Novel Concepts Regarding Calcium Homeostasis during the Transition Period

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Introduction

Adequate circulating calcium (**Ca**) concentrations throughout the transition period are necessary for a productive lactation, but large quantities of Ca are lost from maternal Ca pools into milk and colostrum. A rapid, substantial drop in maternal blood Ca causes 5-10% of cows to be afflicted with clinical hypocalcemia (**CH**) and an additional 50% to suffer from subclinical hypocalcemia (**SCH**). Subclinical hypocalcemia and CH are significant risk factors of early lactation culling/premature removal from the herd (DeGaris and Lean, 2008; Reinhardt et al., 2011; Roberts et al., 2012). Furthermore, SCH increases risks of developing ketosis; displaced abomasum; and metritis; SCH depresses immune function; prolongs the interval until pregnancy is achieved; decreases pregnancy rate; and reduces overall productivity (**Figure 1;** Kimura et al., 2006; Goff, 2008; DeGaris and Lean, 2008; Chapinal et al., 2011; Reinhardt et al., 2011; Chapinal et al., 2012; Martinez et al., 2012). During lactation, dietary Ca is not sufficient to maintain maternal Ca concentrations while supporting milk formation. Therefore, activation of maternal bone Ca mobilization during the dry period is critical for the prevention of post-partum SCH and CH. Using the estimates of Dr. Garrett Oetzel ($300 loss for each treatment of CH, and $125 loss for each treatment of SCH; Guard, 1996) the annual cost to the U.S. dairy industry, which has approximately 9,200,000 cows, is approximately $575,000,000 for SCH and approximately $325,000,000 for CH (NAHMS, 2007; Oetzel, 2013). SCH and CH are detrimental to animal health and welfare, and a formidable economic burden to U.S. farmers (Oetzel, 2013). Currently accepted practices for treatment and prevention of SCH and CH include oral Ca supplementation post-calving and anionic salt supplementation (DCAD) pre-calving (Oetzel, 2004; Oetzel, 2013). However, post-calving Ca supplementations, while critical for treatment, are not sufficient to prevent maternal hypocalcemia and its associated peripartum disorders. Use of anionic salts in the pre-partum period has reduced the incidence of SCH and CH. However, approximately 25% of cows will still be afflicted with SCH (Oetzel, 2004). Additional limitations in the use of anionic salts as a prevention strategy include cost, reduced feed palatability, and the difficulty of finding low potassium forages to include in the diet (Oetzel, 2004; Goff, 2004; Goff, 2008). The lack of adequate therapies targeted towards preventing hypocalcemia leaves a large percentage of the U.S. dairy cow population unprotected and new therapeutic strategies are lacking because the physiological mechanisms of SCH are not fully understood.

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The Onset of Milk Production Drains Ca Pools in Dairy Cows

Colostrum and milk synthesis rapidly depletes Ca from the maternal circulation and therefore Ca must be mobilized from maternal bone to maintain adequate circulating concentrations. Circulating Ca concentrations are tightly regulated and controlled by several hormones including: Vitamin D, calcitonin, parathyroid hormone (**PTH**) and parathyroid hormone related-protein (**PTHrP**). Liberation of Ca from bone stores can only be triggered when circulating Ca concentrations dip below the animal's minimal threshold for Ca, via a classic negative feedback loop. Dietary Ca is insufficient to maintain maternal Ca homeostasis during milk synthesis. This is demonstrated by the fact that a dairy cow will lose 9-13% of her bone mass during the first 30 days of lactation. Bone loss during lactation is an evolutionary strategy of mammals used to support the cow as well as the mammary glands' demand for Ca for milk synthesis (**Figure 2**; Wysolmerski et al., 1995; Wysolmerksi, 2010; Goff, 2014).

The Mammary Gland Functions as an "Accessory Parathyroid Gland" during Lactation

The mammary gland produces the hormone PTHrP, which binds to receptors on bone to drive bone resorption and liberate Ca into the systemic circulation (Wysolmerski et al., 1995; Wysolmerski, 2010). PTHrP is only produced by the mammary gland during lactation. The Ca sensing receptor (**CaSR**) present in the mammary epithelium plays a crucial role in controlling maternal Ca concentrations during lactation. CaSR is highly expressed in the mammary gland during lactation, compared to virgin and pregnant time periods (VanHouten et al., 2003). Mammary PTHrP production is responsible for the mobilization of Ca from the bone during lactation, rather than the typical endocrine regulator of bone, PTH (Wysolmerski et al., 1995; VanHouten, 2005; Wysolmerski, 2010; Wysolmerski, 2012). Our lab made a novel discovery that serotonin is essential for the liberation of Ca from bone during lactation to sustain maternal Ca homeostasis in rodent models. This occurs through induction of PTHrP by the mammary gland (Hernandez et al., 2012; Laporta et al., 2014a, 2014b). Furthermore, we demonstrated that serotonin is critical for the expression of CaSR. This finding indicates that serotonin is crucial for mammary gland sensing of systemic Ca concentrations.

Mammary Gland Coordination with the Skeletal System Liberates Ca During Lactation

The skeletal system maintains its structural and functional roles via communication between two cell types, osteoblasts (**OB**), which are responsible for bone formation, and osteoclasts (**OC**), which are responsible for bone resorption, and thus Ca mobilization. PTH regulates this mechanism under non-lactating conditions. Research in humans and rodents has suggested the PTH action on bone is uncoupled during lactation (Wysolmerski, 2010; VanHouten and Wysolmerski, 2013). PTHrP signals through the same G-protein coupled receptor (PTH1R) that PTH does on the OB to decrease OB cell proliferation and up-regulate genes responsible for OC

differentiation during lactation. In rodents and humans, the mammary gland is the main source of PTHrP found in the circulation (Thiede, 1994; Wysolmerski et al., 1995; Wysolmerski, 2010; VanHouten and Wysolmerski, 2013). Mammary-derived PTHrP, not PTH, is the critical hormone responsible for induction of bone Ca mobilization during lactation (Wysolmerski et al., 1995).

Serotonin Regulates Mammary Gland Physiology During Lactation

Serotonin is synthesized in numerous tissues throughout the body and brain and is incapable of crossing the blood-brain barrier. Serotonin is synthesized from the amino acid L-tryptophan in a two-step process. The first step is production of 5-hydroxytryptophan (**5-HTP**) via the rate-limiting enzyme, tryptophan hydroxylase (**TPH**). The second step is the conversion of 5-HTP to serotonin by aromatic amino acid decarboxylase (Wang et al., 2002). TPH1 is the rate-limiting enzyme for serotonin production in non-neuronal tissues, while TPH2 is used to produce serotonin in neuronal tissues. Our laboratory and others have shown that serotonin regulates milk protein gene expression, as well as the disassembly of tight junctions that occurs during the involution process (Matsuda et al., 2004; Stull et al., 2007; Hernandez et al., 2008; Pai and Horseman, 2008). Furthermore, we have shown that the mammary gland expresses a unique pattern of serotonin receptors in rodent, bovine, and human mammary epithelium (Hernandez et al., 2009; Pai et al., 2009). The epithelial component of the bovine mammary gland expresses at least five serotonin receptor isoforms (5-HT1B, 2A, 2B, 4 and 7; Hernandez et al., 2009). Our lab determined that the 5-HT2B receptor subtype modulates serotonin's regulation of PTHrP production within the mammary gland in a rodent model (Hernandez et al., 2012; Laporta et al., 2013a; Laporta et al., 2014a,b). We also confirmed that circulating serotonin concentrations post-partum are positively correlated with circulating Ca concentrations on the first day of lactation in dairy cows (Laporta et al., 2013b). Furthermore, we showed that serotonin activates expression of various Ca pumps and transporters in the mammary gland to stimulate transport of Ca from blood to milk during mouse lactation (Laporta et al., 2014a). Ca transport into the mammary gland is thought to occur through the Ca^{2+} influx channel (**ORAI1**) and subsequent pumping into the milk by the apical plasma membrane Ca^{2+} ATPase (**PMCA2**; Cross et al., 2014).

Current research in humans and rodents implicates PTHrP in the regulation of maternal Ca homeostasis during lactation. Our laboratory has demonstrated the necessity of serotonin for regulation of Ca transport in the mammary gland during lactation. Furthermore, we have demonstrated that serotonin is necessary for the production of mammary PTHrP during lactation. Mammary PTHrP production is critical to the mobilization of Ca from bone tissue to support lactation. Therefore, delineation of the mechanisms regulating the mammary gland serotonin-PTHrP axis in the dairy cow could lead to development of novel therapeutic interventions to reduce the incidence of SCH and CH in the U.S. dairy cow population.

The following model for the regulation of Ca mobilization from bone by the mammary gland during the transition period has been proposed by our laboratory

(**Figure 3**).

New Ideas About Calcium and Serotonin

Our laboratory recently demonstrated that serotonin is necessary for mammary PTHrP synthesis in lactating rodents and mammary epithelial cells grown in lactogenic culture (Hernandez et al., 2012; Laporta et al., 2013a; Horseman and Hernandez, 2014). We also demonstrated that supplementation of a serotonin precursor, 5-HTP, to rats during the transition from pregnancy to lactation increased the post-parturition circulating serotonin, PTHrP, and Ca concentrations, and also increased total Ca content in milk (Laporta et al., 2013a). Furthermore, we observed increased OC numbers in the femurs collected from rats supplemented with 5-HTP, indicating this response was due to bone Ca mobilization.

In order to better understand the relationship between serotonin and maternal Ca homeostasis, we recently deleted TPH1 in mice.

TPH1 catalyzes the rate-limiting step in non-neuronal serotonin synthesis. TPH1 deficient mice have little to no circulating serotonin. Our goal was to make mice deficient in non-neuronal serotonin and delineate the potential molecular mechanisms underlying serotonin's regulation of Ca homeostasis during lactation. We demonstrated that i.p. injections of 5-HTP to these mice restored and even elevated circulating serotonin concentrations compared to wild-type dams. Our results also demonstrated that total Ca concentrations are decreased in TPH1 null mice and that Ca concentrations can be restored with i.p. injection of 5-HTP. RNA-sequencing analysis of mammary glands collected on d 10 of lactation from wild-type, TPH1 deficient mice and TPH1 deficient mice injected with 5-HTP revealed that serotonin is critical for the cellular response to Ca, along with a variety of further, yet unexplored, regulatory pathways (Laporta et al., 2015). Upon further analysis of the specific Ca pumps and transporters present in the mammary gland we observed that mRNA abundance of several Ca pumps and transporters was reduced in the TPH1 deficient mammary gland and restored by exogenous 5-HTP (Laporta et al., 2014a). These results indicate that peripheral serotonin is critical for maintaining circulating Ca concentrations and mammary gland Ca transport during lactation.

In order to evaluate the utility of the mammary serotonin-PTHrP axis in Holstein dairy cows, we performed several observational studies

In a small study of 42 multiparous Holstein dairy cows, we observed that serotonin and PTHrP concentrations on d 1 of lactation were positively correlated with total Ca concentrations (Laporta et al., 2013b; **Figure 4**). Additionally, we have observed that serotonin concentrations are dynamic over the course of a given lactation, and decrease around the time of calving (d 0-2 lactation), rebounding by approximately ten days into lactation (Moore et al., 2015; **Figure 5**). The overall average serotonin concentration in dairy cows is approximately 1700 ng/ml. However, the concentrations fluctuate dependent on stage of lactation. These results combined

with our rodent data support our hypothesis that serotonin and PTHrP are critical players in the regulation of Ca homeostasis in Holstein dairy cows.

Intravenous (IV) infusion of 5-HTP in late lactation, non-pregnant, multiparous Holstein dairy cows increases circulating serotonin concentrations and alters Ca dynamics

In order to demonstrate the role of serotonin in Ca homeostasis in dairy cows, we performed a preliminary experiment in which we infused 5-HTP IV for one hour daily for four days in late-lactation dairy cows at varying doses (0, 0.5, 1.0, or 1.5 mg/kg) to determine an optimum dose of 5-HTP necessary to produce significant changes in Ca. All three doses of 5-HTP significantly increased circulating serotonin concentrations (Laporta et al., 2015) to a similar extent in the two hours after dosing, with concentrations returning to baseline concentrations observed in the saline controls by two hours after infusion. In addition to serotonin concentrations, we measured circulating total Ca concentrations following the same time course post infusion. While initially counter-intuitive, our data demonstrated that total Ca concentrations decreased in immediate response to 5-HTP treatments (**Figure 6a;** Laporta et al., 2015). In order to determine where the circulating Ca was going after 5-HTP infusion, we measured urine Ca concentrations prior to the start of infusion and two hours after the end of the infusion. Our results indicate that there was a decrease in urine Ca output with higher doses of 5-HTP treatment (**Figure 6b**). This suggests that Ca is not being lost into the urine. Therefore, we measured total Ca concentrations in the milk during the infusion periods and observed that the highest dose of 5-HTP increased total milk Ca concentrations (**Figure 6c**). This supports the hypothesis that serotonin causes transient hypocalcemia by increased Ca transport into the mammary gland and subsequently into milk. Increased Ca transport into the mammary gland during lactation is critical for the stimulation of bone Ca mobilization by PTHrP because transient systemic hypocalcemia.

Use of 5-HTP before calving to prevent hypocalcemia

In order to determine if elevating serotonin concentrations in pre-fresh dairy cows would result in increased post-calving Ca concentrations, we treated multiparous Holstein cows with daily IV infusions of 1.0 mg/kg of 5-HTP beginning 7 d before the estimated calving date until calving. Our data demonstrates that IV infusions of 5-HTP pre-calving increased ($P = 0.04$) post-calving total Ca concentrations compared to saline treated controls (**Figure 7**). Furthermore, we measured deoxypyridinoline (**DPD**), a marker of OC activity and therefore bone resorption, in the urine. These data demonstrate that cows receiving 5-HTP before calving have increased bone resorption on at calving (**Figure 8;** $P = 0.01$). These results support demonstrate that 5-HTP treatment pre-calving can potentially improve post-calving Ca concentrations by increasing bone Ca resorption.

Interrelationship of a negative DCAD and serotonin

Given that 5-HTP treatment pre- calving was capable of increasing post-calving Ca concentrations, we wanted to determine if a common preventative treatment for SCH and CH, negative DCAD, controls Ca homeostasis via a serotonergic mechanism. To this end, we fed Holstein dairy cows a positive DCAD (+130 mEq/kg) diet for 21 days prior to calving or a negative DCAD (-130 mEq/kg) diet for 21 days prior to calving. Upon analysis of circulating serotonin concentrations from 9 days before calving through 6 days post-calving, we determined that a negative DCAD diet increased circulating serotonin concentrations pre-calving ($P = 0.05$; **Figure 9**). This suggests the resulting improvement in post-calving Ca concentrations (data not shown) in the cows receiving a negative DCAD diet pre-calving could be due to serotonin's control of Ca homeostasis. At this time we are unaware if negative DCAD works exclusively through a serotonergic mechanism. Furthermore, we do not know if a negative DCAD diet combined with 5-HTP treatment will have a synergistic effect on post-calving Ca concentrations.

Conclusion

In conclusion, we have demonstrated that serotonin plays a critical role in regulation of maternal Ca transport, maternal Ca homeostasis and mammary PTHrP production in the rodent. Additionally, our data demonstrate that mammary gland Ca transporter expression and induction of PTHrP production by the mammary gland during lactation are key regulators of maternal Ca homeostasis in rodent models. Furthermore, our rodent models indicate that the mammary gland is a significant source of serotonin during lactation. Our observational data in Holstein cows suggests that serotonin, PTHrP, and Ca are interrelated during the early days postpartum. Furthermore, our initial experiment exploring the effects of 5-HTP on maternal Ca homeostasis in late-lactation dairy cows supports the hypothesis that serotonin induces transient hypocalcemia by shuttling Ca into the mammary gland in order to stimulate mammary production of PTHrP, and the elevated PTHrP is critical to stimulate bone Ca resorption. Treating pre-partum Holstein dairy cows with 5-HTP resulted in improvement of post-partum Ca concentrations. Finally, using a current therapeutic intervention for prevention of SCH and CH in the dairy industry, feeding of a negative DCAD diet pre-partum, resulted in the increase of circulating serotonin concentrations. Taken together, our **preliminary** data in rodents and dairy cows support the hypothesis that mammary serotonin is critical to the induction of mammary PTHrP, and PTHrP entering the maternal circulation in turn restores maternal Ca homeostasis during lactation. Further delineation of these molecular pathways could lead to powerful, innovative preventative strategies against SCH and CH in dairy cows.

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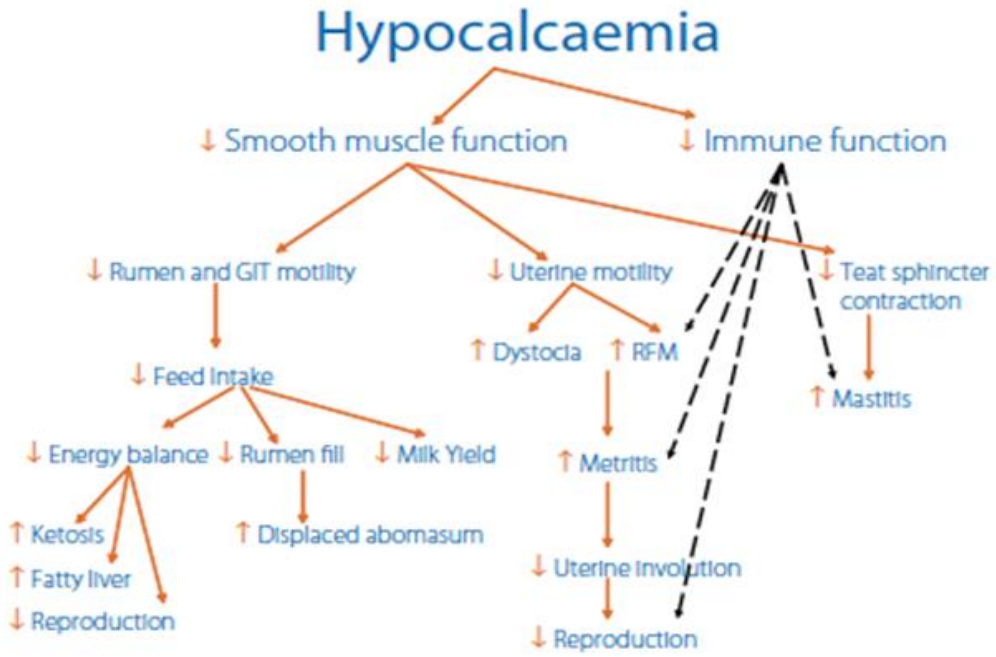

Figure 1. Hypocalcemia is a 'gateway' disease that leads to increased risks of other periparturient diseases. (DeGaris and Lean, 2008).

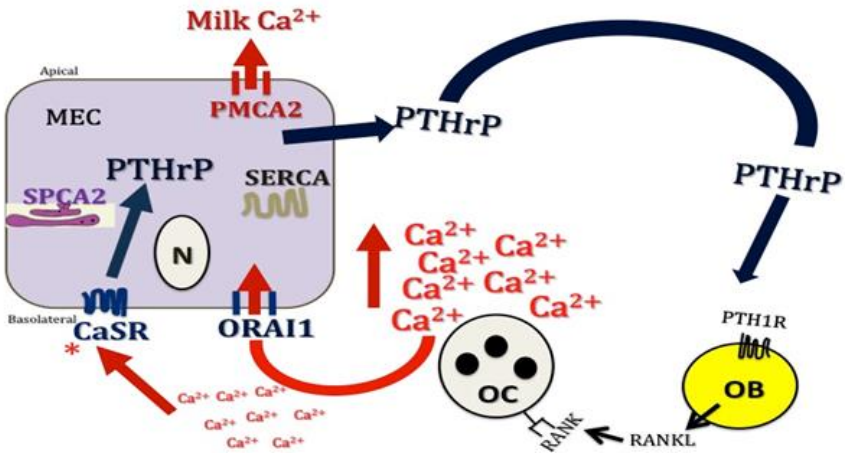

Figure 2. Maternal Ca homeostasis is regulated by the mammary gland-bone axis. During lactation, the Ca sensing receptor (CaSR) on the basolateral side of the mammary epithelial cell (MEC) during lactation detects low blood Ca concentrations due to the increased transport of Ca into the MEC by Ca release-activated Ca channel protein 1 (ORAI1). Ca is either secreted into the milk through the apical plasma membrane Ca ATPase 2 (PMCA2) or sequestered in the Golgi apparatus by secretory pathways Ca ATPase 2 (SPCA2) or endoplasmic reticulum by the sarco(endo)plasmic reticulum Ca ATPase (SERCA). Detection of systemic decreased Ca by CaSR results in parathyroid hormone related-protein (PTHrP) production. PTHrP is secreted into the circulation and will bind its receptor PTH1R on the osteoblast (OB) cell in the bone increasing production of receptor activated nuclear factor kappa B (RANKL), which binds its receptor (RANK) on the osteoclast (OC) cell in the bone tissue, activating Ca liberation from bone.

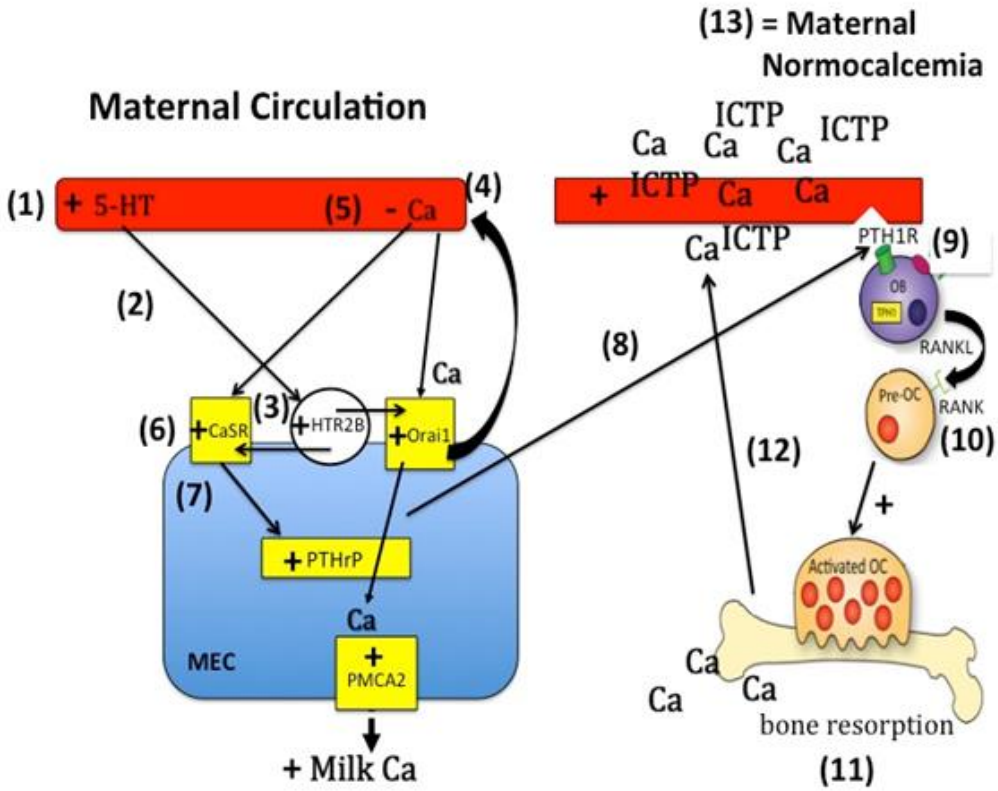

Figure 3. Proposed model of the regulation of maternal Ca homeostasis by serotonin (5-HT). **1)** Increased maternal circulating 5-HT, **2)** stimulates signaling via the 5HT2B receptor on the basolateral side of the mammary epithelium (MEC), **3)** increasing expression of Orai1 and CaSR, **4)** resulting in increased transport of Ca into the mammary gland, which leads to **5)** decreased systemic Ca concentrations, and **6)** this is detected by the CaSR on the basolateral surface of the mammary epithelium, resulting in **7)** stimulation of PTHrP production by the MEC. **8)** PTHrP is then secreted by the MEC and acts through **9)** PTH1R, its receptor present on the OB, stimulating production of RANKL, which binds to the RANK receptor on the OC, **10)** stimulating OC maturation and activation, allowing for bone resorption, **11)** resulting in the liberation of Ca from the bone, and **12)** resulting in increased systemic Ca and type I collagen fragment (ICTP) concentrations (a bone resorption marker),**13)** resulting in normocalcemia.

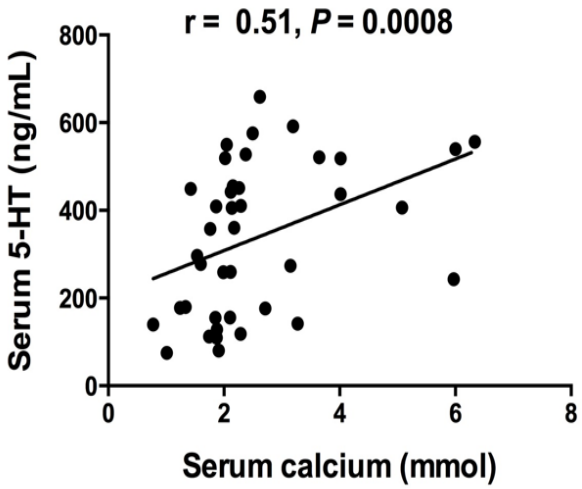

Figure 4. Circulating serotonin (5-HT) and total Ca concentrations are positively correlated on d1 lactation in multiparous Holstein cows (Laporta et al., 2013b).

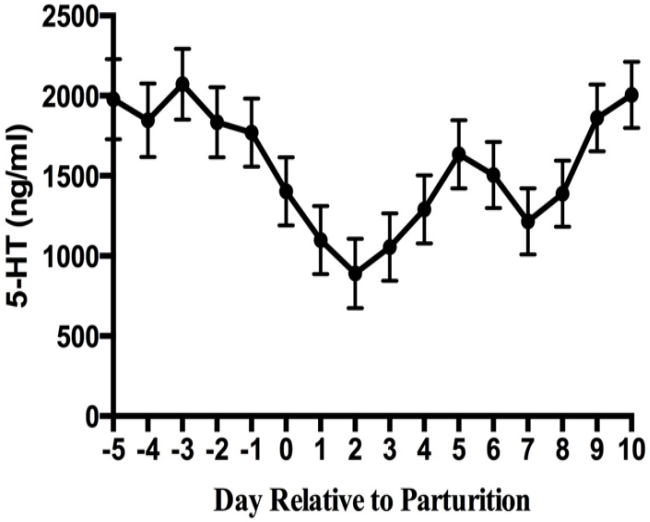

Figure 5. Circulating serotonin (5-HT) concentrations fluctuate around calving in multiparous dairy cows and serotonin decreases around the time of calving. (Moore et al., 2015).

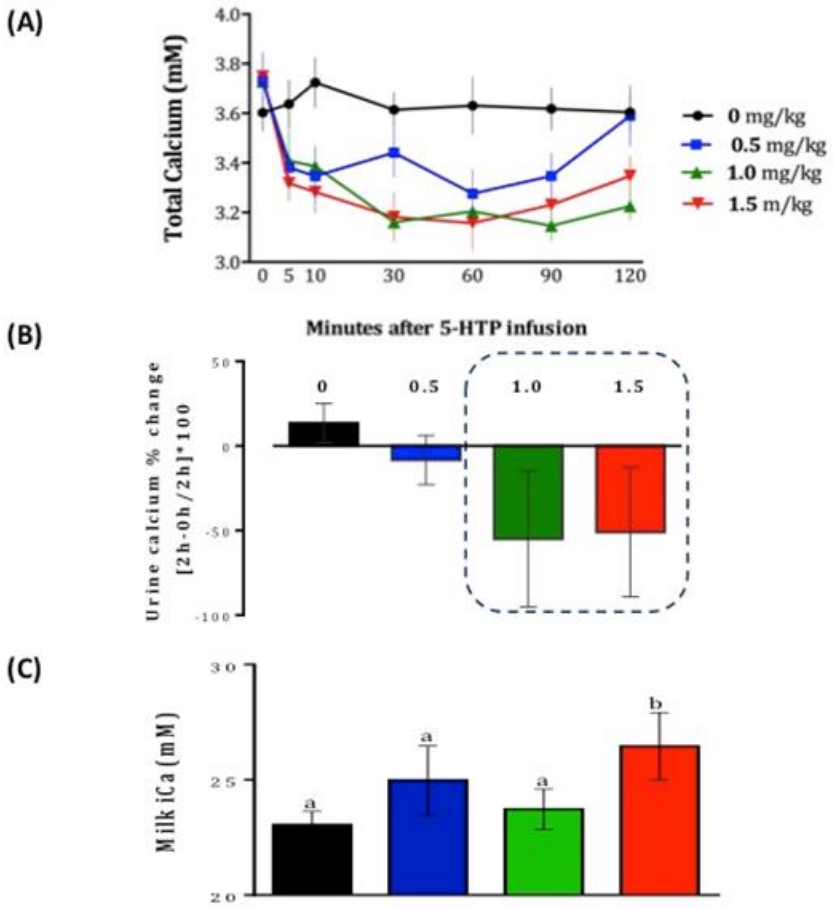

Figure 6. Increasing serotonin concentrations increases flux of Ca to the mammary gland from the circulation **(A)** I.V. infusion of 5-HTP to late lactation dairy cows at varying doses decreases circulating Ca concentrations in the first 2 h after dosing. **(B)** Urine calcium concentrations are decreased after infusion as dose of 5-HTP increases. **(C)** Milk Ca concentrations increase with increased infusions of 5-HTP. (Laporta et al., 2015).

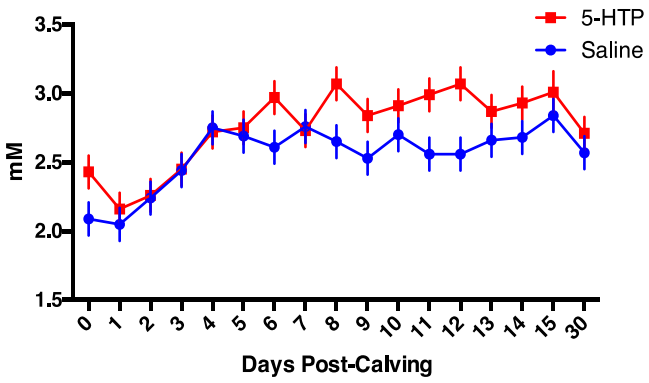

Figure 7. 5-HTP treatment pre-calving increases post-calving circulating Ca concentrations (Weaver et al., unpublished results).

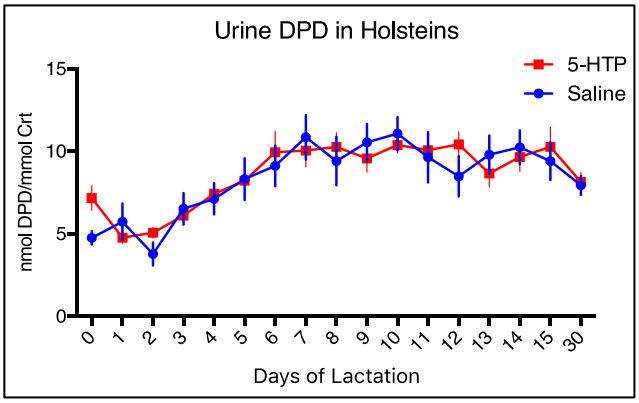

Figure 8. Urine deoxypyridinoline (DPD) concentrations post-calving in cows treated with saline or 5-HTP pre-calving. (Weaver et al., unpublished results).

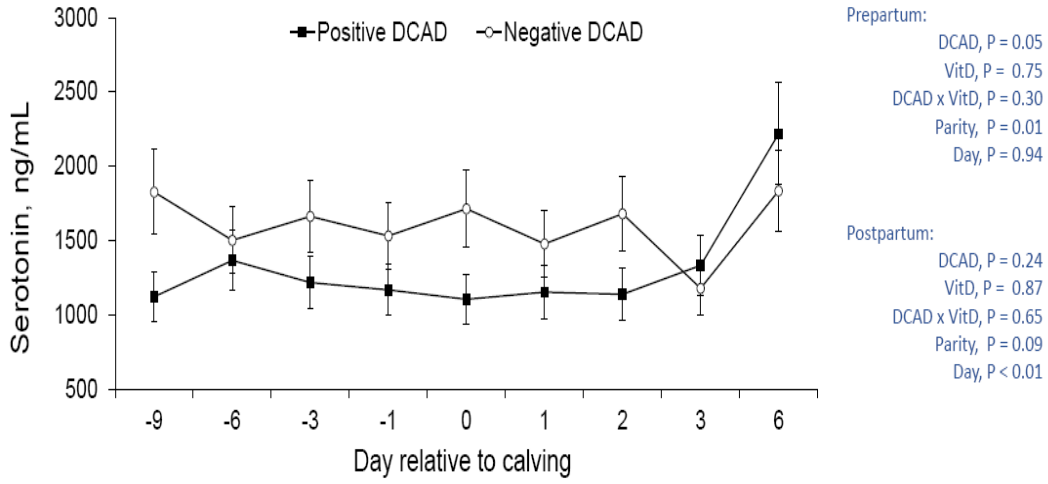

Figure 9. Feeding a negative DCAD for 21 days pre-calving increases circulating serotonin concentrations compared to feeding a positive DCAD for 21 days pre-calving (Martinez, unpublished results).

SESSION NOTES