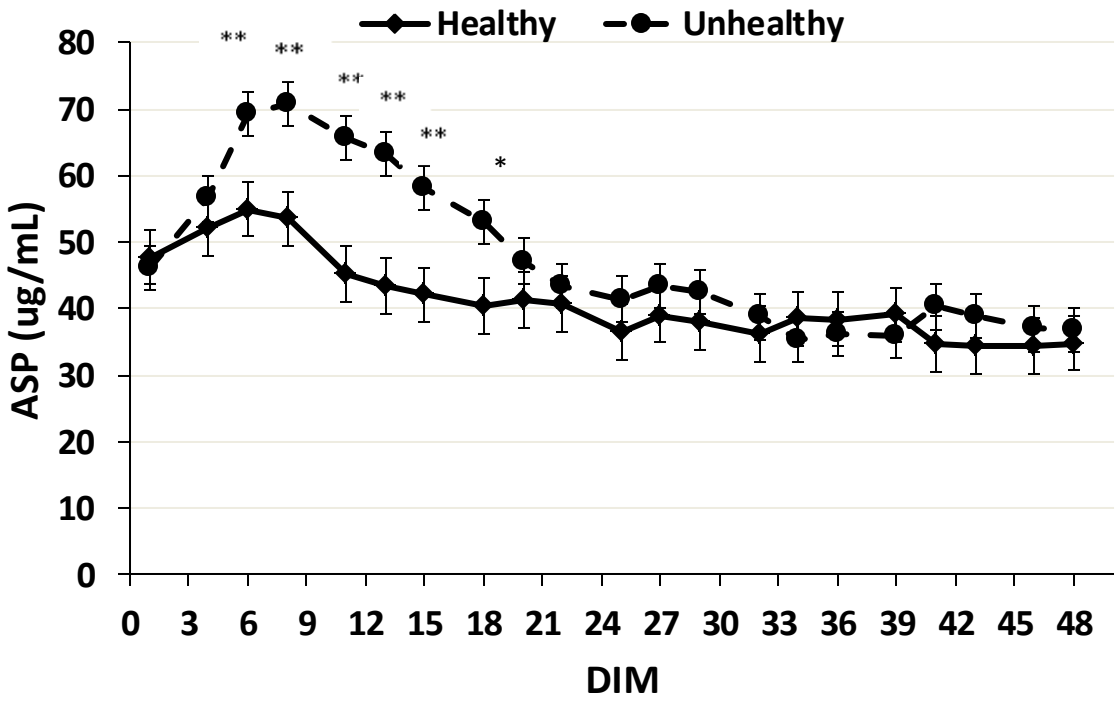

Figure 3-38. Least squares means for concentrations of acid soluble protein of Holstein cows (n = 35) diagnosed as healthy (n = 14) or unhealthy (metritis, mastitis, or retainted fetal membranes, n = 21). Treatment by DIM interaction (P = 0.04). Days in milk with one asterisk indicates that means differed for that day using slice command ($P < 0.05$), with two asterisks differed ($P < 0.001$).

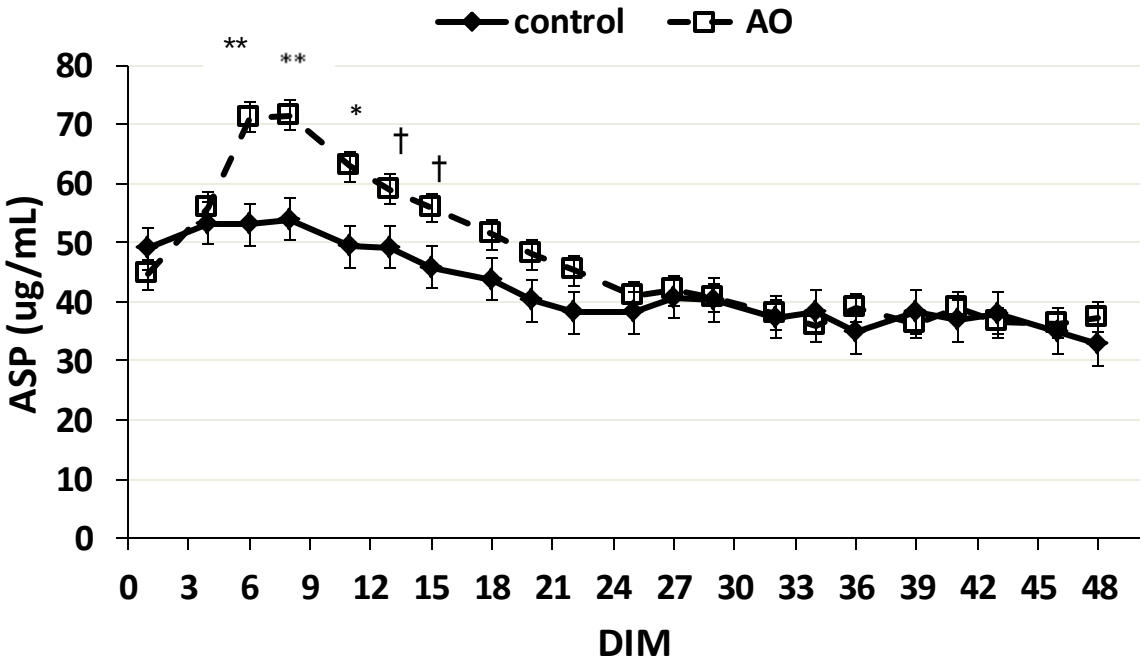

Figure 3-39. Least squares means for concentrations of acid soluble protein (ASP) of Holstein cows (n = 35) fed diets supplemented without (Control) or with synthetic antioxidants (AO). Diet by days interaction ($P = 0.03$). Days in milk with dagger indicates that means differed for that day using slice command ($P < 0.10$), with one asterisk differed ($P < 0.05$), with two asterisks differed ($P < 0.01$).

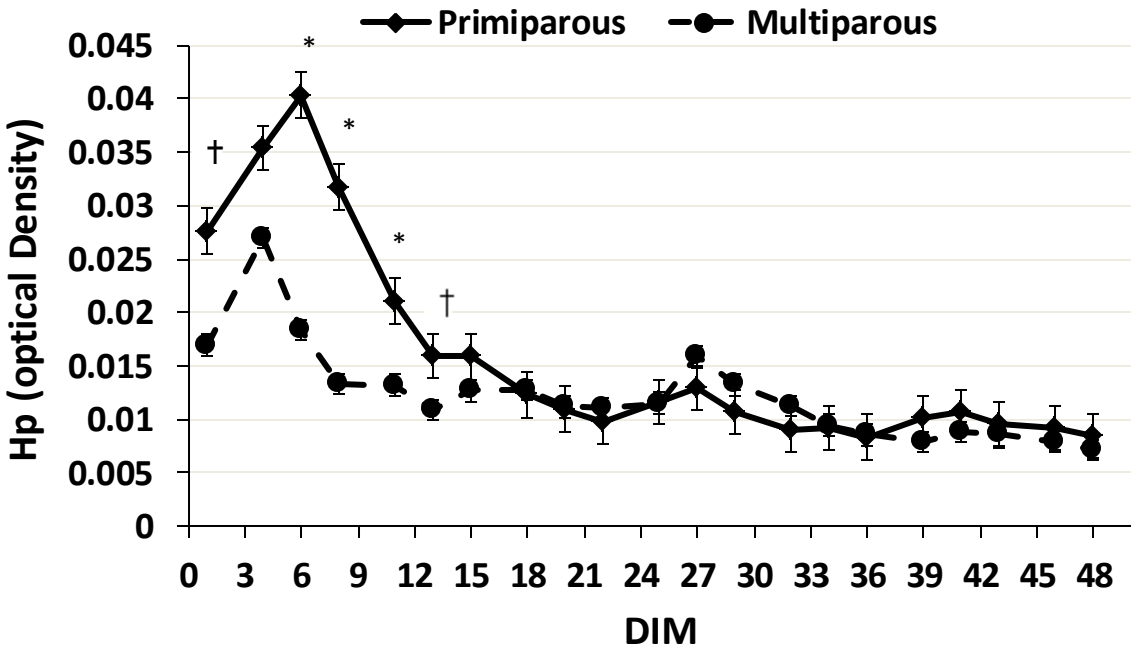

Figure 3-40. Least squares means for concentrations of haptoglobin of primiparous (n = 22) and multiparous (n = 13) Holstein cows fed diets supplemented with or without dietary antioxidant (Agrado Plus) and housed in shaded freestalls equipped with fans and sprinklers or just shade. Parity by DIM interaction (P = 0.09). Days in milk with asterisk indicates that means differed for that day using slice command (P < 0.05), with dagger differed (P < 0.10).

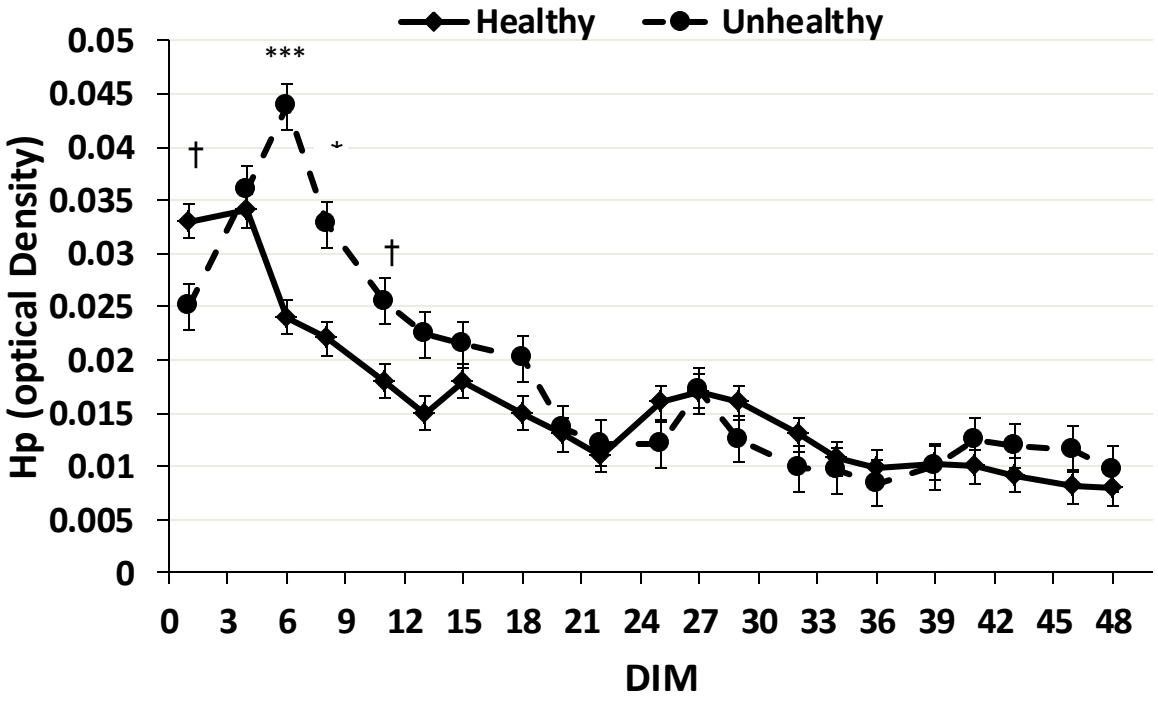

Figure 3-41. Least squares means for concentrations of haptoglobin of Holstein cows (n = 35) diagnosed as healthy (n = 14) or unhealthy (metritis, mastitis, or retainted fetal membranes, n = 21). Treatment by DIM interaction ($P = 0.03$). Days in milk with one asterisk indicates that means differed for that day using slice command ($P < 0.05$), with three asterisks differed ($P < 0.001$), with dagger differed ($P < 0.10$).

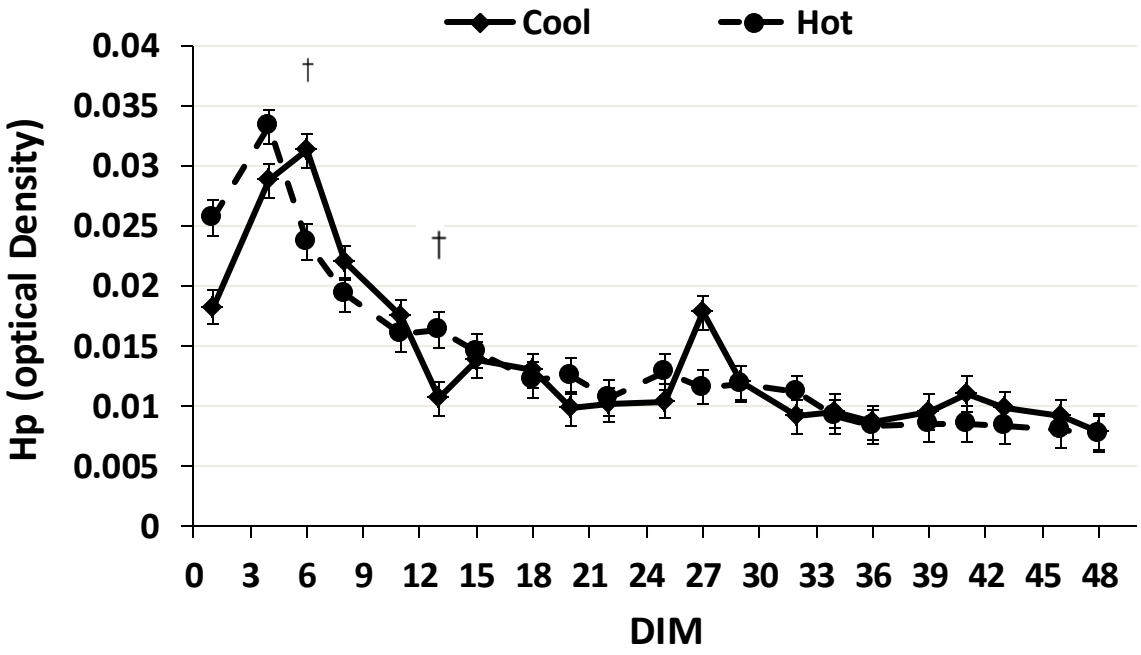

Figure 3-42. Least squares means for concentrations of haptoglobin of Holstein cows (n = 35) fed diets supplemented with or without dietary antioxidant (Agrado Plus) and housed in shaded freestalls equipped with fans and sprinklers (cool) or just shade (hot). Environment by DIM interaction ($P = 0.03$). Days in milk with dagger indicates that means differed for that day using slice command ($P < 0.10$).

Table 3-8. Profile of plasma progesterone of postpartum dairy cows fed with or without synthetic antioxidants (AO) and cooled or noncooled during the prepartum period during the summer season in Florida.

Measure	Treatment							
	Control diet				AO diet[1]			
	Cooled		Non-cooled		Cooled		Non-cooled	
	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous
First ovulation, DIM[2]	21.6 ± 3.8	12.0 ± 8.6	18.7 ± 3.5	21.0 ± 6.1	24.0 ± 3.8	17.0 ± 3.8	23.0 ± 3.5	20.5 ± 6.1
Length of first cycle, d	12.2 ± 2.2	17.0 ± 5.0	15.2 ± 2.0	23.0 ± 3.5	19.5 ± 2.5	20.8 ± 2.2	13.5 ± 2.0	16.5 ± 3.5
Peak progesterone in first cycle, ng/mL	5.0 ± 1.1	10.5 ± 2.5	6.9 ± 1.0	10.6 ± 1.8	5.6 ± 1.3	5.5 ± 1.1	5.7 ± 1.0	6.0 ± 1.8
Number of ovulations	2.0 ± 0.3	1.5 ± 0.5	2.0 ± 0.3	1.5 ± 0.5	1.6 ± 0.3	1.6 ± 0.3	2.0 ± 0.3	0.7 ± 0.4
Accumulated progesterone from 1 to 49 DIM, ng/ml	26.1 ± 8.0	32.0 ± 12.6	35.1 ± 7.3	51.4 ± 12.6	26.8 ± 8.0	41.3 ± 8.0	33.0 ± 7.3	19.0 ± 8.9

[1] Agrado-Plus (Novus Internat. Inc., St. Charles, MO).
[2] Based on 32 of 35 cows ovulating in the first 49 DIM.

Table 3-8. Continuned

Measure	*P* values						
	Diet, Control vs. AO	Cool vs. noncool	Diet by cooling	Parity (P)	Diet by P	Cooling by P	Diet by cooling by P
First ovulation	0.45	0.57	0.81	0.26	0.88	0.27	0.62
Length of first cycle, d	0.74	0.88	0.04	0.06	0.35	0.59	0.88
Peak progesterone in first cycle, ng/mL	0.03	0.54	0.75	0.04	0.05	0.74	0.60
Number of ovulations	0.33	0.68	0.68	0.04	0.81	0.25	0.25
Accumulated progesterone from 1 to 49 DIM, ng/ml	0.36	0.64	0.10	0.40	0.42	0.50	0.15

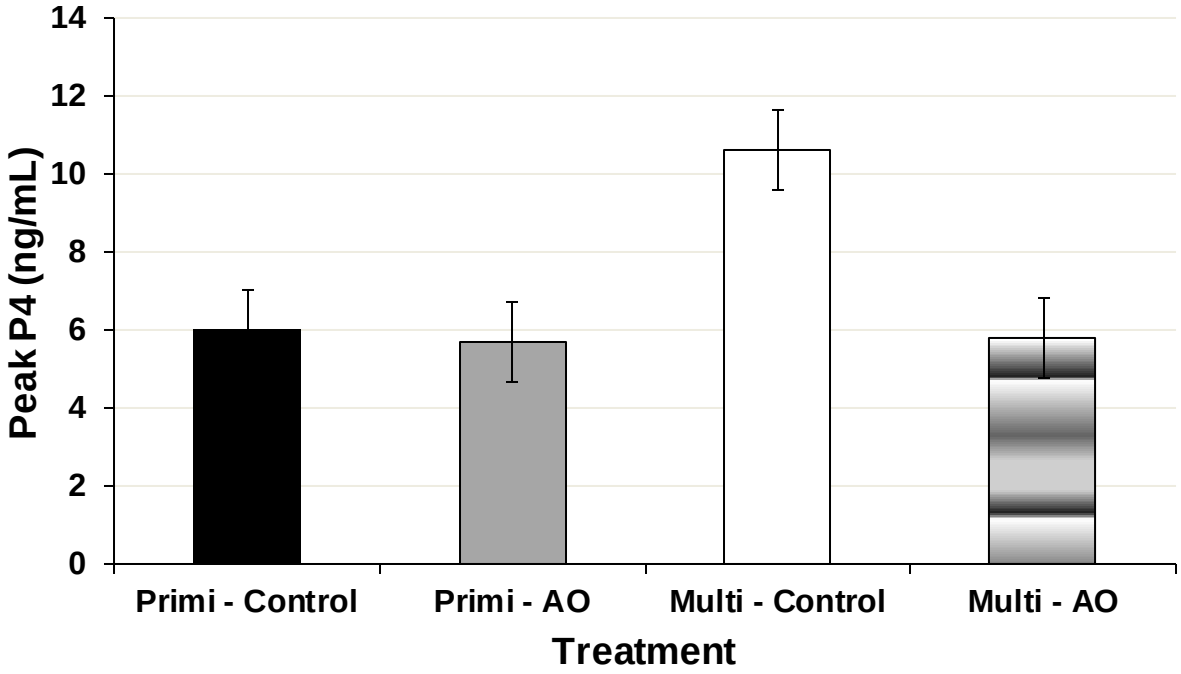

Figure 3-43. Least squares means for mean peak concentration of progesterone (P4) of the first cycle of primiparous (primi, n = 22) and multiparous (multi, n = 13) Holstein cows fed diets supplemented without (Control) or with synthetic antioxidants (AO). Effect of diet by parity interaction (P = 0.05).

Table 3-9. Incidence of postpartum health disorders of dairy cows fed with or without synthetic antioxidants (AO) and cooled or noncooled during the prepartum period during the summer season in Florida.

Measure	Control diet				AO diet[1]			
	Cooled		Non-cooled		Cooled		Non-cooled	
	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous
Number of animals	5	2	6	2	5	5	6	4
Endometritis	1		2		3	2	3	2
Retained fetal membranes					1	1		
Mastitis			2	1	1		2	1
Ketosis	2	2	4	1	3	3	3	4
Displaced abomasum			1				1	1
Milk fever								1

[1] Agrado-Plus (Novus Internat. Inc., St. Charles, MO).

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BIOGRAPHICAL SKETCH

Dan Wang was born in Beijing, China on 1983. She is the daughter of Ruilin Wang and Jinhua Fei. Dan Wang started her Bachelor of Science degree at Capital Normal University in 2002 and graduated in 2006. Later she pursued her Master of Science majoring in microbiology at the same university. But one year later, she married with Zheng (Alex) Fu and moved to Gainesville with her husband in 2007. Dan Wang did a volunteering work in the Department of Animal Sciences in University of Florida from March to June in 2008. Then she started her Master of Science at University of Florida, Department of Animal Science, under the guidance of Dr. Charles R. Staples. Her research focused on the oxidative status of dairy cows during the transition period. After graduation, she is going to stay on her Ph.D. under Dr. Charles R. Staples. Dan Wang has a lovely daughter named Eunice and she really enjoys the life with this baby girl.