GHRELIN CONCENTRATIONS IN MILK AND PLASMA OF DAIRY COWS DURING EARLY LACTATION

by

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ABSTRACT

Ghrelin Concentrations in Milk and Plasma of Dairy Cows
during Early Lactation

by

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Ghrelin is a 28 amino acid octanoylated peptide hormone and is the only naturally occurring peptide with post translation modification by ser3 O-octanoylation. This modification is essential for ghrelin activity and, subsequently, activation of its specific receptor. Active ghrelin binds to growth hormone secretagogue receptor (GHS-R), which stimulates growth hormone release and appetite. Ghrelin is expressed and produced in several tissues, but the gastric mucosa is the major source of circulating ghrelin. Ghrelin is a hormone with multiple functions and diverse biological actions. The acylated ghrelin is the only known biologically active form of ghrelin while the majority of circulating ghrelin is des-acylated ghrelin with no identified function. The aims of the present study were to measure active and total ghrelin in dairy cow’s milk and plasma during early lactation, and to observe changes in the ghrelin concentrations over time. Fifteen Holstein dairy cows were selected randomly from different lactations. Milk and blood samples were taken daily from cows at early lactation (n=10), from day 1 to day 10 in milk, and from cows in mid-lactation (n=5), averaging 227 DIM, for one day. An ELISA assay was
used to measure active and total ghrelin in milk and plasma samples. Other measurements such as milk fat, lactose, protein, SCC, MUN, and milk yield were recorded. Active and total milk ghrelin concentrations were found to be significantly higher in the first day of lactation ($P \leq 0.05$). No significant difference was found in the percentage of active to total ghrelin in milk and blood, suggesting that this constant ratio can be used to estimate active or total ghrelin, if one of them is known, from the same sample. Plasma ghrelin concentrations were not significantly different across days, with a constant percentage of active to total ghrelin. No correlation was observed between milk and plasma active and total ghrelin. Presence of ghrelin in colostrum and milk in measurable amounts of both active and total form (acylated and des-acylated) suggests that it is a critical compound for the metabolic activity of newborn calves and functions transiently to regulate the activity of some physiological processes until the endocrine system of the new calves starts to function independently.
PUBLIC ABSTRACT

Ghrelin Concentrations in Milk and Plasma of Dairy Cows during Early Lactation

Sameer M. Alhojaily

Ghrelin is a hormone produced mainly by the cells lining the gastric mucosa. Ghrelin was first extracted from human and rat stomachs, and identified as an endogenous stimulator of growth hormone release. Ghrelin is synthesized and produced in several tissues, but the gastric mucosa remains the major source of circulating ghrelin. Besides growth hormone release, ghrelin stimulates appetite and plays some major roles in different organs. In several studies, ghrelin was described as a hormone with multiple functions and diverse biological actions. Ghrelin exists in two major forms, active ghrelin and inactive ghrelin, and only the active form binds to the receptor. The majority of total circulating ghrelin is inactive ghrelin with no identified function. The aims of the present study were to measure active and total ghrelin in dairy cow’s milk and plasma during early lactation, and to observe changes in the ghrelin concentrations over time. We are interested in this period of time since the milk during early lactation contains a variety of biologically active hormones that are vital for newborn calves. In this study, fifteen Holstein dairy cows were selected randomly from different lactations. Milk and blood samples were taken daily from cows at early lactation for 10 days, and from some cows in mid-lactation. A laboratory test was used to measure active and total ghrelin in milk and plasma samples. Supplementary measurements such as milk fat, lactose, protein, and milk yield were recorded. Active and total milk ghrelin concentrations were found to be
significantly higher in the first day of lactation during colostrum production. Interestingly, the percentage of active to total ghrelin in milk and blood was constant in all days tested, suggesting that this constant percentage can be used to estimate active or total ghrelin, if one of them is know, from the same sample. However, no correlation was observed between the percentage of milk ghrelin and plasma ghrelin or with other milk components. In conclusion, the presence of ghrelin in colostrum and milk in measurable amounts of both active and total form suggests that it is a critical compound for the metabolic activity of newborn calves and functions transiently to regulate the activity of some physiological processes until the endocrine system of the new calves starts to function independently.
ACKNOWLEDGMENTS

Hours, days, and years are units we use to measure time in our life. However, it is not just a matter of time counting. It is a matter of how much we progress and how much knowledge we acquire in our life time. I count my life by progress I achieve and knowledge I acquire. Doing my master’s degree was a great learning and a prominent change in the journey of knowledge. It was a major step counted for many steps in my life. I am taking this opportunity to express my profound gratitude to everyone who walked me through these steps. I want to thank my major professor, Dr. Allen Young, for his exemplary guidance, monitoring, and constant encouragement during my master’s program. The helpful tips and sincere guidance given by him time to time shall help me walk my first steps in the journey of science and knowledge on which I am about to embark. I want also to thank my committee members, Dr. Lee Rickords and Dr. S. Clay Isom. Moreover, I want to thank my lovely wife and sweet daughters. It was a challenge to be a graduate student and also a father and husband at the same time, but they inspired me to succeed and make the best use of my time. At the end I want to thank my country, Saudi Arabia, who gave me this scholarship and the great chance to continue my higher education in USA, which has the most prominent and leading educational and research institutes in the world.

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## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>Least squares means of concentrations in milk of total and active ghrelin (pg/ml) and percent of active for the first 10 days of lactation and mid-lactation.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>Least square means concentration in milk of active and total ghrelin (pg/ml), and percent active by morning and evening milking for the first 10 days of lactation and mid-lactation.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>24</td>
</tr>
<tr>
<td>Least square means of total and active plasma ghrelin concentrations (pg/ml) in the first 10 days of lactation and mid-lactation.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>28</td>
</tr>
<tr>
<td>The least square means per day of milk yield kg, fat %, protein %, lactose %, SCC (cells/ml; x 1000), and MUN (mg/dL).</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>29</td>
</tr>
<tr>
<td>Correlation coefficients between milk active and total ghrelin (pg/ml) and milk yield, fat%, protein%, lactose%, SCC (cells/ml; x 1000), and MUN (mg/dL).</td>
<td></td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total and active ghrelin concentrations (pg/ml) in milk between colostrum,</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>transitional milk, early lactation mature milk, and mid-lactation milk</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Total and active ghrelin concentrations (pg/ml) in plasma between colostrum,</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>transitional milk, early lactation, and mid-lactation</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Correlation between the percentage of active to total milk ghrelin and the</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>percentage of active to total plasma ghrelin</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Least square means of percent active to total ghrelin in milk and plasma</td>
<td>26</td>
</tr>
<tr>
<td>5</td>
<td>Correlation between active and total milk ghrelin</td>
<td>26</td>
</tr>
<tr>
<td>6</td>
<td>Correlation between active and total plasma ghrelin</td>
<td>27</td>
</tr>
<tr>
<td>7</td>
<td>Correlation between active plasma ghrelin and active milk ghrelin</td>
<td>27</td>
</tr>
<tr>
<td>8</td>
<td>Correlation between plasma total ghrelin and milk total ghrelin</td>
<td>28</td>
</tr>
</tbody>
</table>
INTRODUCTION

Milk is the main source of nutrition for the newborn calves and it contains a variety of biologically active nutrients and hormones that immediately benefit the newborn. Hormones in milk function to transiently regulate some of the physiological activity of some tissues until the neonate’s endocrine systems begin to function independently. Early life nutrition is a critical factor that plays an important role in long-term appetite control and it regulates feeding mechanisms in the central nervous system. The fetus, during gestation, is completely dependent on maternal nutrition and stimulating feeding behavior after birth is critical for survival. There are several factors of orexigenic effect essential in early nutrition that are secreted from the peripheral tissues. Among the known orexigenic factors, ghrelin hormone has been found to be the most powerful appetite stimulator.

Ghrelin is present in human milk in considerable amounts and its mRNA have been identified in the mammary gland tissue (Aydin et al., 2006; Kierson et al., 2006). Ghrelin is a 28 amino acid octanoylated peptide hormone and known to be the only naturally occurring peptide with post translation modification by ser3 O-octanoylation (Kojima and Kangawa, 2010). This modification is essential for ghrelin activity and subsequently, activation of its specific receptor (Date et al., 2000a). Ghrelin binds to growth hormone secretagogue receptor 1a (GHS-R1a) which is expressed extensively in the brain and hypothalamus (Kojima et al., 1999; Kojima and Kangawa, 2005). Ghrelin was first isolated from the rat and human stomach mucosa (Kojima et al., 1999) and it has been identified in several tissues (Gnanapavan et al., 2002; Korbonits et al., 2004), but the gastric mucosa remains the major source of circulating ghrelin. Ghrelin circulates in
the blood in two major forms, acylated (active form) and des-acylated form (Soares and Leite-Moreira, 2008).

Ghrelin was discovered as an endogenous ligand for GHS-R1a and was first identified to stimulate the release of growth hormone, in both in vivo and in vitro (Kojima et al., 1999; Kojima et al., 2001). Further studies later revealed that ghrelin is a hormone with multifunctions and is involved in the regulation of food intake behavior, energy homeostasis, tissue growth and development, reproduction and gastrointestinal functions (Korbonits et al., 2004; Kojima and Kangawa, 2010).

Since ghrelin has an appetite stimulating effect and growth hormone releasing activity, it can potentially moderate the effect of anorexia in newborn calves, increase body weight gain, and potentially improve the resistance to pathological and environmental challenges during early life. Body weight and early life health condition is the best predictor of productive and reproductive performance in the future. In the case of ghrelin hormone, it has been reported that there is a positive relation between the ghrelin concentration in the serum of breast fed infants and its concentration in the breast milk in human (Ilcol and Hizli, 2007). Also, administration of ghrelin, via the stomach tube in piglets, resulted in significant changes in the small intestinal morphometry and mucosal development (Slupecka et al., 2012).

To our knowledge, the effect of ghrelin on calves has not yet been studied and the amount of ghrelin in bovine milk has not been identified in a detailed study. Savino et al. (2011) measured ghrelin level of non-pasteurized cow’s milk to compare with breast milk and some milk formula. The total ghrelin in the cow’s milk was reported to be 2.82 ng/ml. Several studies have identified ghrelin plasma level in cattle (Jennings et al., 2011;
Borner et al., 2013; Field et al., 2013; Ozturk et al., 2013), but none of these have determined the relation between milk ghrelin and plasma ghrelin, or the ratio between acylated (active ghrelin) and des-acylated ghrelin in cattle.

The primary aim of the present study was to investigate the level of ghrelin concentrations in colostrum, transitional milk, early lactation mature milk, and mid-lactation milk and plasma. The colostrum and milk of the first week are particularly important in regard to the development of newborn calves and the function of the gastrointestinal tract. The secondary aim was to determine the percentage of active to total ghrelin in milk and plasma.
LITERATURE REVIEW

Discovery of Ghrelin

The discovery of ghrelin was a surprising consequence of scientists’ studying growth hormone and its releasing bioregulators. That was driven by the desire to find a growth hormone releasing compound that could cure disorders associated with growth hormone deficiency (Aimaretti et al., 2002). The goal was to find either an exogenous or endogenous bioregulator to work on anterior pituitary somatotrope cells to release growth hormone.

Several scientists conducted studies in opioid peptides and found that one of the opioid derivatives (enkephalin) was the endogenous ligand for morphine receptor; therefore several opioid peptides derivatives were synthesized in order to find a more potent and less addictive drug to soothe pain (Kojima et al., 2001). In 1976, Bowers et al. found a modified opioid peptide that had a growth hormone releasing activity, but the effect was minor. Even though the effect was minimal, it triggered a search for synthetic compounds that might mimic growth hormone releasing hormone (GHRH) (Bowers, 2012). Since then, many studies have been conducted to develop and improve a potent synthetic growth hormone releasing compound and several peptides had been developed to stimulate the release of growth hormone from cultured pituitary cells, but their effect was minimal. However, some peptides were found to be more potent such as hexapeptide (GHRP-6), which is one of several synthetic met-enkephalin analogs (Bowers, 2012). This peptide stimulated the releasing of growth hormone in vivo and in vitro in all cultured cells and all species of animals tested. As a result, it became the standard reference compound, even though the developing GHRP-6 did not attract the attention of
the scientific community for further research. This was because researches were focused on studying the role of growth hormone releasing hormone from the hypothalamus to control the release of growth hormone. GHRP-6 was thought to stimulate growth hormone release in a mechanism similar to GHRH, but could not be verified without experimental comparison between them. Comparative analysis of the mechanism of action of GHRH and GHRP-6, demonstrated two different mechanisms, through two different receptors, and two intracellular signaling pathways (Howard et al., 1996).

GHRP-6 and other growth hormone stimulating compounds are known as growth hormone secretagogues (secretagogue is a substance that causes another substance to be secreted) and since they differ in their chemical structure, they are divided into two groups, peptidergic and non-peptidergic. They are known to stimulate growth hormone release in a way distinct from GHRH pathway and bind to a different receptor, known as an orphan receptor (Kojima et al., 2001). The term orphan receptor is a strategy to name any newly identified receptor until identifying its natural or endogenous ligands. MK-0677, a synthetic non-peptide molecule, binds with high affinity to the membrane of cultured cells from rat pituitary gland. It was then purified and the receptor has been identified, which become known as growth hormone secretagogues receptor (GHS-R) (Pong et al., 1996). Since then many researchers have used GHS-R expressing cells to find novel ligands. Several novel bioactive peptides have been used such as nociception, hypocretin, and prolactin-releasing peptides (Bowers, 2012). Because GHS-R was known to be expressed in the pituitary and hypothalamus, experiential studies focused on cell extracts from brain tissue to find an endogenous ligand for GHSR. However, nothing of significance was found.
After screening several tissues other than the brain, Kojima et al. (1999) found very strong activity from stomach tissue extracts that stimulated growth hormone release through GHS-R. After further purification, using reverse-phase high performance liquid chromatography (RPHPLC), the active peptide and the novel endogenous ligand to GHS-R was identified (Kojima et al., 1999; Kojima et al., 2001). They used the term ghrelin, which ghre is derived from the root of the word “grow” in the Proto-Indo-European language (Bowers, 2012).

**Ghrelin Gene and Biochemistry**

Ghrelin is a 28 amino acid octanoylated peptide hormone and is the only naturally occurring peptide with post translation modification by ser3 O-octanoylation (Kojima and Kangawa, 2010). This modification is essential for ghrelin activity and subsequently, activation of its specific receptor. Preproghrelin is a polypeptide precursor for ghrelin and in human is encoded by a single-copy gene (GHRL) that is located on the short arm of chromosome 3 (3p25-26) and organized into four coding exons and three introns (Wajnrajch et al., 2000). Preproghrelin gene codes for a 117 amino acid long peptide which includes a 23 amino acid signal peptide, 28 amino acid mature ghrelin, and a 66 amino acid tail (Bednarek et al., 2000). Ghrelin gene also codes for another bioactive molecule besides ghrelin, obestatin, which is synthesized through alternative splicing or from extensive post-translational modifications. Even though, ghrelin and obestatin are obtained from the same gene, they have opposing effects (Zhang et al., 2005). The sequence of ghrelin amino acid is highly conserved between different species with difference in two amino acid residues between human and rat (Kojima et al., 1999).
Ghrelin has been identified in many species such as human, mouse, swine, frog, fish, and chicken. In general, the chemical structure is similar among species and more important, the active part of ghrelin peptide at the serine residue is conserved between fish and mammals. Interestingly, chicken ghrelin has an opposite effect on feed intake; it reduced food intake (Saito et al., 2002)

**Ghrelin Tissue Distribution**

Ghrelin was first isolated from the stomach and then from different types of endocrine cells in other tissues. Ghrelin cells in the stomach are round or ovoid cells close to the capillaries. Ghrelin is found in the oxyntic gland in the stomach (fundus region which is the acid secreting part of the stomach). In a study conducted on rats, the concentration of ghrelin decreased by 80% in the circulation after surgical removal of the acid producing part of the stomach suggesting that the oxyntic mucosa is the major source of ghrelin (Dornonville de la Cour et al., 2001). A reduction in circulating ghrelin was also seen in a patient following gastrectomy (Leonetti et al., 2003).

Some of ghrelin secreting cells are found in the small and large intestine and produce about 30% of circulating ghrelin, although the majority originate from the stomach (Ariyasu et al., 2001; Krsek et al., 2002; Krsek et al., 2003). There are two types of ghrelin cells in the lower gastrointestinal tract. One type with no contact with the lumen, similar to those in the stomach, and the other one has elongated cells and is open to the lumen (Sakata et al., 2002).

Expression of ghrelin at the mRNA or protein level, or both has also been identified in several tissues other than gastrointestinal tract. The hypothalamic nuclei and their axon terminals have been shown to express ghrelin peptide (Kojima et al., 1999;
Korbonits et al., 2001a; Cowley et al., 2003; Toshinai et al., 2003). Ghrelin expression has been shown in the pituitary (Korbonits et al., 2001a), kidney (Mori et al., 2000), placenta (Gualillo et al., 2001), immune cells (Hattori et al., 2001), the ovary (Caminos et al., 2003; Gaytan et al., 2003), testis (Barreiro et al., 2002), lung (Volante et al., 2002), and in the pancreas (Date et al., 2002). Ghrelin mRNA expression was also identified in tumor tissues at different levels, suggesting that it might play a role in tumor cells development (Korbonits et al., 2001b; Gnanapavan et al., 2002; Iwakura et al., 2002).

**Ghrelin Receptor**

Ghrelin binds to growth hormone secretagogue receptor (GHS-R). There are two different forms of the ghrelin receptor GHS-R–GHS-R1a and GHS-R1b (Kojima et al., 1999; Petersenn et al., 2001). Ghrelin binds only to GHS-R1a which is a G protein-coupled receptor, with seven transmembrane domains (Kojima et al., 1999) while GHS-R1b is a 289 amino acid, five-transmembrane domain, truncated receptor that is unable to bind ghrelin (Howard et al., 1996). GHS-R1a is described as the functional ghrelin receptor and mostly expressed in hypothalamic cells (Zigman et al., 2006; Lopez et al., 2008). GHS-R1a expression is also shown in the pituitary gland and outside the CNS in the thyroid, heart, lungs, intestines, pancreas, adrenal gland, testicles and ovaries (van der Lely et al., 2004). In humans the GHS-R amino acid sequence was found to be about 52% identical to the G protein-coupled receptor for motilin (GPR38) (Feighner et al., 1999). Ghrelin shares some similarity with motilin, both are synthesized in the upper gastrointestinal tract, have growth hormone releasing activity and stimulate gastric motility (Samson et al., 1984; Masuda et al., 2000).
In order for ghrelin to bind to its functional receptor, ghrelin must be post-translationally modified by a specific enzyme, ghrelin O-acyltransferase (GOAT). GOAT facilitates the modification by adding an octanoyl group to the serine residue which is the third amino acid of ghrelin (Gutierrez et al., 2008; Yang et al., 2008). Therefore, there are two forms of ghrelin; octanoylated form (active form) and non-octanoylated form. Non-octanoylated, also called des-acyl ghrelin, circulates in the plasma at higher levels than octanoylated ghrelin (Hosoda et al., 2000b) and has effects other than growth hormone releasing activity because it is unable to bind to GHS-R1a receptor. It has been hypothesized that des-acyl ghrelin has other functions and has an alternative ghrelin receptor that mediates the actions of des-ghrelin (Bang et al., 2007; Beasley et al., 2009).

**Regulation of Ghrelin**

Regulation of ghrelin and its effects happen at different levels. The body regulates ghrelin by modifying the secretion rate from its cells in the stomach, the transcription and translation of the ghrelin gene, expression of the ghrelin receptors and intracellular signaling (van der Lely et al., 2004). The level of ghrelin is not stable in the circulation and fluctuates during the day; being high after fasting and low after food intake. Ghrelin level in the circulation is also affected by the time of the day and reaches its highest level during the night, then declines later in the day (Cummings et al., 2002; Chan et al., 2004). Fasting is the most effective regulator of ghrelin. The increased pulse frequency and pulse amplitude during fasting, in addition to low leptin level in the circulation, create a powerful orexigenic effect and stimulate gastric activity to increase ghrelin synthesis and secretion (Bagnasco et al., 2002). In cows, the level of ghrelin concentration was significantly decreased by about one hour after feeding, and then recovered to the
previous level before feeding (Hayashida et al., 2001). Accordingly, ghrelin level is generally high before food intake and during the night then declines immediately after food intake and during the day. A number of studies have shown that restricting nutrient intake by decreasing DMI of a high-energy diet results in increased plasma ghrelin concentrations (Tschop et al., 2000; Monteleone et al., 2003). In steers, ingredient composition and quantity of DMI did not influence plasma ghrelin concentrations as long as positive energy balance was maintained (Wertz-Lutz et al., 2007).

Physical expansion of the stomach is not the trigger of ghrelin release. In a study in rats, the ghrelin levels decreased when the stomach was filled with 50% glucose solution. On the other hand, no changes were seen when the same volume of water was used to fill the stomach to cause physical expansion (Tschop et al., 2000). Gastric emptying and intestinal absorption of nutrients, especially glucose, are essential to cause a decline in circulating ghrelin (Williams et al., 2003) and suggests an involvement of a post gastric factor.

In human subjects, a significant drop in ghrelin level was seen after eating a diet high in carbohydrates, but no similar effect occur after eating a diet high in fat (Monteleone et al., 2003). The effect of carbohydrates and glucose on ghrelin level may not be direct and could be mediated by other factors, such as insulin. Intravenous infusion of insulin showed an inhibitory effect on ghrelin level in rats (McCowen et al., 2002). A study tested the effect of insulin on ghrelin suppression in human by using different doses and found a positive correlation between the insulin doses and reduction in plasma ghrelin (Anderwald et al., 2003).
Leptin and ghrelin are major hormones involved in the regulation of hunger and satiety. Ghrelin stimulates hunger, while leptin causes satiety. Leptin may also play a role in ghrelin regulation through positive or negative feedback loops and are either direct or indirect. In an experiment with mice, administration of leptin stimulated expression of mRNA of ghrelin in the stomach cells (Toshinai et al., 2001). In contrast, administration of leptin to six patients did not regulate ghrelin over several hours to a few days, suggesting that leptin does not regulate ghrelin levels independently (Chan et al., 2004). Growth hormone has been suggested to have an inhibitory effect on ghrelin level. Administration of a large dose of GH to normal rats resulted in a 50% decrease of ghrelin levels in the plasma (Tschop et al., 2002).

**Physiological Function of Ghrelin**

Ghrelin is a hormone with multiple functions. It is mainly secreted from the stomach mucosa and has paracrine and endocrine effects. It is well known as a peptide with growth hormone releasing activity and appetite inducing factor. In addition to that, the biological functions of ghrelin are much more diverse. It has effects on several systems such as the gastrointestinal tract, energy homeostasis and glucose release, reproduction, enzyme release, cell proliferation and apoptosis, and other functions.

The role of ghrelin in food intake is mediated through the hypothalamus and different pathways that have been suggested to explain how ghrelin induces appetite. The primary pathway is that ghrelin is released into the blood from the stomach, crosses the blood brain barrier, and binds to its receptors in the hypothalamus (Banks et al., 2002). Ghrelin also may reach the brain through the vagal nerve and stimulate the appetite center (Ueno et al., 2005). In addition, ghrelin has a paracrine effect where it is produced locally...
in the hypothalamus and directly affects some appetite center (Cowley et al., 2003). Ghrelin stimulates the activity of neurons expressing NPY, a neuropeptide that acts as a neurotransmitter in the brain, and increases food intake and storage of energy (Kamegai et al., 2001).

Ghrelin plays a significant function in stimulating growth hormone from the hypothalamus. Several studies have shown the in vitro and in vivo GH-releasing activity of ghrelin in rats, pigs and humans (Date et al., 2000b; Hataya et al., 2001; Hashizume et al., 2003). The GH-releasing effect of ghrelin depends on the existence of GHSR-1a which has a different mechanism of action to release growth hormone from somatotrophic cells in the anterior pituitary gland than that of GHRH-R. Ghrelin acts on the GHS-R1a and causes increased intracellular calcium, which stimulates GH release. In some studies, the GH-releasing activity of ghrelin was comparable to that of GHRH when injected intravenously into rats (Kojima et al., 1999; Arvat et al., 2000; Takaya et al., 2000). Furthermore, ghrelin showed three times more stimulation of GH-release than that of GHRH (Arvat et al., 2000). Stimulation of GH release by ghrelin was positively correlated with dose administered both in vitro and in vivo (Peino et al., 2000). Administration of ghrelin intracerebroventricularly increased plasma GH concentration in rats in a dose-dependent manner (Date et al., 2000b). Accordingly, intracerebroventricular injection of ghrelin seems to be a more potent route than intravenous administration. Administration of ghrelin and GHRH showed a synergistic effect on GH secretion that resulted in more GH release than either GHRH or ghrelin alone (Arvat et al., 2001; Hataya et al., 2001).
In the gastrointestinal tract, ghrelin stimulates gastrointestinal motility, accelerates gastric emptying, and regulates gastric acid secretion (Depoortere et al., 2005; Kitazawa et al., 2005; Ariga et al., 2007). Administration of ghrelin, via the stomach tube in piglets, resulted in significant changes in small intestine morphometry and mucosal development and remodeling. These changes were dependent on the dosage of ghrelin administered (Slupecka et al., 2012).

In the cardiovascular system ghrelin was observed to have treatment-potential for severe chronic heart failure (CHF) and cardiac cachexia (Akashi et al., 2009). Also it has been found that early treatment with ghrelin prevented the increase in cardiac sympathetic nerve activity (CSNA) after acute myocardial infarction and improved cardiac function in rats (Schwenke et al., 2008). Ghrelin also showed increased contractile effect on guinea pig papillary muscle, renal artery, and vascular smooth muscle (Mladenov et al., 2006; Dimitrova et al., 2007). Involvement of ghrelin in cardiovascular system homeostasis suggests that ghrelin can be considered as a possible therapeutic target for many pathological conditions associated with cardiovascular damage and remodeling (Isgaard, 2013).

Ghrelin is involved in cell proliferation and apoptosis through different pathways and may function as a protective factor against cells damage. Ghrelin promoted endothelial cells proliferation (Rossi et al., 2008) and inhibited apoptosis of cardiomyocytes and endothelial cells in vitro (Baldanzi et al., 2002). In porcine ovarian granulosa cells, ghrelin reduced apoptosis-related substance-MAP3K5 accumulation and promoted the cell proliferation (Sirotkin et al., 2008). Ghrelin is also involved in the release of some enzymes and has been shown to stimulate amylase release from acinar
cells in rats (Nawrot-Porabka et al., 2007) and increases aromatase activity in porcine follicles after treatment with ghrelin (Rak and Gregoraszczuk, 2008).

Several studies showed that ghrelin gene is expressed in human (Gaytan et al., 2004) and mouse testis (Tanaka et al., 2001), and rat Leydig cells and ovary (Tena-Sempere et al., 2002; Caminos et al., 2003). Also the ghrelin receptor, GHS-R1a, was found in human (Gaytan et al., 2003; Gaytan et al., 2004) and rat (Tena-Sempere et al., 2002; Barreiro et al., 2003) testis tissue. Administration of ghrelin in rodents appeared to inhibit luteinizing hormone (LH) (Fernandez-Fernandez et al., 2004; Martini et al., 2006; Harrison et al., 2008) and gonadotropin-releasing hormone (GnRH) secretion in vivo (Fernandez-Fernandez et al., 2005), while follicle-stimulating hormone (FSH) remained unaffected (Fernandez-Fernandez et al., 2007). Treating pituitary cells in vitro with ghrelin stimulated LH and FSH release (Fernandez-Fernandez et al., 2005; Fernandez-Fernandez et al., 2007). Treatment with ghrelin increased the secretion of prostaglandin F (PGF), and E (PGE) in porcine granulosa cells culture (Sirotkin et al., 2008). Also, ghrelin induced estradiol secretion, cell proliferation and decreases caspase-3 activity in cultured whole porcine follicles (Rak et al., 2009).

**Ghrelin in Milk**

Milk is the main source of nutrition for the newborn calves and it contains a variety of biologically active nutrients and hormones that provide immediate benefit. Ghrelin has been identified in the human breast milk and its mRNA has been found in several human tissues including mammary glands (Aydin et al., 2006; Kierson et al., 2006). Even though the ghrelin hormone is produced and secreted by the mammary gland, it has been suggested it comes mainly from the plasma although, in a lower
amount (Aydin et al., 2006). Another study showed that the level of ghrelin in milk is higher than the plasma (Kierson et al., 2006). The active form of ghrelin hormone also has been identified in the human milk and found to be positively correlated to the serum ghrelin of breast fed infants (Ilcol and Hizli, 2007). No literature has been found regarding the level of ghrelin in cow’s milk. However, there is one indirect reference about total ghrelin in the cow milk about 2.82 ng/ml (Savino et al., 2011). In other animals, ghrelin has been measured in the colostrum and milk of the sow and found to be 6.73 ng/mL and 0.242 ng/mL, respectively (Woliński et al., 2013).

**Acylated and des-acylated ghrelin**

Ghrelin hormone exists in the stomach, hypothalamus and circulation as two major endogenous forms, a form acylated at serine 3 (active ghrelin) and a des-acylated form (des-acyl ghrelin). The acylated form is the active form since acylation is necessary for the binding of ghrelin to the GHS-R1a (Hosoda et al., 2000b). The des-acylated form of ghrelin exists at a higher level in blood than that of the active from (Soares and Leite-Moreira, 2008). Des-acyl ghrelin is the precursor form of active ghrelin. Activation of ghrelin is by a post-translational modification catalyzed by GOAT. It is a polytopic membrane-bound enzyme that attaches octanoate to serine-3 of ghrelin (Yang et al., 2008) and is highly expressed in the stomach, the main source of ghrelin (Gutierrez et al., 2008). Since the majority of circulating ghrelin is des-acylated, not all newly synthesized ghrelin is modified by GOAT. It has been suggested that GOAT modifies most of ghrelin synthesized and des-acylation happens later in the circulation by plasma esterase. Ghrelin is transported in the plasma by binding to high-density lipoproteins (HDLs) that contain a plasma esterase, paraoxonase and clusterin (Beaumont et al., 2003). These enzymes break
the fatty acid chain that is attached to the Ser3 of ghrelin. Therefore, des-acyl ghrelin may exist as either a pre form of non-modified ghrelin or the product of its deacylation by plasma enzymes (Kojima and Kangawa, 2005).

Des-acyl ghrelin does not displace active ghrelin at the binding sites of GHS-R1a in the hypothalamus and pituitary and shows no GH-releasing or other endocrine activities in rats (Hosoda et al., 2000a). Des-acyl ghrelin does not appear to possess any endocrine activities which raises the question as to if there is a specific receptor for des-acyl ghrelin and whether des-acyl ghrelin has specific functions different from that of acyl-modified ghrelin (Kojima and Kangawa, 2005). It has been reported that des-acyl ghrelin shares with active acylated ghrelin some non-endocrine activities, including the modulation of cell proliferation and some adipogenesis effect (Cassoni et al., 2004). These two forms show some dissimilarity and one of the main differences between these two forms is that only the des-acylated form can pass the blood brain barrier (Banks et al., 2002). Several studies suggested that des-acyl ghrelin and acylated ghrelin had the inverse effects on gastric emptying and small intestinal transit and motility through different receptors (Fujino et al., 2003; Chen et al., 2005; Fujimiya et al., 2008). Administration of ghrelin or des-acyl ghrelin increased plasma glucose and were involved in glucose metabolism in humans and rats (Miljic et al., 2007; Broglio et al., 2008; Vestergaard et al., 2008). However, des-acyl ghrelin counteracted the stimulatory effect of acylated ghrelin on glucose release (Gauna et al., 2005). These findings suggest that these two forms of ghrelin are likely to play opposite roles in glucose metabolism. In general, both forms are present in various tissues and seem to have major physiological role. The function of the acylated form (active form) has been studied intensively, and
several of its biological roles have been identified since it has a known receptor. The biological function of the des-acylated form remains unclear although it circulates in the body in greater amounts than acylated ghrelin.

The presence of ghrelin in milk suggests that it is a critical compound for the metabolic activity of newborn calves and functions transiently to regulate the activity of some physiological processes until the endocrine system of the new calves start to function independently.

To our knowledge, the effect of ghrelin on calves has not yet been studied and the amount of ghrelin in bovine milk has not been identified in a detailed study. Savino et al. (2011) measured ghrelin level of non-pasteurized cow’s milk to compare with breast milk and some milk formula. The total ghrelin in the cow’s milk was reported to be 2.82 ng/ml. Several studies have identified ghrelin plasma level in cattle (Jennings et al., 2011; Borner et al., 2013; Field et al., 2013; Ozturk et al., 2013), but none of these have determined the relation between milk ghrelin and plasma ghrelin, or the ratio between acylated (active ghrelin) and des-acylated ghrelin in cattle.

The primary aim of the present study was to investigate the level of ghrelin concentrations in colostrum, transitional milk, early lactation mature milk, and mid-lactation milk and plasma. The colostrum and milk of the first week are particularly important in regard to the development of newborn calves and the function of the gastrointestinal tract. The secondary aim was to determine the percentage of active to total ghrelin in milk and plasma.
MATERIALS AND METHODS

This experiment was conducted at the Utah State University Caine Dairy Research and Teaching facility. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Utah State University.

Fifteen Holstein cows were randomly selected and divided into 2 groups. The first group were cows (n=10) from calving to 10 DIM, and the second group were cows (n=5) from mid-lactation (average DIM = 227). Milk samples were collected twice daily (from AM milking and PM milking) and plasma samples were collected once daily after PM milking. Collection started immediately after calving which was day 1 and stopped after the PM milking of the day 10. Milk and plasma samples were collected twice for one day from group two.

Milk and blood samples were grouped into 4 groups: colostrum (samples from the first two days), transitional milk (day 3 to day 5), early lactation mature milk (day 6 to day 10), and mid-lactation.

Samples Collection and Storage

Blood samples were drawn into evacuated glass tubes containing EDTA and taken to the lab. Plasma was immediately harvested via centrifugation at 2,300 x g for 15 min at 5 ± 2 °C. Collected plasma samples were frozen and stored at -80°C until further analysis.

Milk samples were collected into 100 ml containers and kept refrigerated until taken to the lab. From each milk sample, about 50 ml was transferred into another container containing milk preservative and analyzed for lactose %, protein %, fat %, milk
urea nitrogen (MUN), and somatic cell count (SCC) (Rocky Mountain DHIA lab, Providence, UT). About 10 ml of the remaining milk was transferred into a plastic tube and centrifuged at 4000 x g for 15 min at 5 ± 2 °C. After centrifugation, the fat layer was removed and milk was transferred into another clean tube and stored frozen at -80°C until further analysis.

**Ghrelin Analysis**

Plasma and milk ghrelin concentrations were measured by using two commercially available ELISA kits for active and total forms of ghrelin (Millipore Corp; Billerica, MA, USA). The kit was for rat ghrelin and has been used and validated to detect bovine ghrelin in other studies (Miura et al., 2004; Field et al., 2013). All samples were immediately treated after thawing with 1 mg of AEBSF, a protease inhibitor, for each ml of sample and acidified with HCL to a final concentration of 0.05 N following manufacturer recommendations. AEBSF was used to inhibit the detrimental effect of protease and acid was added to preserve the active side chain of the ghrelin. Extensive testing in our laboratory showed that both are critical in determining ghrelin in milk and plasma. Milk samples were centrifuged again at 3000 x g for 15 after acidification and the liquid phase was used for ELISA test.

Active and total ghrelin ELISA plate were run simultaneously. A standard curve was constructed for each form of ghrelin and two quality control standards were included with each plate. The assay was considered acceptable if both quality control values fell within the range determined by the manufacturer.

An ELISA plate reader was used to read the absorbance of the color change at 450 nm and 590 nm within 5 min of adding ELISA stop solution. Microsoft Excel was
used to construct a graph reference from the measured absorbance on the Y-axis against the concentrations of the known ghrelin standard on the X-axis. The concentration of the unknown samples was calculated by comparing their absorbance to the standards.

**Statistical Analysis**

Data were analyzed using the Proc Corr, Proc Reg, and Proc Mixed procedures of SAS, version 9.3 (SAS, Institute Inc., Cary, NC). Each cow was considered as the experimental unit and analyzed as a repeated measure design over time (DIM). Least squares means were computed and difference between means determined using the Tukey-Kramer multiple comparisons test. Significant difference were determined as $P < 0.05$ and trends defined as $P < 0.1$. 
RESULTS AND DISCUSSION

This study measured the concentrations of active and total ghrelin in colostrum, milk, and plasma during the first 10 days of lactation and at mid-lactation. Ghrelin concentrations on the first day of lactation were significantly higher ($P \leq 0.05$) than other days (Table 1). Concentrations of milk ghrelin on the other days were not statistically different due to the large variations. There were fluctuations in milk ghrelin concentrations between morning and evening milking (Table 2), but no statistical significance was found or clear pattern was demonstrated (Figure 1). This changes in ghrelin concentrations during the day in milk might be influenced by other factors as DMI or ration composition. However, in this experiment all fresh cows were fed the same ration and kept under the same management condition, but feed intake was not calculated.

Table 1. Least squares means of concentrations in milk of total and active ghrelin (pg/ml) and percent of active for the first 10 days of lactation and mid-lactation.

<table>
<thead>
<tr>
<th>Day</th>
<th>Active ghrelin</th>
<th>SEM</th>
<th>Total ghrelin</th>
<th>SEM</th>
<th>Percent active</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>158.8$^a$</td>
<td>13.79</td>
<td>984.0$^a$</td>
<td>97.68</td>
<td>16.6</td>
</tr>
<tr>
<td>2</td>
<td>91.2</td>
<td>13.28</td>
<td>593.0</td>
<td>94.11</td>
<td>15.3</td>
</tr>
<tr>
<td>3</td>
<td>87.6</td>
<td>13.52</td>
<td>514.5</td>
<td>95.72</td>
<td>17.5</td>
</tr>
<tr>
<td>4</td>
<td>66.0</td>
<td>13.79</td>
<td>400.0</td>
<td>97.69</td>
<td>17.2</td>
</tr>
<tr>
<td>5</td>
<td>108.0</td>
<td>13.52</td>
<td>735.3</td>
<td>95.72</td>
<td>16.4</td>
</tr>
<tr>
<td>6</td>
<td>61.5</td>
<td>13.52</td>
<td>467.2</td>
<td>95.72</td>
<td>17.3</td>
</tr>
<tr>
<td>7</td>
<td>75.1</td>
<td>13.52</td>
<td>534.4</td>
<td>95.72</td>
<td>15.2</td>
</tr>
<tr>
<td>8</td>
<td>67.5</td>
<td>13.52</td>
<td>390.4</td>
<td>95.72</td>
<td>19.2</td>
</tr>
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<td>13.52</td>
<td>768.4</td>
<td>95.72</td>
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<td>13.52</td>
<td>455.6</td>
<td>95.72</td>
<td>19.5</td>
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<td>72.1</td>
<td>18.13</td>
<td>528.1</td>
<td>128.42</td>
<td>13.6</td>
</tr>
</tbody>
</table>

$^a$ Superscript indicates significant difference within column ($P < 0.05$)
Table 2. Least square means concentration in milk of active and total ghrelin (pg/ml), and percent active by morning and evening milking for the first 10 days of lactation and mid-lactation.

<table>
<thead>
<tr>
<th>Days in milk</th>
<th>Total ghrelin AM</th>
<th>SEM</th>
<th>Active ghrelin AM</th>
<th>SEM</th>
<th>Percent active AM</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>765.8</td>
<td>165.79</td>
<td>125.8</td>
<td>184.3</td>
<td>23.41</td>
<td>17.0</td>
</tr>
<tr>
<td>2</td>
<td>598.8</td>
<td>165.79</td>
<td>94.1</td>
<td>90.5</td>
<td>23.41</td>
<td>15.3</td>
</tr>
<tr>
<td>3</td>
<td>564.5</td>
<td>165.79</td>
<td>91.4</td>
<td>83.7</td>
<td>23.41</td>
<td>17.7</td>
</tr>
<tr>
<td>4</td>
<td>425.8</td>
<td>165.79</td>
<td>65.7</td>
<td>65.9</td>
<td>23.41</td>
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</tr>
<tr>
<td>5</td>
<td>748.8</td>
<td>165.79</td>
<td>114.8</td>
<td>101.2</td>
<td>23.41</td>
<td>16.0</td>
</tr>
<tr>
<td>6</td>
<td>316.2</td>
<td>165.79</td>
<td>50.3</td>
<td>72.6</td>
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<td>19.0</td>
</tr>
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<td>7</td>
<td>388.4</td>
<td>165.79</td>
<td>64.7</td>
<td>85.6</td>
<td>23.41</td>
<td>15.8</td>
</tr>
<tr>
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<td>165.79</td>
<td>54.8</td>
<td>80.1</td>
<td>23.41</td>
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</tr>
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<td>593.3</td>
<td>165.79</td>
<td>92.2</td>
<td>146.7</td>
<td>23.41</td>
<td>17.3</td>
</tr>
<tr>
<td>10</td>
<td>460.5</td>
<td>165.79</td>
<td>83.3</td>
<td>59.8</td>
<td>23.41</td>
<td>19.0</td>
</tr>
<tr>
<td>227</td>
<td>479.7</td>
<td>222.42</td>
<td>67.3</td>
<td>76.9</td>
<td>31.41</td>
<td>14.6</td>
</tr>
</tbody>
</table>

Figure 1. Total and active ghrelin concentrations (pg/ml) in milk between colostrum, transitional milk, early lactation mature milk, and mid-lactation milk.
One of the objectives of this study was to quantify the concentration of ghrelin in dairy cow’s milk. Based on our findings, concentrations of total ghrelin seem to fall in a range of 0.316 - 1.150 ng/ml. This appears to be lower than what was listed in the Savino et al. (2011) study where the total ghrelin was measured from the milk of 5 different cows to be compared with human breast milk ghrelin. In that study, total ghrelin averaged was 2.82 ± 0.219 ng/ml; although no other details were provided about the lactation status of those cows.

In our study, active and total ghrelin were measured to determine if there is a correlation between them. No significant differences were found in the relationship between active and total ghrelin, determined as a percentage (Table 1), across the first ten days of lactation as well as mid-lactation. This finding, suggests that there is a predictable ratio between active and total ghrelin in milk. The active milk ghrelin was about 17 ± 3 % of total ghrelin. Such a ratio might help in determining the rate of acylation of inactive ghrelin by ghrelin O-acyltransferase (GOAT) (Yang et al., 2008) in ghrelin producing tissues or the rate of des-acylation of active ghrelin by plasma esterase during circulation (Beaumont et al., 2003).

Milk was classified into four groups; colostrum, transitional milk, mature milk of early lactation, and mature milk of mid lactation (Figure 1). The concentrations of total and active ghrelin were higher in colostrum than other groups. This finding suggests that ghrelin in colostrum might play a role in regulating newborn calf’s endocrine function and stimulates appetite. No significant correlation was found between milk volume and the concentration of ghrelin.
The concentrations of active and total ghrelin were also measured in plasma (Table 3). The level of ghrelin of both active and total was higher in the first 2 days of lactation (during colostrum production), but was not significantly different (Figure 2). The slight elevation of ghrelin concentration might be due to appetite stimulation in the first few days of lactation.

**Table 3.** Least square means of total and active plasma ghrelin concentrations (pg/ml) in the first 10 days of lactation and mid-lactation.

<table>
<thead>
<tr>
<th>Day</th>
<th>Total ghrelin</th>
<th>SEM</th>
<th>Active ghrelin</th>
<th>SEM</th>
<th>Percent active</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>696.9</td>
<td>161.89</td>
<td>108.6</td>
<td>22.20</td>
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</tr>
<tr>
<td>2</td>
<td>921.4</td>
<td>161.89</td>
<td>140.6</td>
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</tr>
<tr>
<td>3</td>
<td>612.7</td>
<td>161.89</td>
<td>87.1</td>
<td>22.20</td>
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</tr>
<tr>
<td>4</td>
<td>695.5</td>
<td>161.89</td>
<td>107.5</td>
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</tr>
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<td>84.3</td>
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<td>16.5</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>mid</td>
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<td>217.20</td>
<td>70.3</td>
<td>29.79</td>
<td>14.2</td>
</tr>
</tbody>
</table>

The ratios between active and total plasma ghrelin (Table 3) were not significantly different between days. The percentage of active plasma ghrelin to total was about 15 ± 1.5 %. This conserved ratio could be used to calculate the concentration of total ghrelin if the active concentration is known and vice versa.

No correlation was found between plasma and milk active to total ghrelin percentage (Figure 3). This suggests that the percentage between active and total plasma ghrelin cannot be used to measure the relation between active and total milk ghrelin and vice
versa. As shown in Figure 4, there is a similar pattern between the percentage of active to total between blood and milk ghrelin, but may differ in early lactation milk.

**Figure 2.** Total and active ghrelin concentrations (pg/ml) in plasma between colostrum, transitional milk, early lactation, and mid-lactation.

**Figure 3.** Correlation between the percentage of active to total milk ghrelin and the percentage of active to total plasma ghrelin.
Figure 4. Least square means of percent active to total ghrelin in milk and plasma.

Scatter plot from SAS output shows the relationship between active and total ghrelin in milk and plasma. The correlation was positive ($R^2 = 0.898$) for milk (Figure 5) and plasma ($R^2 = 0.869$) (Figure 6). No significant correlation was found between milk active and plasma active ghrelin or between milk total and plasma total ghrelin (Figure 7) (Figure 8).

Figure 5. Correlation between active and total milk ghrelin.
Figure 6. Correlation between active and total plasma ghrelin.

Figure 7. Correlation between active plasma ghrelin and active milk ghrelin.
Figure 8. Correlation between plasma total ghrelin and milk total ghrelin.

Least square means for milk composition and yield per day are shown in Table 4. No significant correlations were found between active or total milk ghrelin and any milk composition parameter except milk protein percent (Table 5); which was significant ($P < 0.05$) and positive.

### Table 4. The least square means per day of milk yield kg, fat %, protein %, lactose %, SCC (cells/ml; x 1000), and MUN (mg/dL).

<table>
<thead>
<tr>
<th>DIM</th>
<th>Milk yield</th>
<th>Fat %</th>
<th>Protein %</th>
<th>Lactose %</th>
<th>SCC (cells/ml; x 1000)</th>
<th>MUN (mg/dL)</th>
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<td>4.57</td>
<td>498</td>
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</tbody>
</table>
Table 5. Correlation coefficients between milk active and total ghrelin (pg/ml) and milk yield, fat%, protein%, lactose%, SCC (cells/ml; x 1000), and MUN (mg/dL).

<table>
<thead>
<tr>
<th></th>
<th>Milk yield</th>
<th>Fat%</th>
<th>Protein%</th>
<th>Lactose%</th>
<th>SCC, cells/ml, x1000</th>
<th>MUN mg/dL</th>
<th>Total ghrelin</th>
<th>Active ghrelin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk yield</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat%</td>
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<td>-0.251</td>
<td></td>
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<td>-0.118</td>
<td>-0.055</td>
<td>-0.060</td>
</tr>
<tr>
<td>Protein%</td>
<td>-0.251</td>
<td>-0.145</td>
<td>-0.292</td>
<td>0.210</td>
<td>0.025</td>
<td>0.253</td>
<td>0.313</td>
<td></td>
</tr>
<tr>
<td>Lactose%</td>
<td>0.461</td>
<td>-0.292</td>
<td>-0.629</td>
<td>-0.631</td>
<td>0.021</td>
<td>-0.080</td>
<td>-0.075</td>
<td></td>
</tr>
<tr>
<td>SCC, cells/ml, x1000</td>
<td>-0.192</td>
<td>-0.075</td>
<td>0.210</td>
<td>-0.631</td>
<td>-0.194</td>
<td>-0.003</td>
<td>-0.031</td>
<td></td>
</tr>
<tr>
<td>MUN mg/dL</td>
<td>-0.118</td>
<td>0.025</td>
<td>-0.013</td>
<td>0.021</td>
<td>-0.194</td>
<td>-0.048</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td>Total ghrelin</td>
<td>-0.055</td>
<td>-0.085</td>
<td>0.253</td>
<td>-0.080</td>
<td>-0.003</td>
<td>-0.048</td>
<td>0.947</td>
<td></td>
</tr>
<tr>
<td>Active ghrelin</td>
<td>-0.060</td>
<td>-0.099</td>
<td>0.313</td>
<td>-0.075</td>
<td>-0.031</td>
<td>0.019</td>
<td>0.947</td>
<td></td>
</tr>
</tbody>
</table>

*Coefficients that are **bold** are significant at $P < 0.05$.

We saw no correlation between ghrelin and milk fat; however, Kierson et al. (2006) found a direct correlation between total milk ghrelin levels and estimated milk fat content ($r = 0.84$). No one has shown a correlation with milk protein percent. We do not know if this is a direct or indirect effect. Milk volume showed no correlation between active and total milk ghrelin (Table 5).

There was a noticeable variation in ghrelin concentration among cows in the first 10 days of lactation. Several factors such as body condition, health, feed intake, lactation number could be involved in controlling ghrelin concentration in plasma and milk. In this study, we focused on the time factor (day 1 to day 10 and mid-lactation) because all cows were generally similar in age, previous production, and same ration and housed under the same conditions. We did not account for DMI in our experiment which may explain some of this variation. Many dairy cows experience negative energy balance in early lactation due to decreased feed intake which may affect ghrelin concentration.
Regardless of other factors that may contribute to ghrelin concentration, in our experiment ghrelin concentration was significantly higher in colostrum. This was significant and probably plays a role in controlling the appetite of newborn calves and stimulates growth. Consuming ghrelin in colostrum can induce an orexenic effect through ghrelin receptors GHS-R1b that are distributed and highly expressed along the GIT. Oral administration of ghrelin has shown a significant effect on piglets’ growth and development of intestinal mucosa (Ślupecka et al., 2012). Calves at this stage of life are monogastrics and do not develop a rumen until later. No study has shown the effect of oral administration of ghrelin in ruminant newborns, but we assume it is similar to monogastrics. The physiological effect of ghrelin has shown no difference between monogastrics and ruminants.
CONCLUSION

Ghrelin is present in plasma and milk of dairy cows and milk ghrelin concentrations were higher in the first day of lactation in both active and total forms than days 2 to 10 of lactation. Colostrum had a higher concentration of ghrelin when compared with transitional or mature milk of early or mid-lactation. No significant differences was shown in ghrelin level between milk taken at morning and evening milking suggesting no diurnal pattern in milk ghrelin.

The ratio between active and total ghrelin were constant and correlated. Active ghrelin in milk was $17 \pm 3\%$ of total ghrelin. Active and total plasma ghrelin were also constant and correlated with each other. Active plasma ghrelin was $15 \pm 1.5\%$ of total plasma ghrelin. Such a ration can help in estimation of active or total ghrelin if either form is known.

Active and total plasma ghrelin concentrations were simultaneously measured with milk and no correlation was found between milk and plasma active or total ghrelin. This finding suggests that the concentrations of ghrelin in blood and milk are somewhat independent of each other. No relationship was observed between milk active and total ghrelin and milk yield and milk composition except milk protein percent. We conclude that milk ghrelin is found in large concentration in colostrum than other milk and probably is important to calf nutrition.
REFERENCES


Baldanzi, G., N. Filigheddu, S. Cutrupi, F. Catapano, S. Bonissoni, A. Fubini, D. Malan, G. Baj, R. Granata, F. Broglio, M. Papotti, N. Surico, F. Bussolino, J. Isgaard, R.


Sirotkin, A. V., A. Benco, A. Tandlmajerova, D. Vasicek, J. Kotwica, K. Darlak, and F. Valenzuela. 2008. Transcription factor p53 can regulate proliferation, apoptosis and


