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Evaluation of B-mode and color Doppler Ultrasound as Alternative Tools for the Study of Reproduction, Temperament, and Milk production- Related Variables in the Bovine

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Evaluation of B-mode and color Doppler ultrasound as alternative tools for the study of reproduction, temperament, and milk production- related variables in the bovine

By

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An adequate vascular perfusion status is essential not only to maintain life, but to ensure the proper physiology of the different systems that form the animal's body. Due to its role in the transport of oxygen and nutrients toward, and the removal of cellular waste products away from the body's tissues, the circulatory system is responsible for the maintenance of body homeostasis. Production related functions in farm animals are not an exception, and directly depend on sufficient vascular physiology. In the past, the study of blood flow in large domestic species was restricted to highly invasive techniques. However, even when such techniques are the foundation for the actual understanding of vascular dynamics in these animals, their limited feasibility and potential impact over the normal vascular physiology represent significant limitations to these approaches. Recently, the development and application of non-invasive technologies (i.e., Doppler and B- mode ultrasound) to the area of animal sciences has provided the potential for the study of vascular dynamics while, the negative implications aforementioned are avoided. In our studies, these technologies were applied to assess the role of the circulatory system on different production related variables such as: temperament, reproduction, and milk production in the bovine. A tendency toward differences in jugular blood flow was associated with the temperament in beef calves in this study. Also, a significant increase in vasodilation in the uterine arteries of beef cows was found to be associated with a numerically higher reproductive efficiency (i.e., pregnancy rates). Moreover, an increase in blood flow towards the mammary gland in dairy cows was associated with administration of bovine somatotropin. In conclusion, B-mode and Doppler ultrasound resulted in tools able to reflect the essential role of an adequate vascular perfusion in the normal physiology and productive performance in the bovine. However, in real farm scenarios the feasibility of these techniques in large domestic species is limited. Therefore, further specialization of this instrument to the conditions existent in such farm scenarios are recommended to improve its feasibility and to significantly accelerate the rate of knowledge acquisition in this area.

Keywords: Beef cattle, Dairy cattle, Blood flow, Temperament, B-mode ultrasound, Color Doppler ultrasound

DEDICATION

I will like to dedicate this work to all the Puerto Rican people that in one way or the other are the reasons for me to be here today. There are so many people responsible for this achievement that, even when is not my intention to exclude your names, I am afraid it may happen. Therefore, to all of you that contributed to this work, but your names are not here; receive my thanks, my appreciation, and this dedication from the deepest of my heart.

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CHAPTER I

GENERAL INTRODUCTION

Animal production has played an essential role as a source of food for humankind for thousands of years (Clutton-Brock, 1999). During this period of time, selection toward desirable characteristics has resulted in the high producing breeds of livestock that we have today (Zohary et al., 1998). More recently, a continuous increase in the knowledge about the anatomy and physiology in the different domestic species has allowed for the development of production systems that maximize the potential of these animals (Joandet and Cartwright, 1975). Through years of research, a wide range of research tools have been applied for these purposes. However (as a general rule and around the animal research history), for a deeper understanding in the anatomy and physiology fields, highly invasive tools have been required. Even when such tools have provide a significant progress in the understanding of these biological aspects, such a high level of invasiveness may also be accompanied by undesirable and uncontrolled external factors that may potentially bias the obtained results. Therefore, the development and application of non-invasive research tools (i.e., ultrasound) in the study of animal anatomy and physiology has opened the doors for a better understanding of such processes at the same time that such undesirable external influences are avoided or decreased.

Ultrasound as a diagnostic or imaging tool is non-invasive, externally applied, non-traumatic, and safe when used in the correct intensities and cycles (Erikson et al., 1974). After the 1950's, this technology has evolved and specialized to supply accurate anatomical and physiological information to medical practitioners. Since the 70's it has been broadly applied as a diagnostic or imaging tool in multiple branches of human medicine. More recently, this technology has become accessible to the animal science researchers, where it has contributed to the discovery and corroboration of findings in different areas including animal physiology, theriogenology, and regular veterinary medicine. The specialization of this originally human intended research tool in the animal science field will allow further growth in the understanding of these areas in the future.

The first diagnostic ultrasound systems developed during the 1950's-60's were probably tested in the area of animal sciences (Houghton and Turlington, 1992). However, the B-mode ultrasound was really intensively applied in this area for the first time during the 1980's (Ribadu and Nakao, 1999) in different fields including carcass quality measurements (Houghton and Turlington, 1992), theriogenology (Ginther and Matthew, 2004; Herzog and Bollwein, 2007), veterinary medicine, and research. Nevertheless, in other more specific modalities including Doppler ultrasound (even when there is a plethora of publications from studies in humans especially during the 70's), in the animal sciences this approach is relatively new. To our understanding, the first publication about Doppler ultrasound in the area of theriogenology was "Transrectal color Doppler sonography of the *A. uterine* in cyclic mares" by Bollwein and collaborators on Germany in 1998. After this publication, a group of other researchers have added a relatively limited number of studies to this list. However, during all this time this technology has almost exclusively been restricted to the study of the reproductive process in the female, apparently forgetting the essential role of the circulatory system on all the other physiological parameters in the body. Because these technologies have been developed for human use, different factors including their cost and feasibility in real farm scenarios have limited their use (at least in time) in animal science related areas of research. However, as these instruments become accessible to animal practitioners, the combination of different factors including research interest and subsequent market demand, will result in the development of more specialized equipment for the animal sciences area.

The studies presented in this dissertation have applied these technologies (ultrasound modalities) to the study, not only of the reproductive process in the female, but also of other important aspects of the animal anatomy and physiology as well as its relationship with important productive traits. For example, temperament in beef cattle has been found to significantly influence the productive (Voisinet et al., 1997) and reproductive (Phocas et al., 2006) performances. Normal management practices may represent a significant source of stress in temperamental animals (Burdick et al., 2011). In case of stress, these animals respond by increasing their blood flow volume towards the skeletal muscles, heart and brain in order to sustain the "fight or flight" reaction (Sapolsky, 1990). This increase in vascular perfusion is accomplished in part by luminal enlargement of arteries (Smiesko and Johnson, 1993; and Ford, 1995) and veins (Barbera et al., 1999). Therefore, the study of the relationship between temperament and vascular dynamics may provide a better understanding of such an important variable in beef cattle production. Also, for the success of the reproductive process it has been found that an adequate blood perfusion in the female reproductive organs is essential (Ford, 1995; Hsieh et al., 2000; Bollwein et al., 2004). However, even when Doppler ultrasound has been applied to the study of this variable, several factors including the movements of the animals during sampling may limit the feasibility of this technology (Herzog and Bollwein, 2007) in large domestic species. Consequently, the evaluation of alternative techniques that allow for effective and feasible measurement or estimation of this variable under the restrictive conditions existent in the real farm scenarios is imperative. Moreover, in dairy cows, the increase in milk yield observed after exogenous bovine Somatotropin administration [mediated by the induced IGF-I (Sharma et al., 1994) and nitric oxide (Tonshff et al., 1998)], has been associated with an increase in mammary gland blood flow (Boonsanit et al., 2010). The application of this research tool (ultrasound) may provide key information about the effects of such an important drug in the American dairy industry. Consequently, in all these important production related variables the vascular perfusion status plays an essential role and the use of B-mode and / or color Doppler ultrasound may allow for the achievement of a better understanding of such processes in a non-invasive way.

Therefore, the general objectives of this work were to assess the essential role of the circulatory system in multiple important aspects of cattle production in a non-invasive way, while the color Doppler ultrasound technology is evaluated in the difficult conditions existent in different real farm scenarios.

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CHAPTER II

LITERATURE REVIEW

Diagnostic Ultrasound

Ultrasound as a diagnostic or imaging tool is non-invasive, externally applied, non-traumatic, and safe when used in the correct intensities and cycles (Erikson et al., 1974). After the 1950's, this technology has evolved and specialized to supply accurate anatomical and physiological information to medical and veterinary practitioners. From the 1970's it has been broadly applied as a diagnostic or imaging tool in multiple branches of human medicine. More recently, this technology has become accessible to the animal sciences, where it has contributed to the discovery and the corroboration of findings in different disciplines including animal physiology, theriogenology, and general veterinary medicine. The specialization of this human intended research tool in the animal science field will allow further growth in the understanding of these areas in the future.

Ultrasound History

The first human approaches to use ultrasound can be traced back to the 1920's (Erikson et al., 1974). However, its use for diagnostic purposes began during the second half of the twentieth century (McNay and Fleming, 1999). The first approaches of this technology were in the detection of underwater subjects (i.e., submarines) and flags in metals (McNay and Fleming, 1999). In medicine, the first uses of the ultrasound were more of therapeutic than of diagnostic nature (McNay and Fleming, 1999). Erikson et al. (1974) and McNay and Fleming (1999) have summarized some of the more important events that resulted in today's highly used diagnostic ultrasound technology. The following historical events are based on their descriptions.

Before ultrasound was applied in any scenario, several investigations and discoveries created the foundations from which this technology was established. In 1842, Christian Doppler published his observations of the changes observed in the pitch when a source of vibrations is moving toward or away from the observer (what during the 1960's was used to develop today's Doppler ultrasound). In 1877, John W. Rayleigh published "The theory of sound" (Rayleigh, 1877). In 1880, the Curie brothers (Pierre and Jacques) described the piezoelectric effect (Curie and Curie, 1880). This piezoelectric effect is based on particular materials (crystals) that will contract and expand in response to an electrical charge emitting in this way sound waves. It was initially considered as a simple scientific curiosity, but later was found to be of major importance to produce acoustic waves in sea water and was the basis for the ultrasound technology. All this knowledge was then put together resulting in the development of the first applications of this technology.

In the beginning, this technology was considered for industrial and war related purposes. In 1912 after the Titanic sank, a British meteorologist named Lewis F. Richardson suggested the use of sound for the detection of icebergs (Richardson, 1912), an idea that was later successfully demonstrated in 1914 by an American electric engineer named Reginald Fessenden (Hackman, 1984). During the first world war

(1914-1918) as an effort to detect submarines, the first piezoelectric ultrasound transducer was constructed (Biquard, 1972). Later in 1929, ultrasound was suggested as a means to detect flags in materials (Sokolov, 1929). These industrial and war-related applications created a base of understanding that later allowed for the application of this technology in the field of medicine.

Around the middle of the century a series of experiments resulted in the development of the first ultrasound machines with medical applications as well as the first studies in this area. During 1937, Karl Dussik suggested the possible application of ultrasound in medicine (Dusik, 1942). The oldest and simplest type of diagnostic ultrasound machine was developed in 1945 by Floyd Firestone (Firestone, 1945). It was called "Reflectoscope", an A-mode ultrasound instrument that used a time base oscilloscope display of received echo amplitude. In 1948, a young physician trained in radiology named Douglass Howry in collaboration with an engineer named William Bliss developed the "Somascope", the first B-scan instrument. This machine produced higher quality images in comparison with the simple one dimension graphic created by the "Reflectoscope". In 1950, John Wild (known as the father of the ultrasound in medicine), an English surgeon, published "The use of ultrasonic pulses for the measurement of biologic tissues and the detection of tissue density changes" (Wild, 1950). In 1952, Howry and Bliss published "Ultrasonic visualization of soft tissue structures of the body". In 1949, the first A-scope was constructed in Japan by Rokuro Uchida. Uchida later collaborated with other clinicians to develop equipment and applications of ultrasound in clinical practice. In 1954, Inge Edler (a cardiologist) and

Carl Hertz (a physicist) published “The use of ultrasonic reflectoscope for the continuous recording of movements of the heart walls” (Edler and Hertz, 1954).

During the 60’s an increased number of studies added to the development of this technique. Some of the names and titles that significantly contributed to develop the ultrasound as a diagnostic tool include: Dean Fanklin et al. (1969) with “Blood flow measured by Doppler frequency shift of back scattered ultrasound”; Don Baker (1969) with “Pulsed ultrasonic Doppler flowmeter- Biological and engineering applications”; and Pierre Peronneau et al. (1969) with “Doppler ultrasonic pulsed blood flowmeter”. However, even when all this characters were essential for the development of this technology, it is Ian Donald who deserves the title of pioneer in the application of ultrasound as a diagnostic tool in medicine, specially gynecology and obstetrics.

Even though John Wild is considered the father of the ultrasound in medicine, it is Ian Donald (1910-1987) a physician trained in obstetrics and gynecology in London, who established this technology as a diagnostic tool for human gynecology and obstetrics. Donald became a professor at the University of Glasgow during 1954. In 1958, he published “Investigation of abdominal masses by pulsed ultrasound”, one of the central publications in the area during this time. During his 22 years in his position as professor at the University of Glasgow, he established the basics for the ultrasound as the diagnostic imaging tool that we know today in his area. In fact, the instruments developed during this time to apply ultrasonography as a non-invasive diagnostic tool have evolved in to a wide range of specialized instruments that not only allows for the study of the anatomy of the body, but also of its physiology.

During the 70's the use of this technology advanced greatly in human medicine giving rise to a wide range of possible applications in different branches of medicine (Erikson et al., 1974) including neonatology, gastroenterology, and cardiology (Taylor, 1988), and gynecology and obstetrics (Taylor, 1988; McNay and Fleming, 1999) just to mention a few of these application areas.

The first diagnostic ultrasound systems developed during the 1950's-60's were probably tested in the area of animal sciences (Houghton and Turlington, 1992). However, the B-mode ultrasound was really intensively applied in this area for the first time during the 1980's (Ribadu and Nakao, 1999) in different fields including carcass quality measurements (Houghton and Turlington, 1992), theriogenology (Ginther and Matthew, 2004; Herzog and Bollwein, 2007), veterinary medicine, and research. Nevertheless, in other more specific modalities including Doppler ultrasound (even when there is a plethora of publications from studies in humans especially during the 70's), in the animal sciences this approach is relatively new. The first publication about Doppler ultrasound in the area of theriogenology was "Transrectal color Doppler sonography of the *A. uterine* in cyclic mares" by Bollwein and collaborators from Germany in 1998. After this publication, a group of other researchers have added a limited number to this list, most of them in the period of 2005-2007. However, during this time the technology has almost exclusively been restricted to the study of the reproductive process in the female, apparently forgetting the essential role of the circulatory system on all other physiological parameters in the body. Because these technologies have been developed for human use, different factors including their cost and feasibility in real farm scenarios have limited their use. However, as these instruments become accessible to animal practitioners, the combination of different factors including research interest and subsequent market demand, will result in the development of more specialized equipment for the animal sciences area.

Ultrasound Principles

The ultrasound waves from diagnostic purposes originate in a transducer from crystals with piezoelectric properties (Herzog and Bollwein, 2007). These crystals, when activated by electrical pulses, will expand and contract (the piezoelectric effect) resulting in the emission of sound waves. Ultrasound is waves of sound with frequencies above the 20,000 hertz, which is above normal human hearing (Ribadu and Nakao, 1999). These sound waves penetrate the body and are reflected in different levels according to the acoustic impedance of the different tissues. The transducer then receives the echoes reflected from the different tissues creating an image on the screen. In Doppler ultrasound, for example, if the scanned tissue is not moving, the reflected echoes correspond to the waves emitted by the transducer. However, if the structure is moving, a shift in wave frequency will be detected by the transducer. For animal diagnostic imaging purposes, ultrasound frequencies in the range of 1-10 MHz are normally used (Rantanen and Ewing, 1981). More specific frequency values of 3.5, 5.0, and 7.5 MHz are commonly used in the study of the reproductive organs in the cow (Ribadu and Nakao, 1999). In general, the higher the frequency of the transmitted sound waves, the better the image resolution, but the shallower the depth of penetration (Ribadu and Nakao, 1999).

For a better understanding of the next ultrasound modalities (especially Doppler ultrasound), an example has been proposed by Ginther and Matthew (2004). Imagine that you are standing at the side of the road and a car is approaching constantly using it's horn. As the car approaches towards you, the sound of the horn seems to increase. As it passes away from you, the sound seems to decrease. What happens is that as the car approaches you, the sound waves of the horn (which are relatively constant) are amplified by the movement of the car in your direction. As it moves away from you, the sound waves are decreased by the movement of the car in the opposite direction. However, if both, you and the car remain stationary the sound will not change. This example will be used to discuss the B-mode and the Doppler ultrasound in the following sections.

B-mode and Color Doppler Ultrasound

Even when ultrasonography for imaging / diagnostic purposes has evolved in to a wide diversity of instruments and applications, all with a tremendous impact in human and animal medicine and research; the following discussion is based on the two modalities used in the studies contained in this work, B-mode and color Doppler.

B-mode Ultrasound

In B-mode ultrasound, two dimensional (Goldberg and Lehman, 1969) cross sectional images of organs and tissues are obtained (Ostrum et al., 1967) in gray scale. The B-mode ultrasound allows for a direct, non-invasive anatomical visualization, identification, location, and measurement of internal organs (Ginther and Matthew, 2004; Herzog and Bollwein, 2007). Following the aforementioned example of the car, in the B-mode both (the car and the listener) are static (Ginther and Matthew, 2004). In this case, the transducer emitting the sound waves and the tissue being scanned are relatively static. The observed gray scale image is the result of the difference between the sound waves emitted by the transducer and the echoes received back. In this case, dense tissue (as bone) will reflect a higher degree of echoes than a soft tissue resulting in a white spot (white pixels) in the produced two dimensional image. The other extreme (liquids) will allow more sound waves to pass through, resulting in a decrease in the echoes returning to the transducer. In this case, a black spot (black pixels) will be observed in the image. Tissues of intermediate density (i.e., muscles) will appear in different grades of gray in the image. The combination of all these gray scale tones will provide an accurate two dimensional visualization that will allow non-invasive evaluation of the scanned tissue or organs.

Color Doppler Ultrasound

Color Doppler ultrasound (Ginther and Matthew, 2004; Herzog and Bollwein, 2007) is a relatively new variation of the ultrasound technology that allows not only for anatomical visualization, but also for non-invasive physiological measurements in blood vessels to be obtained. Following the aforementioned car example, if the car is approaching to the listener the horn sound will be increased. In terms of ultrasound waves, if the scanned target tissue (i.e., red blood cells) is moving towards the transducer, the echoes received back by the transducer will be higher than the sound waves originally emitted. In other words, there will be a positive frequency shift. However, if the target tissue is moving away from the transducer, the shift will be negative (smaller amount of received echoes; the sound of the horn decreases in the car example). Color Doppler ultrasound is able to capture these movements by differences in the echoes of the emitted ultrasound beam. If the shift is positive, the tissue will appear in red color on a gray scale B-mode image; if negative, the color will be blue. These properties have allowed Doppler ultrasound to emerge as a useful diagnostic tool of the cardiovascular system. It can potentially be used for the measurement of vascular dynamics all around the body. With this modality, not only a color anatomical illustration of the blood vessels can be observed on a B-mode image, but also the speed and direction of the blood flow over time can be recorded in a graphic format. The values in this graphic allow for the determination of different parameters to access the vascular perfusion status of different organs including volume and resistance to blood flow. Therefore, color Doppler ultrasound presents the advantage that it not only provides anatomical visualization (as B-mode) of the scanned tissue, but also physiological information.

Ultrasound Parameters

Color Doppler ultrasound can be used to obtain quantitative and semi-quantitative measurements of the vascular status in different vessels all around the body (Herzog and Bollwein, 2007). In the studies presented in this dissertation, the blood flow was characterized by the blood flow volume (BFV; mL/min), the Resistance Index (RI), and the Pulsatility Index (PI). In order to obtain reliable BFV values, longitudinal sections of blood vessel of interest for relatively long periods of time must be obtained (Herzog and Bollwein, 2007). Also, the insonation angle (the angle between the ultrasound beam and the direction of the blood flow in the vessel) must be maintained between 30°-60° (the smaller the better), and the gate size must be adjusted to the luminal diameter size of the vessel (Herzog and Bollwein, 2007). When using color Doppler ultrasound, in order to decrease the variability in the obtained results, multiples measures in the site of interest are normally obtained (i.e., triplicate sampling) and the average is the value used for statistical analysis purposes. For example, after obtaining three uniform consecutive pulse waves, the image is frozen and the blood flow is characterized in each one of the pulse waves (See Fig. 1 B). The BFV may be calculated by the ultrasound machine by means of the following formula:

$$BFV = TAMV * \pi * (D * 0.1/2)^2 * 60; \text{(Bollwein et al., 2002)} \qquad \text{(Eq. 1)}$$

where the time average maximum velocity *(TAMV)= (TAMF * C) / (F * cos α)*, *C* is the ultrasound propagation speed, *F* is the transmitted wave frequency, *α* is the insonation angle, and *D* is the diameter of the vessel.

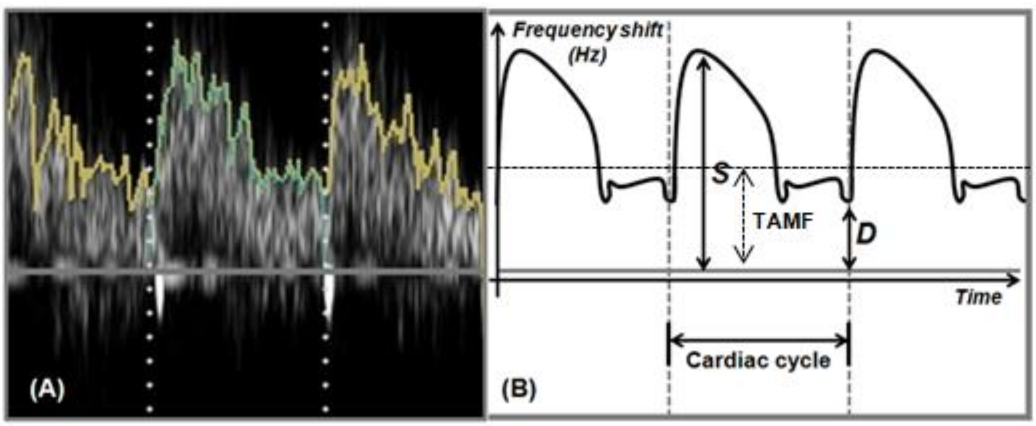

Figure 1 (A) Real time arterial color Doppler ultrasound spectral waveforms during three consecutive cardiac cycles in a beef cow; (B) Schematic representation of the same color Doppler spectral waveforms.

Notes: Note the calipers (two white (A) and gray (B) dotted vertical lines) selecting the waveform of interest. The frequency shifts represented by *S* and *D* are the maximum during systole and minimum during diastole, respectively. The time average maximum frequency (TAMF) is represented by a vertical dashed double arrow.

While BFV is considered a quantitative value of vascular perfusion, the RI and PI are considered semi-quantitative estimators that do not require control of the angle of insonation or the imaging of longitudinal sections of the vessel (Herzog and Bollwein, 2007).

The RI is a measure of the impedance to the blood flow distally to the sampling point (Herzog and Bollwein, 2007). The RI is calculated by the machine using the following formula:

$$RI = (S-D)/S; \text{ (Herzog and Bollwein, 2007)} \qquad \text{(Eq. 2)}$$

where *S* is the maximum systolic frequency shift and *D* is the end diastolic frequency shift of the cardiac cycle (Fig. 1 A).

In blood vessels where the flow is completely stopped during diastole, the RI cannot be applied because for simple mathematical reasons (the blood speed at diastole will be zero), it will always assume an unreal value of one. In such cases, the PI (a similar semi-quantitative value of distal vascular impedance) is employed. The PI increases if the vascular bed distal to the sampling point constrict whereas the proximal vascular bed remains unchanged (Bollwein et al., 2002). In general, the greater the PI value, the greater the distal impedance to blood flow and vice versa. The PI is also calculated by the ultrasound machine by means of the following formula:

$$PI = (S-D)/TAMV, \text{ (Herzog and Bollwein, 2007)} \qquad \text{(Eq. 3)}$$

where *S* is the peak systolic velocity, *D* is the end diastolic velocity, and *TAMV* is the time averaged maximum velocity of the cardiac cycles. Because the denominator of the equation is an average of blood speeds during the cardiac cycle, the PI can be used in blood vessels with zero velocity during diastole.

However, the control of the angle of insonation and the requirement of longitudinal sections of the vessel essential to measure BFV (Herzog and Bollwein, 2007) may be highly restrictive in real farm conditions. Moreover, even when the RI and the PI are estimators not subjected to these restrictions, all Doppler parameters require a relatively large sampling period without physical movements of the target vessels, which is extremely difficult to achieve when working with beef cattle. Consequently, any alternative index of vascular perfusion not restricted by these limitations deserves special attention. The BFV in arteries (Smiesko and Johnson, 1993; and Ford, 1995) and veins (Barbera et al., 1999) is directly regulated by changes in luminal dimensions. Therefore, the measurement of the dimensions (i.e., diameter) of the vessel from frozen B-mode ultrasound may represent a feasible estimator of blood flow unaffected by the aforementioned restrictions.

Blood Flow and Reproduction

Insufficient blood perfusion to the female reproductive organs has been found to be an important reason for sub- or in-fertility problems in different species including the equine (Bollwein et al., 2004 a), human (Hsieh et al., 2000), and bovine (Ford, 1995). Alterations in the blood flow dynamics to the female reproductive organs have been reported to be essential for follicular development, ovulation, implantation, and early pregnancy maintenance. Even after pregnancy is achieved, adequate materno-fetal vascular exchange is essential for successful continuation of gestation (Jauniaux et al., 2000). Therefore, the following paragraphs present a summary of the literature available in regard to the crucial role of an adequate vascular perfusion to the female reproductive organs and its role in reproduction.

Human

Extensive research in humans corroborates the essential role of vascular dynamic changes for the success of the reproductive process. An increase in blood flow to the reproductive organs is so important for reproduction that it has been establish that low daily doses of aspirin supplementation in women undergoing *in vitro* fertilization results in a significant increase in the volume of blood flow to the uterus and ovaries which subsequently and significantly increases: the number of follicles, the number of oocytes retrieved, serum estradiol concentrations, and the implantation and pregnancy rates (Rubistein et al., 1999). In fact, it has also been reported that women with poor uterine perfusion present higher rates of embryo implantation failure than their normally perfuse counterparts (Goswany et al., 1988). Moreover, Kurja et al. (1991) reported complete infertility in a group of women whose uterine blood flow completely stop at the end of the diastolic phase of the cardiac cycle. However, the essential role of vascular perfusion does not finish when pregnancy is achieved. Growth retardation of the fetus (a birth weight that is under the 10^{th} percentile of the normal predicted fetal weight for gestational age; Vandenbosche et al., 1998) has been associated with reduced uterine artery perfusion during pregnancy (Trudinger et al., 1985). Therefore, in the literature there is plenty of evidence about the potential improvement of human reproduction by altering the vascular dynamics to the female reproductive organs.

Bovine

More recently, the application of Doppler ultrasound to large domestic species has corroborated the potential to improve the reproductive process by altering the vascular dynamics. In cows, an increase in blood flow to the ovaries has been reported to occur in parallel with an increase in corpus luteum volume and function (progesterone production; Acosta et al., 2003; Matsui and Miyamoto, 2009). Moreover, the structural, secretory, and functional changes that occur in the pre-ovulatory bovine ovaries are closely associated with an increase in its blood flow status (Acosta et al., 2003). In fact, a close relationship has been observed between blood flow to the ovaries and follicular growth, atresia, and ovulation in cows (Matsui and Miyamoto, 2009). As in humans, the role of an adequate blood perfusion on the success of the reproductive process does not end once pregnancy is achieved. It has been reported that as soon as d 14-18 post-mating, pregnant cows presented a 2-3 fold increase in blood flow to the gravid uterine horn in comparison with the non-gravid horn and with non-pregnant cows (Ford et al., 1979). Therefore, there is also potential for improvement of the reproductive function in the bovine by altering blood flow dynamics.

Equine

Also recently, Doppler ultrasound technology has been intensively applied to the study of equine's reproduction providing information about the essential role of vascular perfusion changes in this species. An increase in blood flow resistance, which is negatively correlated with volume of blood flow, in the arteries that supply the reproductive organs has been signaled as a cause of sub-fertility in mares (Bollwein et al., 2004 a). In fact, in these mares, supplementation with aspirin or captropil (a vasodilator) increases the blood flow to the ovaries resulting in an increase in the progesterone production by the corpus luteum (Bollwein et al., 2004 a). In mares, as in other species, an increase in blood flow to the ovaries in parallel with corpus luteum function (progesterone production) has been observed during diestrus (Bollwein et al., 2002).

Also, during estrous an increase in blood flow in the ovarian and uterine arteries has been reported (Bollwein et al., 2002). As in the human and in the bovine, the importance of adequate blood perfusion for reproductive success does not finish once pregnancy is achieved. Bollwein et al. (2003) reported an increase in the uterine arteries blood flow during the second week of gestation in comparison with cyclic mares. Bollwein et al. (2004 b) reported that the week of gestation is positively correlated with the uterine blood flow volume during pregnancy in mares. In other words, as gestation advances, the volume of blood flowing through the uterine arteries is increased in order to supply the increasing demands of the developing new organism. Therefore, alteration of the blood flow dynamics also represents a potential way to improve the reproductive process in equines.

Pregnancy Rates and Blood Flow

To our understanding, studies about the role of aspirin (acetylsalicylic acid) supplementation over the pregnancy rates in the bovine have not been reported. However, other drugs from the same family (non steroidal anti-inflammatory drugs; NSAIDs) have been evaluated in this regard. Using 100 animals, Elli et al. (2001) found that an ibuprofen lysinate injection (5 mg/kg BW) 1 hour before embryo transfer significantly increased the pregnancy rates in heifers. They found pregnancy rates of 82% (41/50) and 56% (28/50) for the ibuprofen lysinate and control groups, respectively. Merrill et al. (2004 and 2007) have reported in different studies with hundreds of Angus crossbred cows that treatment with flunixin meglumine (1.1 mg/kg of body weight, approximately 14 days post artificial insemination) resulted in pregnancy rates of 68-73% and 58-64% ($P < 0.05$) for treated and control animals, respectively. Therefore, the partial inhibition of the COX enzymes by these drugs has significantly improved the reproductive process, probably through an enhancement in the vascular perfusion status in the female reproductive organs.

Aspirin (Acetylsalicylic Acid)

Aspirin is an analgesic, antipyretic, anti-inflamatory, and antiplatelete (inhibit thrombus formation) drug (Friend, 1974) widely used by humankind and classified as a non-steroidal anti-inflammatory drug (NSAID; Cerhan et al., 2003). Its active ingredient, acetylsalicylic acid has the capacity to inhibit the cyclooxygenases also known as PGHS-1 and -2; COX-1 and -2; or Cyclooxygenase-1 and -2 (Meade et al., 1993). The cyclooxygenases are the enzymes required for the synthesis of different compounds essential for the maintenance of the homeostasis in the organism (Jenkins, 1988; i.e., prostaglandins and thromboxanes) in a variety of processes including reproduction (Reese et al., 2001) and inflammation responses (Espey, 1994). The COX enzymes (Fig. 2) catalyze the formation of PGH2 from arachidonic acid (Gibb et al., 2000). Then, in reactions catalyzed by several specific synthases (i.e., PGE_2 Synthase, $PGF_{2\alpha}$ Synthase, Thromboxane Synthase, etc.) PGH2 is converted to the different prostaglandins and thromboxanes (Gibb et al., 2000). In a variety of cells all around the body as well as in the platelets in the circulating blood, it has been reported that aspirin (acetylsalicylic acid) irreversibly inactivates the cyclooxygenase activity of the COX enzymes by acetylating a Ser residue (Serine 529) and consequently blocking the enzyme's active site (Schor, 1997; Castella-Lawson et al., 2001; Nelson D. L., and M. M. Cox, 2005).

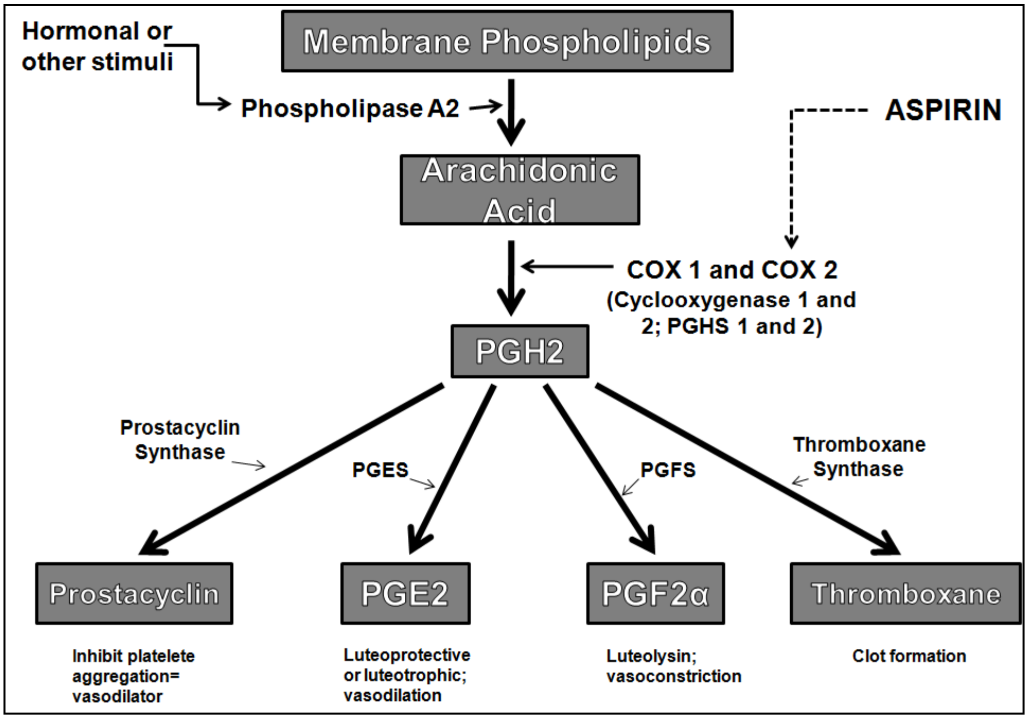

Figure 2 Summarized schematic representation of aspirin (acetylsalicylic acid) mode of action at the cellular level.

Notes: The enzyme Phospholipase A2 catalize the production of arachidonic acid from the phospholipids present in the cell membrane. Subsequently, the COX enzymes catalyze the reaction where PGH2 is produced from arachidonic acid. The PGH2 is then converted to different homeostasis-require compounds (i.e., prostaglandins and thromboxanes) through the action of specific synthases (i.e., PGE_2 Synthase, Thromboxane Synthase, etc.). One of these essential compounds is Thromboxane A_2, which is essential for platelete activation and recruitment during blood clot formation after injury or trauma to a blood vessel and has vasoconstrictor properties. Acetylsalicylic acid inhibition of the COX enzymes consequently results in the inhibition of the synthesis of Thromboxane A_2, which therefore result in a decrease in vascular resistance.

To understand how the inhibition of the synthesis of these molecules may affect the vascular perfusion in the body it is necessary to understand the basic role of Thromboxane A_2 in the blood clot formation.

During the event of injury to a blood vessel, a blood clot (thrombus or plug) formation is essential to avoid excessive bleeding (Rivera et al., 2009). In summary, this process occurs in three stages: initiation, extension, and stabilization of the thrombus (Rivera et al., 2009). During the ***initiation stage***, when the extracellular matrix components are exposed (i.e., collagen, fibronectin, laminin, etc.), platelets will adhere to the blood vessel wall creating a monolayer (Benjamin et al., 1999; Rivera et al., 2009). Second, in order to stop the bleeding, more platelets need to be recruited and aggregated from the circulating blood (***extention stage***; Rivera et al., 2009). The third phase is the ***stabilization stage*** where platelets come in a so close contact that they can "communicate" with each other (transfer of molecules) and avoid the diffusion of plasma factors (i.e., plasmin) that may affect the integrity of the plug until the damaged tissue is repaired (Rivera et al., 2009). For this aggregation to occur, platelets need to be activated so they can adhere to each other. This activation is mediated through the action of platelet agonist compounds produced or secreted by already activated platelets (Benjamin et al., 1999; Rivera et al., 2009). One of the molecules that plays an essential role in the activation and recruitment of platelets during a thrombus formation is Thromboxane A_2, a potent platelet agonist with vasoconstrictor properties (Benjamin et al., 1999; Rivera et al., 2009).

Therefore, the aspirin inhibition of the COX enzymes results in the blockade of Thromboxane A_2 synthesis (Rivera et al., 2009) with a subsequent inhibition of its vasoconstrictors and thrombus formation (platelet aggregation; Hennekens et al., 1989) properties, decreasing in this way the resistance to the blood flow in the vessels. Such a decrease in the vascular resistance may facilitate the flow dynamics in the vessels resulting in an enhanced vascular perfusion rate in the different organs of the body including the reproductive ones.

Temperament and Stress

In cattle, temperament has been defined as the behavioral response presented by the animal towards human contact or new environments (Fordyce et al., 1988 as reviewed by Burdick et al., 2011b). While some animals may be barely influenced by these experiences, others may react in a completely nervous and aggressive way. These aggressive, nervous animals are often referred to as "temperamental". For temperamental cattle, the normal management practices used in the production systems may result in a significant source of stress (Burdick et al., 2011b). Stress has been defined as a "disruption in homeostasis" (Miller and O'Callaghan, 2002). During such an event (stress or homeostasis disruption), the Hypothalamus-Pituitary-Adrenal (HPA) system is activated (Miller and O'Callaghan, 2002). This stress response is responsible for the physiological and metabolic changes required to cope with such a homeostatic challenge (Miller and O'Callaghan, 2002). In the presence of a stressor, the HPA axis initiates the release of corticotrophin releasing hormone (CRH) from the hypothalamus (Miller and O'Callaghan, 2002). The released CRH results in the subsequent release of adrenocorticotropin hormone (ACTH) from the anterior pituitary to the general circulation (Miller and O'Callaghan, 2002). The ACTH acts on the adrenal cortex resulting in the release of glucocorticoids (i.e., cortisol) also in to the blood circulation (Miller and O'Callaghan, 2002). These glucocorticoids are able to regulate the CRH release from the hypothalamus through a negative feedback mechanism (Miller and O'Callaghan, 2002).

One of the effects of the increase in the concentrations of glucocorticoids in the blood is an increase in blood glucose, necessary for providing tissues with a source of energy to respond to such a stressful situation (Miller and O'Callaghan, 2002). Also, increased cortisol concentrations have been associated with decreased volume of blood flow towards the skin (Walker at al., 1979) and visceral organs (Panaretto, 1974), while the volume of blood flow is increased in the central nervous system (Ahs et al., 2006) and skeletal muscles (Larsson et al., 1995). In fact, it has been established that during the "fight or flight" reaction, blood containing glucose and oxygen is redirected from the skin and visceral organs towards the central nervous system, heart, and skeletal muscles in order to sustain such a response (Sapolsky, 1990). Therefore, it may be reasonably assumed that the temperament of the animal has potential to interfere with other physiological processes including those regulating animal's production and reproduction.

Cattle Temperament and Performance

Normal management practices may represent a significant source of stress for temperamental animals (Burdick et al., 2011b). Such practices have been reported to negatively impact the animal's productivity (Burdick et al., 2011b), reproduction (Phocas et al., 2006) and meat yields (Fordyce et a., 1988) in temperamental bovines. It has been found that temperamental animals present lower daily body weight gains than their calmer counterparts (Voisinet et al., 1997; Hoppe et al., 2010). Moreover, Phocas et al. (2006) reported a trend toward a better reproductive and calving performance as well as higher maternal abilities in calm cattle in comparison with temperamental animals. Even more, carcasses from temperamental cattle has been reported to require more trims due to bruised tissue during transportation to the slaughter house as well as a tendency to produce less tender meat than calm animals (Fordyce et a., 1988). Therefore, due to its potential negative impact over the beef cattle industry, temperament is a variable that has received special attention during time. In fact, special effort has been applied to the development of classifications methods that allow for identification of different temperaments in cattle in an efficient and repeatable way.

Temperament Classification Methods

Because temperament has the capacity to influence the productive (Fordyce et a., 1988; Voisinet et al., 1997) and reproductive (Phocas et al., 2006) performance in cattle, multiple methods that allow for the classification of the animals as calm, intermediate or temperamental have been developed. For the purpose of this dissertation, only the ones that applied to our study will be described; including the exit velocity (EV), the pen score (PS), and the temperament score (TS). The EV is the rate at which the animals traversed a distance of 1.83 m after released from a working chute (expressed at m/s; Burrow et al., 1988; Curley et al., 2006). Because temperamental animals are expected to leave the chute in a faster way than calm ones, the greater the numerical EV value (greater velocity), the more temperamental the classification this animal receives, and vice versa. For the PS, calves are confined in small groups (n = 3 to 5) in a pen and their response to a human presence is visually evaluated (Hammond et al., 1996). Several scales have been applied in order to assign a "temperament value" to the animal. In our study, the PS was based on a scale of 1 to 5, where 1 represented a completely calm animal (docile, allows human approach), and 5, an extremely temperamental one (very aggressive, "crazy", often runs at fences, gates and even humans). The EV can be assessed visually or measured by means of infrared sensors in an instrument (FarmTek Inc., North Wylie, TX). When the EV is determined by infrared sensors (as in our study) as the distance traveled by the animal as a function of time, this classification method provides objective values. However, the PS depends on the visual opinion of an observer, and therefore, may be considered a subjective value or classification method. Other disadvantages of both methods are that while there is always potential for the EV to be influenced by uncontrolled external factors, the PS may be highly dangerous for the observer, especially when approaching highly excitable cattle. Subsequently, both values (EV and PS) have been combined by means of a simple mathematical equation in order to obtain an index that combines the best attributes of both classification methods while an attempt is made to minimize the negative aspects in each one. As a result of this combination, the TS has been developed (Burdick et al., 2010). The TS is calculated by means of the following formula:

$$TS = (EV + PS)/2, \text{(Burdick et al., 2010).} \quad \text{(Eq. 4)}$$

After the TS is calculated, the calves can be classified in different temperament groups following a similar approach to the one used by Burdick et al. (2011a). Animals under one standard deviation (SD) lower than the mean TS for the studied population are considered "calm"; whereas those animals above one SD over the mean TS are classified as "temperamental". The remaining animals in the studied population are considered "intermediate".

Conveniently, beef cattle have been commonly classified for temperament during the pre-weaning and weaning periods, when animals are normally handled (i.e., for vaccinations and weaning related purposes, respectively). Therefore, for these classifications to be useful later in the life of the animal, a high degree of repeatability over time is essential. In fact, Burdick et al. (2011a) reported that the EV may allow for temperament classifications early in life (i.e., pre-weaning) that are viable indicators of this variable later in the life of the animal, especially in temperamental cattle. Curley et al. (2006) reported positive significant correlations between serial measurements of EV (r > 0.31; $P < 0.02$) and PS (r > 0.31; $P < 0.01$) in Brahman bulls. Moreover, Café et al. (2011) reported correlations between 0.41-0.52 ($P < 0.001$) for serial EV measurements in Brahman cattle. Therefore, a considerable degree of repeatability has been established in some of these classification methods. However, because there is always space for some uncontrolled external factor to affect these classification methods, the determination and implementation of physiological variables that may act in its support have also received special attention.

Physiological Indices of Temperament

As aforementioned, due to the possible several disadvantages that the already existent temperament classification methods may present, the measurement of physiological variables such as serum cortisol concentrations (SC) and rectal temperature (RT) has been applied as supportive values.

Temperamental cattle have been found to present greater SC than calm animals (Curley et al., 2006; Stahringer et al., 1990). Stahringer et al. (1990) reported greater ($P < 0.04$) SC in temperamental than in calm Brahman heifers and a positive correlation between this hormone and the TS (r = 0.65; $P < 0.0006$). Curley et al. (2006) also observed, in yearling Brahman bulls, greater ($P = 0.008$) SC in temperamental than in calm animals. In their study, cortisol was positively correlated with the PS (r = 0.29; $P < 0.05$) and the EV (r = 0.26; $P < 0.05$). Therefore, SC may be considered a viable and reliable physiological index that may help not only to assess the stress response, but also in the corroboration of the temperament classification of the animal.

Burdick et al. (2010) reported greater RT in temperamental than in calm Brahman bulls as a response to transportation. Normal management practices may represent a significant source of stress for temperamental cattle (Burdick et al., 2011b). During the stress response or as a result of high physical activity levels (as evidenced by greater EV values), temperamental animals will make physiological adjustments to redirect a significant portion of their volume of blood flow from the visceral organs toward the skeletal muscles and central nervous system (Sapolsky, 1990). Those changes in the blood flow dynamics from the core body to the peripheral organs are essential to sustain such an increased rate in muscular activity which may result in energy loss as heat and a subsequent increase in core body temperature. Therefore, the RT may be also considered a reliable physiological index that may help in the assessment of the stress response, as well as in the corroboration of the temperament classification of the animal.

Bovine Somatotropin

Somatotropin (growth hormone) is a protein hormone that is normally produced in the anterior pituitary gland and transported in the blood to specific target tissues in the body where it regulates important metabolic processes (Bauman, 1992). When exogenously administered to lactating dairy cows, the bovine somatotropin (bST) "orchestrate" a wide variety of metabolic changes in the body tissues which subsequently result in a more efficient use of nutrients for milk synthesis, enhancing milk yield and persistency (Bauman, 1992). Bovine somatotropin can be extracted from bovine pituitary glands from slaughterhouses (with a low efficiency as was done in the past) or can be produced by recombinant DNA technology from bacterial ribosomes (as is commercially done; Bauman, 1992). Therefore, the recombinant version of this hormone (rbST) has been commercially available for years (i.e., Posilac by Monsanto) for use in dairy production farms where it enhances milk production and persistency in part due to an increase in vascular perfusion to the mammary gland.

A series of studies performed during the 80's and 90's using direct infusions to the pudic arteries (the main arteries supplying the right and left sides of the mammary gland independently) in sheep and goats helped to achieve a better understanding of how exogenous bST is able to increase blood flow in the mammary gland. Mc Dowell et al. (1987) found no increase in mammary gland blood flow as a result of bST infusion to one pudic artery in lactating goats and sheep in comparison to the control side (infused with saline solution as a placebo). However, Prosser et al. (1993 and 1994) reported that IGF-I infusion to one pudic artery of lactating goats resulted in both, a milk yield and a blood flow increases in the infused side of the mammary gland in comparison with the control side (saline solution as a placebo). Later, it was reported that infusion of diethylamine NONOate [a nitric oxide (NO) donor] to one pudic artery of lactating dairy goats induced a rapid and sustained increase in blood flow to the infused side of the mammary gland in comparison with the control side (Lacasse et al., 1996). However, infusion of N-nitro-arginine (an inhibitor of NO synthesis) decreased the blood flow in the infused side by 35%, but re-infusion of arginine (the NO precursor) to the already N-nitro-arginine treated side reestablished the blood flow values (Lacasse et al., 1996). Therefore, the increase in mammary blood flow observed after exogenous bST administration is not directly mediated by the growth hormone, but by the effect of the IGF-I that is produced as a response to the bST treatment and by a subsequent production of NO.

Administration of exogenous bST in lactating dairy cows has been found to increase milk yield, serum IGF-I concentrations, and hepatic IGF-I mRNA (Sharma et al., 1994). In fact, as a result of bST administration to dairy cows, an increase in IGF-I concentrations can be measured in the animal's biological fluids (Daxemberger et al., 1998) including plasma (Schams et al., 1991), serum (Sharma et al., 1994) and milk (Zhao et al., 1994). This IGF-I produced in the liver because of the action of bST, subsequently target different tissues in the body including the mammary gland (Sharma et al., 1994).

Insulin-like growth factor I has been reported to play an essential role in the regulation of the cardiovascular system function (Vecchione et al., 2001) because of its vasodilatory effects (Perticone et al., 2008). In human kidneys, IGF-I stimulates a rapid increase in NO production (Tonshff et al., 1998). Therefore, the vasodilatory effects of IGF-I may be mediated through NO and some vasodilatory prostaglandins (Tonshff et al., 1998). Moreover, Vecchione et al. (2001) have reported that the vasodilation produced by IGF-I in rats is blunted by the inhibition of endothelial NO Synthase. In fact, vascular tone has been reported to be regulated by the effect endothelial NO (Vecchione et al., 2001) which regulates the cyclooxygenase (COX) activity with a subsequent prostaglandins production (Salvemini, 1997) including PGE_2 which is known to be a potent vasodilator (Clyman et al., 1978).

Nitric oxide, as a biological messenger, is produced in one cell, diffuse through cell membranes, and regulates the function of other cells (Dixit and Parvizi, 2001). It regulates biological activity through reversible reactions with available functional groups (i.e., Fe^{2+} and thiols) in some proteins which results in the release of a ligand with subsequent activation of the protein (i.e., enzymes; Salvemini et al., 1993; Dixit and Parvizi, 2001). Therefore, it has been reported that NO act at the cellular level by inducing an increase in the production of second messengers including guanosine 3’5’-monophosphate (cGMP; Dixit and Parvizi, 2001). High concentrations of cGMP have been reported to result in Ca^{2+} efflux from smooth muscle cells which facilitates blood flow in blood vessels because of the induced smooth muscle relaxation (Van Der Horst et al., 2003). Also, because most of the NO effects are mediated through interaction with iron containing enzymes and the COX isozymes (enzymes that catalyse the production of prostaglandins) contain an iron-heme center at its active site, they are potential targets for NO induced activation (Salvemini et al., 1993). This NO induced activation of the cyclooxygenase isozymes (COX enzymes) results in the up regulation of prostaglandins synthesis including PGE_2 (Salvemini et al., 1993; Dixit and Parvizi, 2001) which is a well known vasodilator (Clyman et al., 1978).

Therefore, the increase in blood flow to the mammary gland observed after exogenous bST administration may be an effect of the IGF-I (mostly from liver origin) induced NO production. The COX enzymes activation and the secondary messengers activated by NO may play an essential role in this effect mostly by the production of vasodilatory prostaglandins that may regulate the diameter in the blood vessels of the mammary gland.

Infrared Thermography

Body temperature is a variable highly studied in a wide range of scenarios in animal science due to its ability to reflect the physiological status of the animal. However, traditional temperature recording devices require direct human-animal interaction which may introduce considerable bias in the obtained results. Therefore, alternative body temperature recording instruments that are not subjected to these limitations may provide more reliable thermal information in the study of different physiological processes in large domestic animals. Infrared thermography cameras measure the infrared radiation emitted by an object, including animals, which allows for surface temperature calculations while avoiding direct interaction between the assessed surface and the human technician (Speakman and Ward, 1998). Specialized cameras create images that reflect the thermal differences in the animal's anatomy by different shades in a color scale (McCafferty, 2007). Because of this ability to assess animal thermal status, infrared thermography allows not only for the assessment of different physiological processes, but also to diagnose disease and injuries (McCafferty, 2007).

As previously mentioned, for studies that require the determination of the thermal status in animals, the infrared radiation detected by infrared thermography cameras needs to be converted in to an estimation of the surface temperature (McCafferty, 2007). In order to obtain such surface temperature estimations, an emissivity value needs to be provided (McCafferty, 2007). Emissivity refers to the ability of an object to emit radiation, in other words, the heat that is dissipated from a surface through infrared radiation (Hildebrandt et al., 2010). Emissivity values of 0.98 and 0.98-1.00 have been established for bare skin and for dry fur in mammals (Monteith and Unsworth, 1990 as reviewed by McCafferty, 2007).

As aforementioned, probably, the most noticeable advantage of this technology when assessing the thermal status in animals is that it is non-invasive in nature, and it does not require direct human-animal contact (McCafferty, 2007). Moreover, this technology allows for considerably flexibility in regard the angle between the camera and the measured surface positions. For a surface with an emissivity of 0.98, if maintained bellow 90º, this angle will not considerably affect the margin of error observed in the obtained temperature values (Watmough et al., 1970).

Subsequently, as a research tool, infrared thermography represents a potential option for the study of different physiological processes that involve thermal changes in large domestic species; and what is more important, it may do it in a non-invasive way and with a minimum or non-existent degree of interaction with the animal.

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CHAPTER III

EVALUATION OF B-MODE ULTRASOUND UTERINE ARTERIAL DIAMETER AS AN ALTERNATIVE INDEX OF BLOOD FLOW TO THE REPRODUCTIVE TRACT IN BEEF COWS

Abstract

The use of B-mode ultrasound measurement of the uterine arterial diameter (UAD) and color Doppler ultrasound Resistance Index of the right external iliac artery (RIEIA) were evaluated as alternative indices of the blood perfusion status of the reproductive tract in 38 cycling Angus crossbred cows. The ultrasound measurements and jugular blood samples were collected at days (d) -10.5, -2.5, 0, 3, 6, 10, 16, 20, 25, and 32 (d 0 = artificial insemination d). The promoting effects of acetylsalicylic acid over the blood flow and the hormonal profiles [13,14-dihydro-15-keto prostaglandin E_2 (PGEM) and progesterone (P_4)] during the estrous cycle and early pregnancy were measured and used to evaluate these techniques. The UAD presented intra- and inter-individual Intraclass Coefficients of Correlation of 0.49 and 0.68 (moderate to good reproducibility), respectively. In general, acetylsalicylic acid supplementation resulted in an increase (P = 0.01) of the UAD. However, this drug did not affect overall serum P_4 concentrations (P = 0.12), but tended to increase serum PGEM concentrations (P = 0.06) in pregnant animals. In the same group (pregnant cows), positive correlations were observed between UAD and the serum PGEM concentrations (r = 0.48; P = 0.0003; d -10.5-32);

and serum PGEM and P4 concentrations ($r = 0.33$; $P = 0.02$; d 6-32). However, the RIEIA and the UAD were not correlated ($P = 0.37$). In general, in the present study the use of the UAD obtained from frozen B-mode ultrasound images was a technique sensitive enough to be associated with important vasodilatory changes related to the supplementation of low daily doses of acetylsalicylic acid and the hormonal profiles during the different stages of the estrous cycle and the early pregnancy with a moderate to good reproducibility. According with our results, the RIEIA may not be considered an useful estimator of the reproductive tract vascular status in beef cows.

Keywords: Acetylsalicylic acid, Beef cow, Uterine arteries diameter, Blood flow

Introduction

Adequate blood perfusion to the female reproductive organs has been found to be essential for the success of the reproductive process in different species including the equine (Bollwein et al., 2004); human (Hsieh et al., 2000); and bovine (Ford, 1995). For this reason, Doppler ultrasound has been broadly used to study blood flow dynamics in the female reproductive tract of different large domestic species. However, this technology requires real-time live measures and in beef cattle the continuous movements of the animal (i.e., temperamental cows) greatly limits the applicability of this research tool. Also, in order to obtain volume of blood flow values, control of the angle between the ultrasound beam and the blood flow (insonation angle) is essential (Herzog and Bollwein, 2007), which represents a considerable level of difficulty especially in relatively small diameter vessels like the uterine arteries. Consequently, the establishment of alternative techniques that allow for effective and feasible measurement or estimation of this variable under those restrictive conditions is imperative. Because the volume of blood flowing in a vessel is directly and positively associated with its dimensions (Paniagua et al., 2001), B-mode measurements of the uterine arterial diameter (UAD) from frozen ultrasound images deserves special attention as such an alternative research tool.

Low daily doses of acetylsalicylic acid supplementation have resulted in increased blood perfusion to the genitals in women (Hsieh et al., 2000) and mares (Bollwein et al., 2004). Also, in ruminant reproductive vessels, prostaglandin E_2 (PGE_2) induces vasodilation (Reynolds, 1986) with a subsequent increase in blood flow which stimulates the development of healthy functional luteal structures (Arosh et al., 2004) able to produce high concentrations of progesterone (P_4) during the luteal stage of the estrous cycle and during pregnancy. Therefore, the differences in blood perfusion (and vessel dimensions) to the bovine reproductive organs associated with supplementation of low daily doses of ASA, the estrous cycle, and early pregnancy and the respective hormonal profiles may allow for the evaluation of such an alternative technique.

To our understanding, the UAD has not been intensively used to study and evaluate the changes in blood perfusion of the reproductive organs in beef cows. Therefore, the objectives of the present study were: (i) to study the effects associated with acetylsalicylic acid supplementation and the hormonal profile changes that occur over time within the different stages of the estrous cycle and early pregnancy in beef cows; (ii) to use those effects and changes to evaluate the feasibility of B-mode measurements of the UAD as an index of the blood flow to the reproductive organs in beef cows; (iii) to estimate the reproducibility of this technique; and (iv) to study if the blood flow in major arteries in the body (i.e. external iliacs) can be used as an index of the perfusion of the reproductive organs.

Materials and Methods

Animal Handling

Thirty-eight cycling multiparous Angus crossbred cows at the Mississippi Agricultural and Forestry Experiment Station, Brown Loam Branch, Raymond, MS were utilized in this experiment in compliance with the Institutional Animal Care and Use Committee of Mississippi State University (IACUC Approval Number 10-011). The study was conducted in 3 trials of 54 days (d) each between June and December 2010. Water, hay, and a mineral block were provided *ad libitum*. Animals were fed and kept in pens with a soil floor and artificial shade close to the working facilities where all the samples were collected.

Treatments

After an adaptation period to of two weeks, animals were divided in two treatment groups. One of the groups [ASA group; n = 19; 4.21 ± 1.18 yr old; 576.67 ± 43.73 kg of BW (mean ± SD)] received 2,500 mg/d/cow of acetylsalicylic acid top dressed in the feed (5 g of aspirin in powder with molasses flavor; AniPrin F, A. H. C Products Inc, Winchester, KY), from d -9.5 to 45 (d 0 = d of AI). The remaining group [Control group; n = 19; 4.32 ± 1.25 yr old; 560.41 ± 49.40 kg BW (mean ± SD)] received 5 g/d/cow of dry molasses in flakes as a placebo top dressed in the feed during the same period. Briefly, cows were individually penned at 07:00–08:00 and at 19:00–20:00 h everyday and 1 kg of sweet feed (12% C.P., Crossroad all-stock CRS sweet 12, Land O'Lakes

Purina, Shoreview, MN) containing half of the daily treatment was administered at both periods in a 58.5 L rubber feeder.

Sampling

Using cattle handling facilities close to the feeding pens, cows were restricted in a hydraulic chute and sampled (ultrasound and jugular blood) during d 0, 3, 6, 10, 16, and 20 (d 0 = d of AI; Fig. 3) in representation of the estrus, luteal (early I and II, mid and late), and follicular phases of the bovine estrous cycle, respectively (Miyamoto et al., 2000 and Murakami et al., 2001). Additionally, two pre- (d -10.5 and -2.5) and two post- (d 25 and 32) estrous cycle additional samples were recorded.

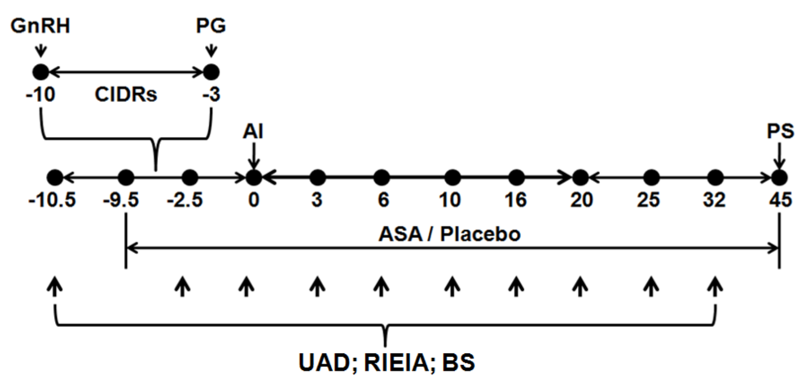

Figure 3 Experimental treatments and sampling schedule.

Notes: All cows were synchronized using an Eazi-Breed CIDR insert (1.38 g of progesterone; Pfizer Animal Health, New York, NY) from d -10 to -3 (d 0 = AI d). As part of the synchronization protocol, cows were treated with GnRH (100 µg, i.m.; Cystorelin, Merial, Athens, GA) and PG (25 mg, i.m.; Lutalyse, Pfizer Animal Health, New York, NY) at d -10 and -3, respectively. All cows were artificially inseminated by the same technician and with semen from the same proven fertility bull. All cows were AI at 72 h post CIDR removal and treated with GnRH (100 µg, i.m.; Cystorelin, Merial, Athens, GA). Treatments (ASA, 2500 mg/d of acetylsalicylic acid in powder with molasses flavor per cow; and Control, 5g/d of dry molasses in flakes in a per cow basis) were administered as a top dress in the food twice daily at 07:00-08:00 h and 19:00-20:00 h from d -9.5 to 45 (d 0 = AI d). B-mode uterine arterial diameter (UAD), Resistance Index of the right external iliac artery (RIEIA) and blood samples (BS) were recorded on d -10.5, -2.5, 0, 3, 6, 10, 16, 20, 25, and 32 (vertical arrows; d 0 = AI d). Pregnancy status (PS) was verified by transrectal ultrasonography at 45 d post-AI.

Synchronization and Artificial Insemination

All cows received an Eazi-Breed CIDR insert (1.38 g of progesterone; Pfizer Animal Health, New York, NY; Fig. 3) from d -10 to -3 (d 0 = AI d). Animals were also treated with GnRH (100 µg, im; Cystorelin, Merial, Athens, GA) and $PGF_{2\alpha}$ (25 mg, im; Lutalyse, Pfizer Animal Health, New York, NY) on d -10 and -3, respectively. All cows were artificially inseminated (AI) at 72 h post CIDR removal, followed by a second GnRH treatment. All cows were AI by the same technician and with semen from the same proven fertile bull. Pregnancies were confirmed at d 45 post-AI by transrectal ultrasound examination (SonoSite, M-Turbo ver. 1.2.6. equipped with a L52X/10-5 MHz transducer, Bothel, WA) by the same technician.

Doppler and B-mode Ultrasound

The dimensions of the uterine arteries, the main blood supply to the uterus, were measured by B-mode ultrasound (Fig. 4) in a subsample of 16 cows randomly selected (n = 8 per treatment). To find the uterine arteries, a similar approach to the one used by Bollwein et al. (2000) was adopted. Briefly, by means of transrectal sonography and with the ultrasound probe oriented dorsally, the abdominal aorta was easily identified. By moving the probe distally along the abdominal aorta, first the external, and second the internal iliac arteries branches were located. Each internal iliac artery branches to a common stem for the umbilical and the uterine arteries. Following this description, cross-sectional B-mode ultrasound images of both uterine arteries, with the probe oriented to the side of interest (right or left) immediately frontal to the body of the ilium and close to its bifurcation with the umbilical artery, were obtained. From the frozen images the dimensions, including diameter, were measured using spherical calipers (Fig.

4). Because of the differences in arterial luminal dimensions during the systolic and diastolic phases of the cardiac cycle, three consecutive measurements were recorded in each uterine artery (Honnes et al., 2008) always beginning with the right side and the average (per side) was used for the statistical analysis. All Ultrasound measurements were carried out by the same operator using a SonoSite, M-Turbo ver. 1.2.6. equipped with a L52X/10-5MHz transducer in B-mode (Bothel, WA). During the complete sampling period, the uterine artery had overall mean cross-sectional values of diameter, area, and circumference of 4.48 ± 0.03 mm, 10.84 ± 0.25 mm^2, and 12.06 ± 0.08 mm, respectively.

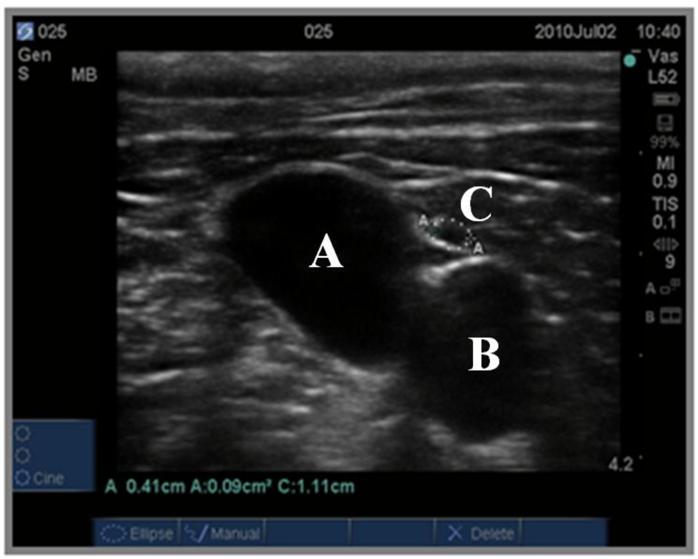

Figure 4 Cross-sectional B-mode ultrasound image of the external iliac vein (A), the external iliac artery (B), and the uterine artery (C) in a cycling beef cow.

Notes: Dimensions (diameter, area, and circumference) of the uterine artery appear at the bottom left of the image in green font. Note the white dotted spherical caliper around the uterine artery.

Following a similar approach to the one previously described to find the uterine arteries, cross-sectional color Doppler ultrasound (SonoSite, M-Turbo ver. 1.2.6. equipped with a L52X/10-5MHz transducer, Bothel, WA) was performed to monitor the blood flow in the right external iliac artery in a randomly selected subsample of 12 cows (n = 6 per treatment group). After obtaining three uniform consecutive pulse waves, the image was frozen and the blood flow was characterized by the Resistance Index (RI). The RI is a measure of the impedance to the blood flow distally to the sampling point (Herzog and Bollwein, 2007). The RI (Fig. 5) was calculated by the machine using the following formula:

$$RI = (S-D)/S; \text{ (Herzog and Bollwein, 2007)} \qquad \text{(Eq. 5)}$$

where *S* is the maximum systolic frequency shift and *D* is the end diastolic frequency shift of the cardiac cycle (Fig. 5). Measures of RI in the right external iliac artery (RIEIA) were also performed in triplicate from three uniform consecutive pulse waves and the average was used in the statistical analysis.

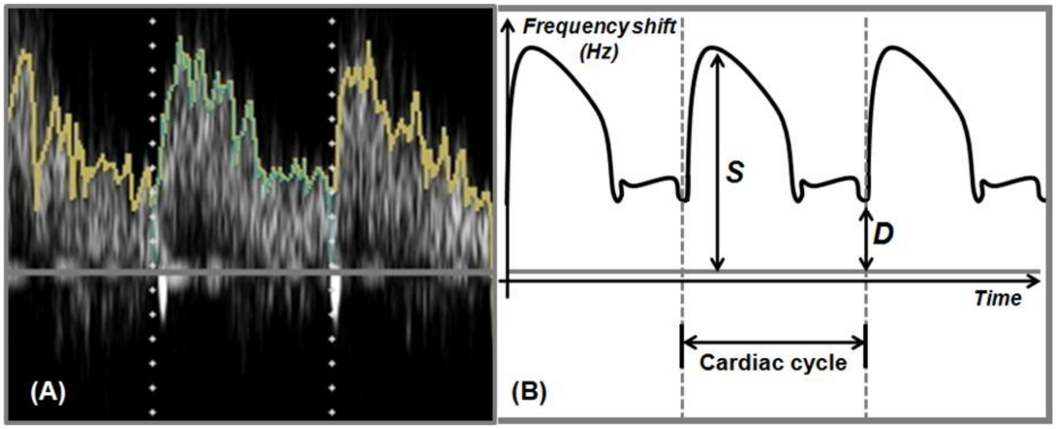

Figure 5 (A) Real time color Doppler spectral waveforms of a right external iliac artery during three consecutive cardiac cycles in a beef cow; (B) Schematic representation of the same color Doppler spectral waveforms.

Notes: Note the calipers (two white (A) and gray (B) dotted vertical lines) selecting the waveform of interest. The frequency shifts represented by *S* and *D* are the maximum during systole and minimum during diastole, respectively.

Blood Samples and Hormones

On the same subsample of 16 cows where B-mode UAD was measured, jugular vein blood samples (8.5 mL in Vacutainer, SST, Clot Activator; Becton Dickinson, Franklin Lakes, NJ) were collected each sampling day immediately after all other variables were recorded. Blood samples were centrifuged within 2 h after collection at 2,800 x *g* for 25 minutes at 5°C and the serum was recovered and stored in individual vials at -20°C until analysis. Serum P_4 concentrations (in duplicate) were measured by RIA (Coat-A-Count Progesterone, Siemens Healthcare Diagnostics, Los Angeles, CA). Tubes containing 0, 0.1, 0.5, 2, 10, 20, and 40 ng/ml of P_4 were used for calculation of the standard curve. An intra-assay CV of 3.5% was obtained. *In vivo* PGE_2 is quickly converted to its metabolite, 13, 14-dihydro-15-keto prostaglandin E_2 (PGEM). Therefore PGEM concentrations (in triplicate) were measured by enzyme immunoassay kits

(Cayman Chemical Co., Ann Arbor, MI). Wells containing 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, and 50 pg/ml of PGEM were used for calculation of the standard curve. Non-extracted samples were diluted 1:2 with assay buffer immediately before the assay was performed. Intra and inter-assay CVs of 13.06 and 13.47% were obtained, respectively.

Statistical Analysis

All statistical analyses were performed using SAS (SAS Inst., Inc., Cary, NC). Normality was tested using the Shapiro-Wilk statistic of the Univariate procedure (PROC UNIVARIATE of SAS). No differences ($P > 0.05$) were found between trials; therefore the data from the three trials were combined for the statistical analysis. Also, because differences in diameter between the right and left uterine arteries were not found ($P = 0.31$), an average of both sides was used in the statistical analysis. The UAD, RIEIA, P_4, and PGEM were analyzed by the General Linear Model procedure (PROC GLM of SAS). All models include: treatment, sampling day, pregnancy status, and the respective interactions as independent variables. Differences between means were assessed by the LSD test of SAS. Serum P_4 concentrations in non-pregnant and pregnant cows (individually) were subjected to regression analysis with respect to sampling day to determine the best fit curve using linear and quadratic models in PROC GLM in SAS. The correlation procedure (PROC CORR of SAS) was used to generate Pearson correlations to determine the relationship between UAD, RIEIA, P_4, and PGEM. The Chi Square test of the FREQ procedure of SAS was used to study the relationship between the treatments and the pregnancies in the present study. Significant differences were detected at a P-value ≤ 0.05, whereas a P-value between 0.06 and 0.10 was considered a tendency.

Reproducibility of the Uterine Artery B-mode Diameter Measurements

A SAS macro previously published by Lu and Shara (2007) was modified to study the reproducibility of the use of B-mode ultrasound measurements of the UAD as an index of blood perfusion to the reproductive organs. The Intraclass Coefficient of Correlation (ICC), one of the most used measures of reproducibility or reliability in quantitative variables (Lu and Shara, 2007), and the respective lower and upper confidence intervals were calculated. Briefly, the ICC assumes values in the range of 0-1, the closest to 1, the highest the reproducibility or reliability of the measure (Jung et al., 2011). Nagy et al. (2009) has described as good reliability an ICC value in the range of 0.5-0.6. Intraclass coefficient of correlation values in the ranges of 0.50-0.59, 0.60-0.79, 0.80-0.89, and 0.90-1.00 have previously been classified as moderate, good, very good, and excellent agreement or reproducibility, respectively (Weber et al., 2009).

Results and Discussion

Reproducibility of the Uterine Artery B-mode Diameter Measurements

Doppler ultrasound has broadly been used to study the blood flow in the female reproductive tract of different species. However, this technology requires real-time live measures which are considerably difficult to obtain in real farm scenarios where animals move during sampling (i.e., beef cattle). Moreover, in order to obtain volume of blood flow values, control of the angle between the ultrasound beam and the direction of the blood flow (insonation angle) is essential (Herzog and Bollwein, 2007) which is also complex in the farm environment. All these conditions are even harder to achieve in relatively small diameter vessels like the uterine arteries. Therefore, this tool may not be feasible for repeatable use in some of the real scenarios present in animal production including excitable beef cattle. For these reasons, in the present study the measure of the UAD from frozen B-mode ultrasound images as a direct index of vasodilation, and indirect of blood flow, was evaluated.

The Intraclass Coefficient of Correlation, one on the most used measures of reproducibility or reliability in quantitative variables (Lu and Shara, 2007) and the respective lower and upper confidence intervals were calculated in the B-mode UAD data. The intra-individual reproducibility of the UAD measurements, expressed as ICC, was 0.49 and ranged between 0.40 and 0.57 ($P = 0.003$). For the inter-individual reproducibility, an ICC of 0.68 with a confidence interval between 0.61 and 0.74 ($P < 0.0001$) were obtained. Nagy et al. (2009) has described as good reliability an ICC value in the range of 0.50-0.60, while Weber et al. (2009) classified ICC values in the ranges of 0.50-0.59, 0.60-0.79, 0.80-0.89, and 0.90-1.00 as of moderate, good, very good, and excellent agreement or reproducibility, respectively. Therefore, even when more attention is needed in order to improve this technique; it may offer a viable and feasible way to assess vasodilation as an index of blood flow in the reproductive tract of beef cows with moderate to good reproducibility and more adapted to real farm conditions.

Uterine Arteries Diameter

There were no differences ($P = 0.31$) between the right and left side UAD, therefore, an average of both sides was used for the statistical analysis. The overall UAD was greater ($P = 0.01$) in the ASA than in the Control cows (Fig. 6; 4.67 ± 0.04 and 4.54 ± 0.04 mm, respectively). Specifically, the UAD were larger in the ASA than in Control cows during the d -2.5 (pre-estrus; 4.61 ± 0.12 vs. 4.23 ± 0.12 mm; $P = 0.03$); d 0 (estrus; 4.52 ± 0.12 vs. 4.10 ± 0.10 mm; $P = 0.04$); and d 10 (mid luteal phase; 4.86 ± 0.18 and

4.41 ± 0.16 mm, respectively; *P* = 0.05). In the Control group, there was a reduction in UAD (*P* = 0.02) from d -10.5 to -2.5 (4.91 ± 0.11 to 4.23 ± 0.12 mm, respectively). From d -2.5 to 0 there was no change (4.23 ± 0.12 and 4.10 ± 0.10 mm, respectively; *P* = 0.38). After the AI (d 0), the UAD increases (*P* = 0.02) until d 32 (4.10 ± 0.10 to 4.98 ± 0.07 mm, respectively). In the ASA group, the UAD was maintained constant (*P* = 0.34) from d -10.5 to 6 (4.63 ± 0.12 to 4.59 ± 0.10 mm, respectively). After d 16 there was a general increase (*P* = 0.05) in the overall UAD for both treatments combined until d 32 (4.68 ± 0.07 to 4.99 ± 0.07 mm, respectively).

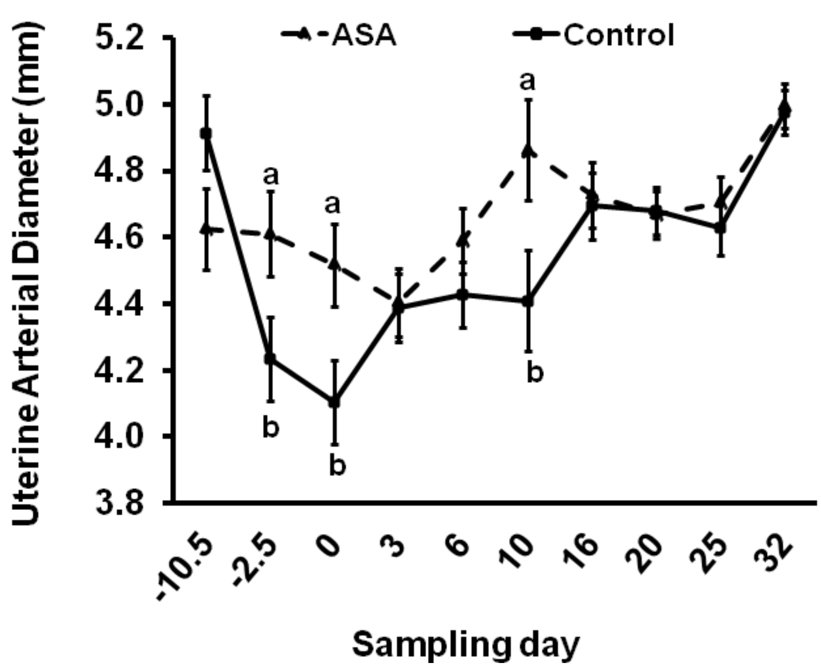

Figure 6 Uterine arterial diameter in beef cows as affected by low daily doses of acetylsalicylic acid supplementation.

Notes: Data presented as means ± SEM (mm). [a,b]Means within a sampling day with unlike superscripts differ (*P* < 0.05). ASA group cows (dash line; triangular dots) received 2,500 mg/d/cow of acetylsalicylic acid in 5 g of aspirin with molasses flavor; Control group cows (straight line; square dots) received 5 g/d of dry molasses in flakes as a placebo on a per animal basis. Treatments were administered from d -9.5 to 45. A sampling day effect was also observed (*P* = 0.01) in both treatments (d 0 = AI d).

A positive correlation ($r = 0.48$; $P < 0.01$) between the diameter and the volume of blood flow in the uterine arteries of pregnant cows has been previously reported in the literature (Bollwein et al., 2002). In fact, the dimensions of a vessel are positively and directly affected by the volume of blood flow (Paniagua et al., 2001). Therefore, in the present study the measurement of the UAD was evaluated as an index of vasodilation and, therefore, of the perfusion status to the reproductive organs during the estrous cycle and early pregnancy in beef cows. The time average maximum velocity (TAMV) is a Doppler ultrasound parameter that has been used in the study of the uterine perfusion because of its direct and positive association with volume of blood flow (Bollwein et al., 2000). Bollwein et al. (2000) reported a trend in the uterine arteries TAMV during the bovine estrous cycle similar to the one observed in UAD in our study. They found lowest and highest TAMV values during d 0-1 of the present, and d -3 to -1 of the next estrous cycle, respectively. Honnens et al., (2008) also reported a significant increase in the TAMV of the uterine arteries during the estrous cycle in cows. In the present study, during a similar period (from d 0-20) the Control cows presented an increase in UAD of 0.58 mm ($P = 0.02$). In the ASA group, during the same period the UAD increased only 0.15 mm ($P = 0.02$). This increment represents a 14 and 3.3% increase in UAD from d 0-20 for Control and ASA cows, respectively. Because by d 0 the ASA cows had already greater UAD ($P = 0.04$) than the Control ones, the smaller percent of increase in UAD in the ASA group may be the result of the already present vasodilatory effect of acetylsalicylic acid.

Resistance Index in the Right External Iliac Artery

From d 0-32 the overall RIEIA values did not change (*P* = 0.54; Fig. 7) over time. However, ASA cows presented greater (*P* = 0.04) mean overall RIEIA than the Control ones (0.78 ± 0.02 and 0.72 ± 0.02, respectively). Specifically, ASA cows presented greater RIEIA than the Control group during d 0 (estrus; 0.79 ± 0.06 vs. 0.61 ± 0.09; *P* = 0.04), 10 (mid luteal phase; 0.78 ± 0.06 vs. 0.61 ± 0.07; *P* = 0.03); and 16 (late luteal phase; 0.82 ± 0.05 vs. 0.63 ± 0.06; *P* = 0.03).

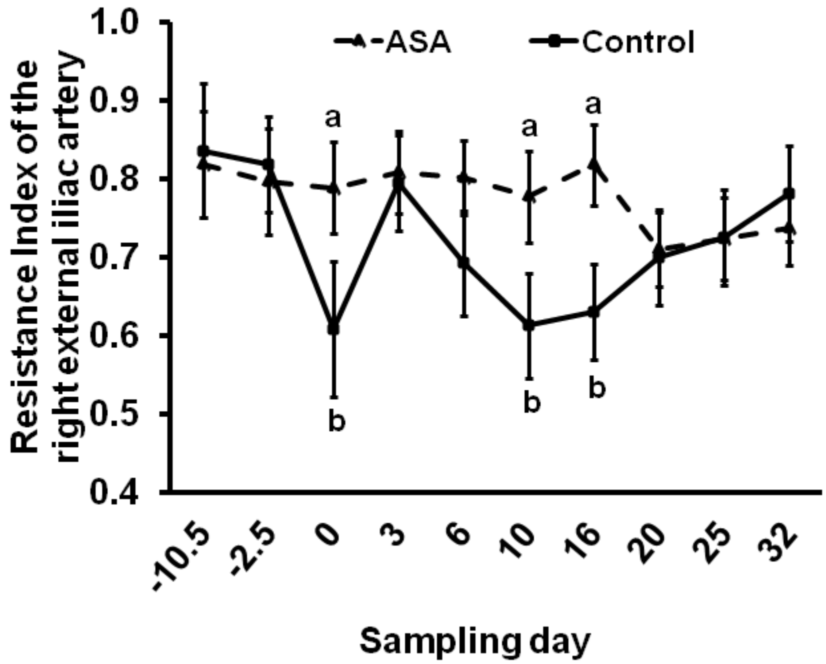

Figure 7 Resistance index in the right external iliac artery in beef cows as affected by low daily doses of acetylsalicylic acid supplementation.

Notes: Data presented as means ± SEM. [a,b]Means within a sampling day with unlike superscripts differ (*P* < 0.05). The ASA group cows (dash line; triangular dots) received 2,500 mg/d/cow of acetylsalicylic acid in 5 g of aspirin with molasses flavor; Control group cows (straight line; square dots) received 5 g/d of dry molasses in flakes as a placebo on a per animal basis. Treatments were administered from d -9.5 to 45 (d 0 = AI d).

The RI is a measure of the impedance to the blood flow distal to the sampling point and is negatively associated with the volume of blood flow (Bollwein et al., 1998 and 2002). Because of the acetylsalicylic acid properties, lower RIEIA values could be reasonably expected in the ASA group. In fact, the anticoagulant effect of acetylsalicylic acid has been found to significantly decrease the Pulsatility Index (a similar measure of distal blood flow resistance) in the uterine arteries of the mare (Bollwein et al., 2004). However, in our study, acetylsalicylic acid supplementation resulted in greater ($P = 0.04$) RIEIA values. This unexpected results in the RIEIA observed in the present study may be attributed to two possible reasons: first, to our relatively small population size; and second, to differences in blood flow regulation associated with the luminal size of the vessel. Zimmermann et al. (1997) also reported significantly higher RI values in the utero-placental arteries of pregnant women (24-36 weeks) treated with low daily doses of acetylsalicylic acid as a prophylactic for problems associated with inadequate blood flow to the reproductive tract during gestation. They were not able to attribute this phenomenon to any physiological explanation and concluded that it must be the result of chance due to a small population size. In bovine studies, the relatively long period of time required to sample one animal (20-40 min) greatly limits the amount of observations obtained in a homogeneous period of time. Therefore, the trends in RIEIA observed in our study may be attributed to the influence of any external uncontrolled factor. Moreover, vascular flow in large diameter vessels is mostly influenced by the cardiac output (Fowler, 2000). Therefore, the changes in blood perfusion due to such a low daily dose of acetylsalicylic acid (2,500 mg/d/cow) may be diluted in the circulation volume and velocity of a large diameter artery like the right external iliac. However, flow in small vessels (including capillaries) is mostly influenced by the blood viscosity (Fowler, 2000). Hagedorn et al. (1992) reported that 300 mg of acetylsalicylic acid administered orally were able to decrease blood viscosity and probably increase blood perfusion in horses. In a 10 year study of gastrointestinal bleeding involving 538 human patients, Nwose and Cann (2010) concluded that the viscosity of the blood has a significant negative relationship with the salicylic acid concentrations (an acetylsalicylic acid metabolite) in the blood. Thus, the possible increase in blood flow associated with the acetylsalicylic acid anticoagulant effect, may not substantially affect perfusion in the external iliacs. Therefore, our findings suggest that the blood flow dynamics in the right external iliac artery of beef cows may not be a direct index of the perfusion status in the smaller blood vessels that supply the female reproductive tract. However, studies involving a larger population size may decrease the observed variability in this variable and therefore, help to clarify this trend.

Pregnancies

Pregnancy rates of 73.68% (14/19) and 52.63% (10/19) were detected at 45 d post-AI in the ASA and Control cows, respectively ($P = 0.18$). Using 100 animals, Elli et al. (2001) found that ibuprofen lysinate (a non steroidal anti-inflammatory drug; NSAID) injection (5 mg/kg BW) 1 h before embryo transfer significantly increases the pregnancy rates in heifers. They found pregnancy rates of 82 (41/50) and 56% (28/50) for the ibuprofen lysinate and control groups, respectively. Merrill et al. (2004 and 2007) have found in different studies with hundreds of Angus crossbred cows that treatment with flunixin meglumine (another NSAID; 1.1 mg/kg of BW approximately 14 d post-AI) resulted in pregnancy rates of 68-73 and 58-64% ($P < 0.05$) for treated and control animals, respectively. Because pregnancy rate is a variable that can only assume two possible values, larger data sets (than the one evaluated in our study) are required in order to achieve significant statistical differences. However, the numeric similarities between our results and the values published by others (using other NSAID) suggest that the significant increase in B-mode UAD measured in the ASA group may potentially be affecting the reproductive process through an increase in volume of blood flow. Therefore, B-mode UAD deserves attention as a possible index of the blood perfusion to the reproductive organs in beef cows. Also, acetylsalicylic acid deserves consideration as a possible mean to enhance the reproductive performance in cows.

Serum Progesterone Concentrations

In our study, supplementation with 2,500 mg/d of acetylsalicylic acid was not enough to affect ($P = 0.12$) serum P_4 concentrations. However, Bollwein et al. (2004) reported a significant increase in the plasma P_4 concentrations in mares supplemented with the same daily dose of acetylsalicylic acid. The therapeutic dose of acetylsalicylic acid recommended in food animals by the International Veterinary Information Service is 200 mg/kg of BW per d (George, 2003), which is approximately 48 times greater than the dose used in our study. Subsequently, the small dose used in this study may not be enough to achieve a significant change in P_4 production in cattle. Therefore, these conflicting results on the effects of acetylsalicylic acid supplementation over the systemic P_4 concentrations may be the results of some inter species dissimilarities in the responsiveness of the CL to an increase in blood flow, or in the metabolism of acetylsalicylic acid. For this reason, further studies using higher doses of this drug in the bovine may be required.

However, as expected, there was an interaction ($P < 0.0001$; Fig. 8) between sampling day and pregnancy status for serum P_4 concentrations. No differences ($P = 0.15$) in the concentration of this hormone were observed between pregnant and non-pregnant cows before d 16. Serum P_4 concentrations sharply increased ($P < 0.0001$) in all cows from d 3-16. In the non-pregnant cow group the serum P_4 concentrations decreased ($P = 0.001$) from d 16-25 until a second increase ($P = 0.02$) was observed at d 32. However, pregnant animals maintained steady high ($P = 0.92$) serum P_4 concentrations after d 16. From d 16-32, pregnant cows had greater ($P < 0.0001$) serum P_4 concentrations than the non-pregnant ones.

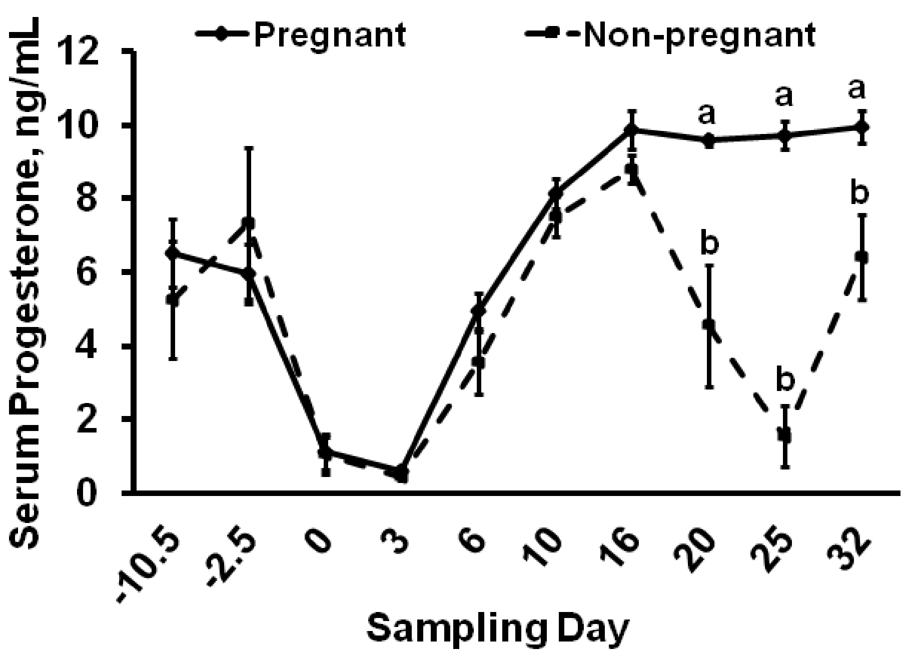

Figure 8 Serum progesterone concentrations during the sampling period in cows that resulted non-pregnant (dash line; square dots) and pregnant (straight line; diamond dots) at 45 d post-AI.

Notes: Data presented as LSmeans ± SEM (ng/ml). [a,b]LSmeans within a sampling day with unlike superscripts differ ($P < 0.0001$); Sampling day x pregnancy status ($P < 0.0001$; d 0 = AI d).

In non-pregnant cows, the change in serum P_4 concentrations from d 3-25 was best described by the following quadratic relationship ($P < 0.0001$) with a maximum peak value at d 16:

$$P_4 = -41.99 + 15.01\ d - 1.13\ d^2, \qquad \text{(Eq. 6)}$$

where P_4 is serum progesterone concentrations and d is sampling day. In pregnant cows, serum P_4 concentrations from d 3-25 were also best described by the following quadratic relationship ($P < 0.0001$):

$$P_4 = -28.69 + 9.83\ d - 0.62\ d^2, \qquad \text{(Eq. 7)}$$

where P_4 is serum progesterone concentrations and d is sampling day. Because the maximum serum P_4 concentration in the period between d 3-25 was observed for the first time at d 16 in pregnant and non-pregnant cows, the data set was further subdivided and analyzed in two sub-periods: d 3-16 and d 16-25. During these sub-periods, regression analyses were performed in both segments of the curve for pregnant and non-pregnant cows, individually. Both periods in non-pregnant cows were best described by linear relationships ($P < 0.0001$; Table 1). In pregnant animals, the period from d 3-16 was also described by a linear regression ($P < 0.0001$; Table 1). However, in pregnant cows, the regression line for serum P_4 concentrations upon sampling day during the sub-period of d 16-25 was not significantly different from zero ($P = 0.78$; Table 1).

Table 1 Coefficients of linear regression equations describing the serum P_4 concentrations in non-pregnant and pregnant cows during the sub-periods of sampling days 3-16 and 16-25.

Pregnancy status[a]	Sampling days	Intercept	*P*-value	Day Slope	*P*-value
Non-pregnant	3-16	-10.90	< 0.0001	2.90	< 0.0001
	16-25	34.04	< 0.0001	-3.64	= 0.0002
Pregnant	3-16	-11.14	< 0.0001	3.10	< 0.0001
	16-25	10.31	< 0.0001	-0.07	= 0.78

All linear regressions were significantly different from zero except the one for pregnant cows during sampling d 16-25. [a]Pregnancy status was based on d 45 post-AI pregnancy check.

Such an increase in serum P_4 concentration observed in pregnant and non-pregnant cows in our study after d 3 can be explained by the presence of a functional corpus luteum. In the bovine, ovulation occurs at approximately 26 h after the onset of estrus (Pinheiro et al., 1998) and gives rise to the development of the corpora lutea. Donaldson and Hansel (1965) reported that by d 4 of the estrous cycle, an early functional corpus luteum is already in existence in the bovine ovary. The postovulatory rise in P_4 associated with the presence of the luteal structure has been reported to begin at d 4-5 of the bovine estrous cycle (Mann et al., 2006) or around 5 d after ovulation (Opsomer et al., 1998). Therefore, the lack of differences in serum P_4 concentrations between pregnant and non-pregnant cows during d 3-16 may be explained by the presence of a functional corpus luteum in the ovaries of both groups of cows.

Also, in the bovine, maternal recognition of pregnancy (in the presence of a viable conceptus) or luteolysis (in the absence of the same) occurs at d 15-17 of the estrous cycle (Arosh et al., 2004). During this period and in the absence of a conceptus signal (Robinson et al., 2008 and Arosh et al., 2004), $PGF_{2\alpha}$ secretion by the endometrium will result in luteolysis (Robinson et al., 2008; Hansen et al., 1999; Mann et al., 1999) with a subsequent decrease in P_4 concentrations until a new estrous cycle begins. Therefore, the significant interaction between sampling day and pregnancy status observed in the present study may be explained by the fact that in cows that did not conceive or that underwent luteolysis during d 15-17 of the estrous cycle, the serum concentrations of P_4 significantly decreased after d 16; while in the pregnant cow's ovaries, a corpus luteum remained functional. This may also explain why in the non-pregnant group a second significant increase in serum P_4 concentrations associated with a new estrous cycle was observed between d 25-32. However, pregnant cows reached a maximum peak in serum P_4 concentrations of 9.87 ± 0.51 ng/ml at d 16 and maintained it for the remaining sampling period. Humblot et al. (1988) reported that after the initial rise in P_4 concentration observed in pregnant cows during the first three weeks of gestation, P_4 concentrations remain stable throughout the first third of gestation. This may also explain why even when both groups (non-pregnant and pregnant cows) serum P_4 concentrations fit a quadratic regression line from d 3-25, after d 16 pregnant cows regression line slope is not significantly different from zero.

Serum 13,14-dihydro-15-keto Prostaglandin E_2 Concentrations

The acetylsalicylic acid supplemented daily dose did not appear to affect (P = 0.40) the overall serum PGEM concentrations. However, in pregnant cows this drug resulted in a tendency (P = 0.06) toward greater serum PGEM concentrations in ASA cows. Pregnant ASA and Control cows presented overall serum PGEM concentrations of 86.84 ± 3.20 and 80.10 ± 2.76 pg/ml, respectively. In non-pregnant cows, serum PGEM concentrations in the Control group did not differ from those in the ASA group (P =

0.45). Acetylsalicylic acid exerts its effect through the inhibition of the COX enzymes (Meade et al., 1993) which catalyze the production of different compounds essential for the reproductive process (Espey, 1994) and the maintenance of the homeostasis in the body (Reese et al., 2001). Some of these compounds have vasoactive (vasodilation and vasoconstriction) properties including the prostaglandins and thromboxanes. In fact, the increase in blood flow associated with the acetylsalicylic acid inhibition of some of those compounds (vasoconstrictors, i.e. thromboxanes; Valdes et al., 2009) has been successfully used to assist reproduction in women with low vascular perfusion to the reproductive organs (Hsieh et al., 2000). However, in ruminants PGE_2 has luteotrophic and luteoprotective properties (Arosh et al., 2004) which makes it essential for the reproductive process and its complete inhibition may result in failure of the same (Reese et al., 2001). Therefore, complete inhibition of this hormone may result in impairment of the reproductive process in the bovine. However, our results suggest that daily supplementation with 2,500 mg of acetylsalicylic acid did not negatively affect jugular vein serum PGEM concentrations and, therefore, reproduction in beef cows.

In our study, there were no differences ($P = 0.81$) in overall serum PGEM concentrations between pregnant and non-pregnant cows. Therefore, the data from both research groups were combined to study the possible effect of sampling day over serum PGEM concentrations (Fig. 9). A sampling day effect ($P = 0.01$) was observed over the overall serum PGEM concentrations. From d -2.5 to 3 the overall serum PGEM concentrations were maintained steady ($P = 0.89$). From d 3-6 there was a decrease ($P = 0.04$) in the concentration of this hormone followed by a subsequent increase ($P = 0.002$) from d 6-20. After d 20 the serum PGEM concentrations were kept high ($P = 0.11$)

throughout the remaining sampling period. This overall increase in serum PGEM concentrations observed from d 6-20 may also occur simultaneously with the presence of a functional corpus luteum on the cow's ovaries. In fact, Cerbito et al. (1994) found greater ($P < 0.01$) PGE_2 concentrations in bovine endometrial tissue homogenates obtained during the luteal than during the follicular phase. During the luteal phase, the PGE_2 vasodilatory effects may promote the corpus luteum development and function in the bovine (Reynold, 1986). Reynold (1986) suggested that the increase in ovarian P_4 secretion during the days crucial for pregnancy maintenance may act as an amplifier of the conceptus vasodilatory "signal" (i.e., PGE_2). Thus, the observed tendency toward higher overall serum PGEM concentrations in pregnant animals in the ASA group may play an important physiological role in the conceptus survival and any further increase in vascular perfusion associated with acetylsalicylic acid supplementation may reinforce this effect.

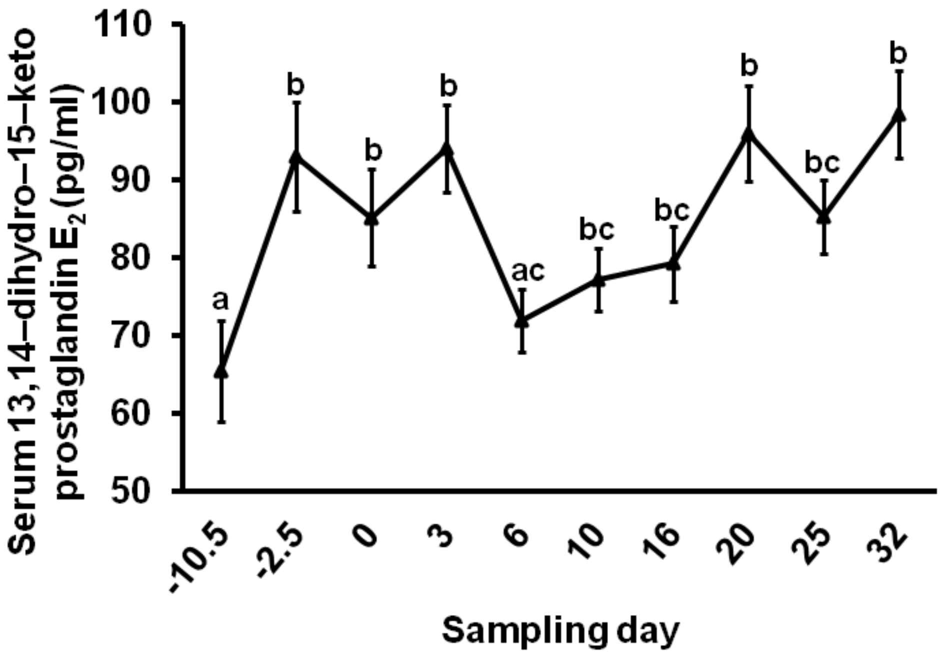

Figure 9 Overall serum 13,14-dihydro-15-keto prostaglandin E_2 concentrations during the sampling period.

Notes: Data presented as means ± SEM (pg/ml). [a,b,c]Means with unlike superscripts differ ($P = 0.01$; d 0 = AI d).

Relationships Between Serum Progesterone and 13,14-dihydro-15-keto Prostaglandin E_2 Concentrations, B-mode Uterine Arterial Diameter, and Resistance Index in the Right External Iliac Artery

A positive correlation ($r = 0.33$; $P = 0.02$) was observed between PGEM and P_4 in pregnant cows during d 6-32. There was also a low, but positive and significant correlation between serum PGEM and P_4 at this period ($r = 0.26$; $P = 0.01$) when pregnant and non-pregnant cows were analyzed together. However, in cows that resulted non-pregnant at 45 d post-AI, PGEM and P_4 were not related ($r = -0.02$; $P = 0.65$) during the same period.

There was a negative correlation between the RIEIA and the serum PGEM concentrations in the complete dataset (ASA and Control cows combined; $r = -0.21$; $P = 0.006$) and in the cows receiving the placebo ($r = -0.34$; $P = 0.001$). However, the RIEIA and serum PGEM concentrations in the ASA group cows were not related (r = -0.17; P = 0.13).

The UAD was positively correlated with the jugular serum PGEM concentrations [r = 0.24 (P = 0.004) and 0.48 (P = 0.0003); for the complete data set and for cows that resulted pregnant at 45 d post-AI, respectively]. In the group of cows that resulted non-pregnant at 45 d post-AI there was no relationship between serum PGEM concentrations and UAD (r = 0.19; P = 0.73).

During d 15-17 of the bovine estrous cycle the volume of blood flowing to the corpus luteum increases in parallel with the systemic P_4 concentrations (Reynolds, 1986). During this period, the conceptus is responsible for the production of vasodilatory signals that ensure its own survival (Reynolds, 1986). Prostaglandin E_2, a well known vasodilator (Neisius et al., 2002), has been signaled as one of those signals secreted in the early gravid uterus (Reynolds, 1986). In fact, high positive correlations (r = 0.60-0.70) have been previously reported between P_4 and PGE_2 during the estrous cycle in dairy cows (Cerbito et al., 1994). Therefore, during the luteal phase of the bovine estrous cycle, PGE_2 and P_4 may present a direct positive relationship. In our study, a positive significant relationship was observed between serum PGEM and P_4 concentrations during d 6-32 in pregnant cows. However, in non-pregnant cows there was no relationship between both hormones. Moreover, the significance of the low correlation values observed when all cows (pregnant and non-pregnant) were combined for analysis may be attributed to the hormonal concentrations in the pregnant cow group. Thus, our results support the idea that the PGE_2 vasodilatory effects may play a crucial role in sustaining a healthy functional corpus luteum able to maintain high P_4 concentrations and sustain early pregnancy in the bovine. This conclusion is reinforced by the fact that in the same animals (pregnant cows), PGEM directly and positively correlates ($r = 0.48$, $P = 0.0003$) with the UAD.

In the present study, Control group cows ($r = -0.34$, $P = 0.001$) and the complete group of cows (including ASA and Control animals; $r = -0.21$, $P = 0.006$) showed negative correlations between jugular vein serum PGEM concentrations and RIEIA. Besides the discrepancies in our RIEIA results, it has been established that the RI has a negative relationship with volume of blood flow (Bolwein et al., 2002). In other words, the higher the volume of blood flow in certain vessel, the lower the RI values and vice versa. The external iliac arteries branch directly from the abdominal aorta and supply a substantial part of the caudal anatomy of the animal (Budras and Habel, 2003). Therefore, because PGE_2 is well known for its vasodilatory effects (Neisius et al., 2002), it may be reasonably expected that an increase in the concentrations of this hormone in the systemic blood may directly result in a decrease in the general RI at least in the main vessels in the body (i.e., external iliacs).

Relationships between the Uterine Arterial Diameter and Resistance Index in the Right External Iliac Artery in Beef Cows

In our study, even when acetylsalicylic acid treatment resulted in higher UAD ($P = 0.01$) and RIEIA ($P = 0.04$), no significant relationship was found between both variables ($P = 0.37$). A possible explanation for this is the fact that even when the external iliac arteries supply a considerable portion of the caudal anatomy of the animal (Budras and Habel, 2003), the bovine uterine and umbilical arteries originate from a common stem that branches directly from the internal iliac arteries (Bollwein et al., 2000). Also, as aforementioned, the main factors affecting blood flow in large (i.e., external iliac) and small (i.e., uterine blood supply) vessels are the cardiac output and the blood viscosity, respectively (Fowler, 2000). Consequently, the blood flow dynamics in these two vessels is not directly connected and may be predominantly influenced by different factors. Thus, even when the large luminal dimensions and the location of the external iliac arteries allow for feasible Doppler ultrasound measurement, the obtained values may not serve as a good index of the vascular perfusion status in the reproductive organs of beef cows.

Conclusions

In general, the present study suggest that the B-mode ultrasound UAD measurement is a research tool sensitive enough to be associated with the vasodilatory effects of acetylsalicylic acid and with the hormonal profiles during the different stages of the estrous cycle and early pregnancy with a moderate to good reproducibility. Therefore, it is concluded that, in real farm conditions where Doppler ultrasound use may be restricted by the aforementioned limitations, the use of B-mode UAD represents a promising research tool for monitoring blood perfusion changes in the reproductive organs of beef cows. Also, based in our results, the RIEIA may not be a reliable predictor of the perfusion status in the uterine arteries in the bovine. Future studies must be directed to: (i) the improvement of the reproducibility of this technique, and (ii) to the comparison of the B-mode UAD with the already established Doppler ultrasound measurements in calm and docile cattle.

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CHAPTER IV

EVALUATION OF PULSATILITY INDEX AND DIAMETER OF THE JUGULAR VEIN AND SUPERFICIAL BODY TEMPERATURE AS PHYSIOLOGICAL INDICES OF TEMPERAMENT IN WEANED BEEF CALVES: RELATIONSHIP WITH SERUM CORTISOL CONCENTRATIONS, RECTAL TEMPERATURE, AND SEX

Abstract

The relationship between temperament, Pulsatility Index and diameter of the jugular vein, and body temperature was assessed in Angus crossbred calves (262.73 ± 24.94 days old). Temperament scores were used to classified calves as calm (n = 31), intermediate (n = 32), or temperamental (n = 28). Blood samples were collected for serum cortisol concentration analysis. Rectal, skin and hair coat temperatures were also recorded. The Pulsatility Index and luminal diameter corrected by BW (diameter) of the right jugular vein were measured via Doppler and B-mode ultrasound, respectively. Temperament and sex groups [heifers (n = 35) and steers (n = 44)] were compared. Temperament and sex did not interact to affect any of the studied variables. Cortisol (P = 0.016; 4.05 ± 0.30 and 2.97 ± 0.18 μg/dl) and rectal temperature (P = 0.012; 39.26 ± 0.13 and 38.87 ± 0.09°C) were greater in temperamental than in calm calves, respectively. Hair temperature was lower (P = 0.052) in calm than in temperamental calves (24.71 ±

0.60 and 26.07 ± 0.52°C, respectively). Calm and temperamental calves did not differ in skin temperature (P = 0.837; 33.29 ± 0.49 and 33.61 ± 0.45°C, respectively) or diameter (P = 0.204; 0.052 ± 0.002 and 0.052 ± 0.002 mm/kg, respectively). Temperamental calves tended to have greater Pulsatility Index values than calm calves (P = 0.095; 1.97 ± 0.22 and 1.67 ± 0.11, respectively). No differences were observed in cortisol (P = 0.104; 3.88 ± 0.27 and 3.36 ± 0.21 µg/dl) or skin temperature (P = 0.905; 33.60 ± 0.39 and 33.66 ± 0.43°C) between heifers and steers, respectively. Heifers had greater rectal (P = 0.002; 39.22 ± 0.10 and 38.84 ± 0.08°C) and tended to have greater hair temperatures (P = 0.093; 26.91 ± 0.57 and 25.66 ± 0.49°C) than steers, respectively. However, rectal temperature was not associated to skin temperature (P = 0.123), Pulsatility Index (P = 0.719), or diameter (P = 0.650). Nor was cortisol related to skin temperature (P = 0.710), Pulsatility Index (P = 0.266) or diameter (P = 0.548). Therefore, under the conditions existent in the present study, rectal temperature and cortisol were able to reflect physiological differences associated with temperament in beef cattle whereas the superficial temperatures and the Pulsatility Index and diameter of the jugular vein did not. Arterial hemodynamic (i.e., carotids) and other superficial temperature recording methods may need to be evaluated in future studies.

Keywords: Beef calves, Body temperature, Blood flow, Temperament

Introduction

There is increasing evidence to support the restrictive role that temperament exerts on cattle productivity. Temperamental cattle have been reported to exhibit decreased average daily gains (Voisinet et al., 1997b) and reproductive performance (Phocas et al., 2006), production of less tender meat with more dark cutters (Voisinet et al., 1997a), and increased bruise trims due to injuries during transportation (Fordyce et al., 1988) in comparison with their calm counterparts. Moreover, handling of these excitable animals represents a greater risk of injury to both, cattle and human workers (Burrow, 1997). Consequently, different methods have been developed for the classification and study of temperament in cattle. However, some of these classification methods are subjective in nature (i.e., pen score) or may be influenced by uncontrolled external factors (i.e., exit velocity) (Curley et al., 2006).

Three of the commonly reported responses to management in temperamental cattle are increases in serum cortisol concentrations (Curley et al., 2006), rate of muscular activity (Bass et al., 2010; Burdick et al., 2011a), and rectal temperatures (Gruber et al., 2010). Greater than normal values of both, cortisol concentrations (Browning et al., 1998; Hemsworth et al., 2000; Cooke et al., 2009) and rectal temperature (Collier et al., 1981; Finch, 1986; Hahn, 1999), have been further associated with impaired productivity in cattle. Therefore, cortisol concentrations and rectal temperatures measurements have been applied to support the already existent temperament classification methods. However, though reliable, cortisol concentration results are not available immediately and rectal temperature assessment normally requires some kind of direct human-animal interaction; which may potentially increase bias, especially in temperament studies where exposure to humans or novel stimuli play a critical role (Burdick et al., 2011b). Consequently, evaluation of alternative physiological parameters as possible indicators of temperament in cattle is imperative.

To sustain the greater physical activity rate observed in temperamental cattle during management (Bass et al., 2001), an increased blood flow volume towards the skeletal muscles, heart and brain is essential (Sapolsky, 1990). Such increased vascular perfusion is greatly mediated by luminal enlargement of arteries and veins and a subsequent decrease in vascular resistance (Smiesko and Johnson, 1993; Ford, 1995; MacAllister and Vallance, 1996). Color Doppler and B-mode ultrasound may represent feasible, non invasive research options for the characterization of vascular dynamics and tone in temperament studies, respectively (Bollwein et al., 2002; Aiken et al., 2007). Due to its proximity to the skin and the accessibility of the calf neck after restriction in a chute, the jugular vein may represent a feasible option for hemodynamic assessment in cattle. Additionally, the use of temperature recording devices that do not require direct contact may counteract any possible human-animal interaction effect. However, in cattle, temperament (Voisonet et al. 1997), vascular dynamics (Huntington et al., 1989), and body thermal insulation (i.e., subcutaneous fat accumulation; Marchello et al., 1970; Klastrup et al., 1984; Choat et al., 2006) have been reported to also be affected by gender, which suggest the need for additional intersex comparison in these possible alternative temperament physiological parameters.

Therefore, the objectives of this investigation were to (i) evaluate currently used physiological indices of temperament such as cortisol concentrations and rectal temperature and their relationship with temperament classifications, and to use these variables to assess the potential of the jugular vein Pulsatility Index and diameter, and of superficial body temperatures as alternative indicators of temperament in weaned Angus crossbred calves, as well as (ii) to evaluate if the sex of the calf may influence these physiological differences.

Materials and Methods

Animals

Ninety-one weaned Angus crossbred calves (223.36 ± 33.24 kg of BW and 262.73 ± 24.94 days of age; Table 2) at the Mississippi Agricultural and Forestry Experiment Station, Brown Loam Branch, Raymond, MS were utilized in this experiment in compliance with the Institutional Animal Care and Use Committee of Mississippi State University (IACUC Approval Number 11-044). Calves included bulls (n = 12), heifers (n = 35), and steers (n = 44) raised in the same herd. Steers were castrated at first day of life and immediately implanted with zeranol [Ralgro, Intervet / Merck Animal Health, Summit, NJ; effective life of 100-120 days (ZoBell et al., 2000)] as part of the regular management practices in the farm. The study was conducted during the month of December 2010, with air temperature values ranging from 0.18 to 10.46°C. Water and hay were provided *ad libitum*. Calves were fed and kept in pens with a soil floor and artificial shade close to the working facility where all the samples were collected.

Table 2 Description of the body weights, age, pen scores, exit velocities, and temperament scores for the different gender groups and temperament classifications at weaning in the Angus crossbred calves used in this study.

		Temperament classification			
		Calm	Intermediate	Temperamental	*P*-Value[b]
Heifers	Body weight, kg	222.95 ± 11.36	214.62 ± 7.39	219.26 ± 6.75	0.797
	Age, d	272.33 ± 7.17	265.67 ± 9.54	263.36 ± 6.19	0.706
	Pen score	1.79 ± 0.11	3.04 ± 0.07	4.50 ± 0.13	<.0001
	Exit velocity, m/s	1.20 ± 0.06	2.70 ± 0.18	4.10 ± 0.15	<.0001
	Temperament score	1.50 ± 0.06	2.87 ± 0.08	4.30 ± 0.12	<.0001
	n	12	12	11	
Steers	Body weight, kg	211.96 ± 7.90	239.94 ± 9.22	221.62 ± 9.43	0.084
	Age, d	257.20 ± 5.24	260.67 ± 7.12	255.86 ± 6.72	0.861
	Pen score	1.60 ± 0.11	2.80 ± 0.14	4.39 ± 0.20	<.0001
	Exit velocity, m/s	1.18 ± 0.07	2.46 ± 0.13	4.12 ± 0.16	<.0001
	Temperament score	1.39 ± 0.06	2.63 ± 0.05	4.25 ± 0.09	<.0001
	n	15	15	14	
Bulls	Body weight, kg	232.73 ± 20.47	238.00 ± 14.20	220.30 ± 29.66	0.834
	Age, d	278.00 ± 3.11	268.20 ± 14.29	250.66 ± 4.91	0.310
	Pen score	1.25 ± 0.14	3.00 ± 0.16	3.83 ± 0.17	<.0001
	Exit velocity, m/s	1.49 ± 0.17	2.42 ± 0.13	4.72 ± 0.30	<.0001
	Temperament score	1.37 ± 0.07	2.71 ± 0.12	4.28 ± 0.08	<.0001
	n	4	5	3	
P-Value[a]	Body weight	0.520	0.117	0.984	
	Age	0.105	0.857	0.592	
	Pen score	0.080	0.348	0.259	
	Exit velocity	0.111	0.439	0.223	
	Temperament score	0.343	0.039	0.950	

Note: Data presented as means ± SEM. [a]*P*-Values for sex groups within temperament classification. [b]*P*-Values for temperament classifications within each sex group. Body weight and age were determined during experimental days (69-71 days post-weaning). Pen scores and exit velocity were measured 28 days pre-weaning and at weaning and used to calculate the temperament scores. Steers were castrated at first day of life and immediately implanted with zeranol [effective life of 100-120 days (ZoBell et al., 2000)].

Temperament Classifications

Calves were evaluated for exit velocity (Burrow et al., 1988) and pen score (Hammond et al., 1996) at two different periods: 28 days before weaning and at weaning (192.73 ± 24.94 days of age). For the assessment of exit velocity and pen score calves were temporarily separated from their dams (physically and visually). Briefly, for the exit velocity, the rate at which the calves traversed a distance of 1.83 m after release from a working chute (expressed in m/s) was recorded. The greater the numerical exit velocity value, the more temperamental the calf, and vice versa. For the pen score, calves were confined in small groups (n = 3) in a pen (4 x 12 m) and their response to a human presence was visually evaluated. The pen score was based on a scale of 1 to 5, where 1 represented a completely calm calf (docile, allows human approach), and 5, an extremely temperamental calf (very aggressive, "crazy", often runs at fences, gates, and even humans). All pen score evaluations were performed by the same technician. The average value of each temperament classification method at both periods (28 days before and at weaning) was used to calculate a temperament score by means of the following formula:

$$\text{Temperament score} = (AEV + APS)/2, \text{ (Burdick et al., 2010)} \qquad \text{(Eq. 8)}$$

where AEV is the average exit velocity and APS is the average pen score. Calves were classified in different temperament groups following a similar approach to the one used by Curley et al. (2006) and Burdick et al. (2011a). Calves under 1 SD lower than the mean temperament score were considered calm (n = 31; < 1.58 ± 0.20); whereas those calves above 1 SD over the mean temperament score were classified as temperamental (n = 28; > 3.94 ± 0.34). The remaining were considered intermediate (n = 32; 2.73 ± 0.25). The overall mean temperament score for our calf population was 2.76 ± 1.18 (mean ± SD). Calves BW and ages were evenly distributed between temperament groups (Table 1). Heifers and steers were also distributed uniformly between temperament groups (Table 2).

Body Weight and Temperature

The calves were moved to working facilities 69-71 days post-weaning and handled through the sorting and crowding pens, and the crowding tub. Sampling periods had a total duration of approximately 10 min per calf. After obtaining individual BW in a weighing crate, calves were restricted in a hydraulic squeeze chute and the rectal temperature was recorded using a digital thermometer (GLA M500, GLA Agricultural Electronics, San Luis Obispo, CA) with a stainless steel probe of 11.43 cm of length completely inserted in the rectum. The probe was kept in the rectum until a stable measurement was obtained (60-90 s). However, human contact affects cattle behavior (Fordyce et al., 1988), which may influence different physiological variables including rectal temperature. Therefore, alternatively, in the present study the hair coat and the skin temperatures (after clipping the hair) were recorded over the right jugular groove region at a center distance between the brisket and the ear of each calf using an infrared laser thermometer (Extech, Model # AC107, Waltham, MA) at an angle and a distance of approximately 90° and 70 cm from the neck surface, respectively.

Doppler and B-mode Ultrasound

Ultrasound jugular vein dimensions measurements were performed similarly to the B-mode approach described by Aiken et al. (2009) in the caudal artery of beef heifers. Immediately after recording body temperatures, a cross sectional ultrasound image of the right jugular vein was obtained by placing the transducer in a transverse position over the right jugular groove region at the same location where superficial temperatures were recorded. Because vasoconstriction and vasodilation affect vessel diameter during the different stages of the cardiac cycle, the luminal dimensions of the right jugular vein were measured in triplicate by means of spherical calipers from frozen B-mode ultrasound images (Fig. 10 A) and the average luminal diameter was recorded. In order to eliminate any possible differences associated with BW, the average luminal diameter value of each calf was standardized with its respective BW (diameter) by means of the following equation:

$$\text{Diameter, mm/kg} = \text{AD/BW}, \qquad \text{(Eq. 9)}$$

where AD is average cross-sectional diameter (mm), and BW is body weight (kg). For the complete population of calves sampled, at the anatomical position aforementioned the right jugular vein had overall mean cross-sectional values of diameter, area, and circumference (not corrected by BW) of 11.02 ± 0.19 mm, 26.97 ± 1.69 mm^2, and 28.59 ± 0.42 mm, respectively.

Following the same approach, cross-sectional color Doppler ultrasound was performed in each calf to monitor the vascular dynamic in the right jugular vein (Fig. 10 B). After obtaining at least 3 uniform consecutive pulse waves, the image was frozen and the Pulsatility Index was calculated by the machine in triplicate using the following formula:

$$\text{Pulsatility Index} = \text{(S-D)/TAMV; (Herzog and Bollwein, 2007)} \qquad \text{(Eq. 10)}$$

where S is the peak systolic velocity, D is the end diastolic velocity, and TAMV is the time averaged maximum velocity of the cardiac cycles. The average diameter and Pulsatility Index values (from the triplicate samples) were used in the statistical analysis. All Ultrasound measurements were carried out by the same operator using a SonoSite, M-

Turbo ver. 1.2.6. equipped with a HFL 38X/13-6 MHz transducer (SonoSite Inc., Bothell, WA).

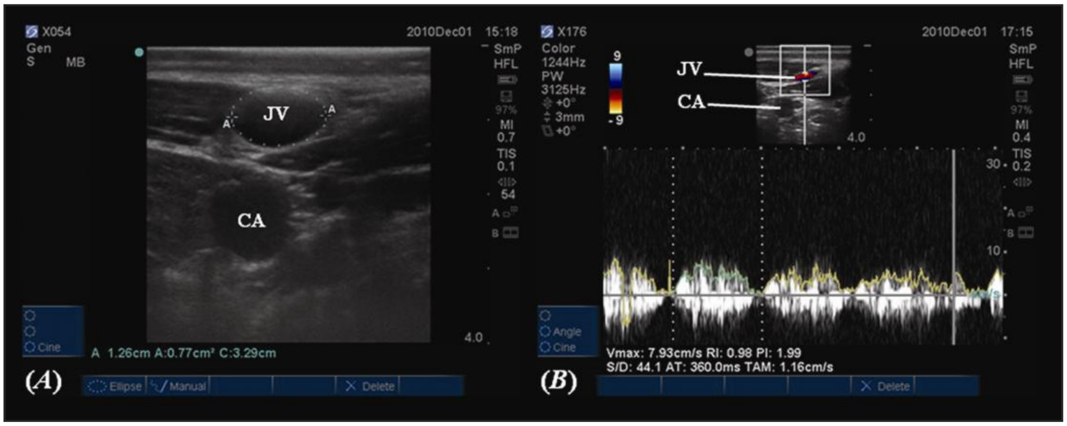

Figure 10 (A) Cross sectional B- Mode ultrasonographic image of the bovine jugular vein (JV) and carotid artery (CA) in the central portion of the right jugular groove. (B) Color Doppler ultrasound flow velocity spectral curve and blood flow parameters measured in a cross section of the bovine right jugular vein.

Notes: (A) Note the spherical caliper around the jugular vein. Values at the bottom (green font) are diameter (cm), area (cm^2), and circumference (cm). (B) Note that in the jugular vein the flow is steadier, opposite to the well defined systolic peaks characteristics of arterial blood flow. The vertical calipers (dotted vertical lines) establish the spectral cycle to be evaluated and the calculated parameters appear in the bottom left of the image.

Blood Samples and Hormone Analysis

Jugular vein blood samples (8.5 mL in Vacutainer, SST, Clot Activator; Becton Dickinson, Franklin Lakes, NJ) were collected from each calf immediately after all the measurements were recorded. Blood samples were centrifuged within 2 h after collection at 2,800 x *g* for 25 minutes at 5°C and the serum was recovered and stored in individual vials at -20°C until analysis. Serum cortisol concentrations (in duplicate) were measured by RIA (Coat-A-Count Cortisol, Siemens Healthcare Diagnostics, Los Angeles, CA).

Tubes containing 0, 1, 5, 10, 20, and 50 μg/dl of cortisol were used for calculation of the standard curve. An intra-assay CV of 6.94% was obtained.

Statistical Analysis

All statistical analyses were performed using SAS (SAS Inst., Inc., Cary, NC). Normality was tested using the Shapiro-Wilk statistic of the UNIVARIATE procedure in SAS. The rectal, hair coat and skin temperatures, as well as the diameter and Pulsatility Index of the right jugular vein, and serum cortisol concentrations were analyzed by the General Linear Model procedure (PROC GLM in SAS). All models include: body weight, sex, temperament classification, and sex by temperament interactions as independent variables. Due to the low number of bulls in our dataset, sex comparisons were carried out only between steers and heifers (Table 2). Differences between means were assessed by the LSD test of SAS. The correlation procedure (PROC CORR of SAS) was used to generate Pearson correlations coefficients to determine the relationship between the rectal, hair coat, and skin temperatures, the diameter and Pulsatility Index of the right jugular vein, the serum cortisol concentrations, and the temperament scores. Unless otherwise expressed, results are reported as means ± standard error of the mean (SEM). Significant differences were detected at a *P*-Value ≤ 0.05, whereas a *P*-Value > 0.05 and < 0.10 was considered a tendency.

Results and Discussion

Temperament Classifications

In our study, the Pearson correlation coefficients for the temperament classification methods repeated over time (on day 28 pre-weaning and on weaning day) were 0.73 ($P < 0.0001$), 0.76 ($P < 0.0001$), and 0.84 ($P < 0.0001$) for pen score, exit velocity, and temperament score, respectively. Furthermore, our temperament score based temperament classifications made during the peri-weaning period were positively correlated with the rectal temperature ($P = 0.004$) and the serum cortisol concentrations ($P = 0.005$; Table 3) measured 69-71 days post-weaning. These values suggest a good repeatability over time for the temperament classifications used in the present study.

Table 3 Pearson correlation coefficients for diameter (corrected for BW) and Pulsatility Index in the right jugular vein; rectal, hair coat, and skin temperatures; serum cortisol concentrations; and temperament scores for the 91 weaned Angus crossbred calves used in this study.

	Pulsatility Index	Rectal temperature	Hair coat temperature	Skin temperature	Cortisol concentrations	Temperament score
Diameter[a]	-0.14 0.172	0.05 0.650	0.18 0.093	0.11 0.299	-0.06 0.548	0.02 0.876
Pulsatility Index		0.04 0.719	-0.36 0.0004	-0.25 0.017	-0.11 0.266	0.18 0.097
Rectal temperature			0.22 0.038	0.16 0.123	0.13 0.224	0.30 0.004
Hair coat temperature				0.43 < 0.0001	0.13 0.235	0.16 0.132
Skin temperature					0.04 0.710	0.03 0.767
Cortisol concentrations[b]						0.29 0.005

Note: All temperament groups pooled together. [a]Diameter = right jugular vein luminal diameter divided by the body weight of the calf; [b]Cortisol concentrations measured from jugular vein blood serum. Values are correlation coefficients over *P*-Values.

Also, as expected, our temperament score values were highly associated with the values observed in pen score ($r = 0.94$; $P < 0.0001$) and exit velocity ($r = 0.95$; $P < 0.0001$). Even when exit velocity is considered an objective method, it may be subjected to the influence of uncontrolled external factors. For example, highly aggressive cattle may exit the chute at low speed searching for an opportunity to attack the human technicians or may simply slip and fall while attempting to escape fast, which may reflect a wrong temperament classification. Moreover, pen score is considered a subjective method due to its dependence on the personal opinion of a human observer. Therefore, our temperament score based classifications provided similar information about the temperament status of the calves than the pen score and exit velocity alone, while providing a way to dilute any possible bias in individual pen score and exit velocity measurements.

Similarly, others have also reported associations between sequential temperament assessments in both, *Bos indicus* and *Bos taurus* beef cattle. Curley et al. (2006) reported positive correlations between three consecutive exit velocity ($r > 0.31$; $P < 0.02$) and pen score measurements ($r > 0.31$; $P < 0.01$) made 30 days apart in 66 yearling Brahman bulls. Café et al. (2011) found correlations of up to 0.52 ($P < 0.001$) and up to 0.55 ($P < 0.001$ to $P < 0.9$) for four consecutive exit velocity measurements made approximately 9.3 weeks apart in Brahman and Angus steers, respectively. Turner et al. (2011) reported an exit velocity repeatability of 0.51 ($P < 0.001$) in 144 *Bos taurus* beef animals (466-554 days of age) in four consecutive temperament assessments, each one made 3-19 days apart from each other. In fact, Burdick et al. (2011a) concluded that the use of exit velocity may allow for early temperament classifications (i.e., during the pre-weaning period) which are viable indices of this variable later in the animal's life, especially in temperamental cattle. Consequently, in the present study the temperament classifications made at the peri-weaning period based on the temperament score values were used in the evaluation of alternative physiological indicators of temperament in beef calves.

Calves Distribution

No differences in age ($P = 0.782$) or BW ($P = 0.534$) were observed between temperament groups. Calm, intermediate, and temperamental calves were 265.74 ± 4.00, 263.72 ± 5.22, and 258.25 ± 4.17 days old; and 218.90 ± 6.30, 230.14 ± 5.83, and 220.55 ± 5.92 kg of BW, respectively. Moreover, no differences in age or BW were observed when assessing each temperament group individually ($P > 0.05$; Table 2). Also, no overall differences in BW ($P = 0.56$) or age ($P = 0.212$) were observed between sex groups. Bulls, heifers, and steers were 231.82 ± 10.64, 218.94 ± 4.99, and 224.58 ± 5.30 kg of BW; and 267.08 ± 6.53, 267.23 ± 4.45, and 257.95 ± 3.62 days old, respectively. Additionally, no overall BW effects were observed in rectal ($P = 0.128$), hair coat ($P = 0.310$), and skin ($P = 0.525$) temperatures; diameter ($P = 0.615$) and Pulsatility Index ($P = 0.376$) of the right jugular vein; or serum cortisol concentrations ($P = 0.126$). Nor were overall sex-related differences in the pen score ($P = 0.537$), exit velocity ($P = 0.937$), or temperament score ($P = 0.851$) values observed in the present study.

Sex and Temperament Interaction

Sex and temperament did not interact to significantly affect any of the examined variables in the present study. Therefore, the simple effects for both independent variables will be presented and discussed in the present manuscript.

Temperament and Serum Cortisol Concentrations

In the present study, serum cortisol concentrations were greater ($P = 0.016$; Fig. 11) in temperamental than in calm calves. Calves in the intermediate temperament group did not differ in cortisol concentrations from those in the calm and temperamental groups. These results are in agreement with multiple previous studies that have found greater cortisol concentrations in temperamental than calm beef cattle in both, *Bos taurus* and *Bos indicus* animals. Gruber et al. (2010) found that *Bos taurus* heifers and steers that are excitable during management in the chute present greater ($P < 0.05$) serum cortisol concentrations than their calm counterparts. Cooke et al. (2011) reported that temperamental *Bos taurus* cows had greater ($P < 0.01$) plasma cortisol values than calm cows. Curley et al. (2006) also observed, in 66 yearling Brahman bulls, greater ($P = 0.008$) serum cortisol concentrations in temperamental than in their calm counterparts. Using 24 calves, Stahringer et al. (1990) reported greater ($P < 0.04$) serum cortisol concentrations in temperamental than in calm Brahman heifers. Moreover, in our study, cortisol was positively associated with pen score ($r = 0.24$; $P = 0.020$) and exit velocity ($r = 0.30$; $P = 0.004$). Consequently, a similar relationship was also observed between cortisol and temperament score ($P = 0.005$; Table 3). Curley et al. (2006) reported similar cortisol associations with pen score ($r = 0.29$; $P < 0.05$) and exit velocity ($r = 0.26$; $P < 0.05$) in yearling Brahman bulls. In *Bos taurus* heifers and steers, Gruber et al. (2010) reported correlations of 0.20 ($P < 0.05$) between pen score values and cortisol concentrations as a result of management in a chute. Therefore, in agreement with previously published results, our serum cortisol values were able to differentiate between temperamental and calm calves. Consequently, this physiological index may help in the evaluation of other alternative physiological indicators of temperament in cattle (i.e., vascular dynamics and superficial body temperature).

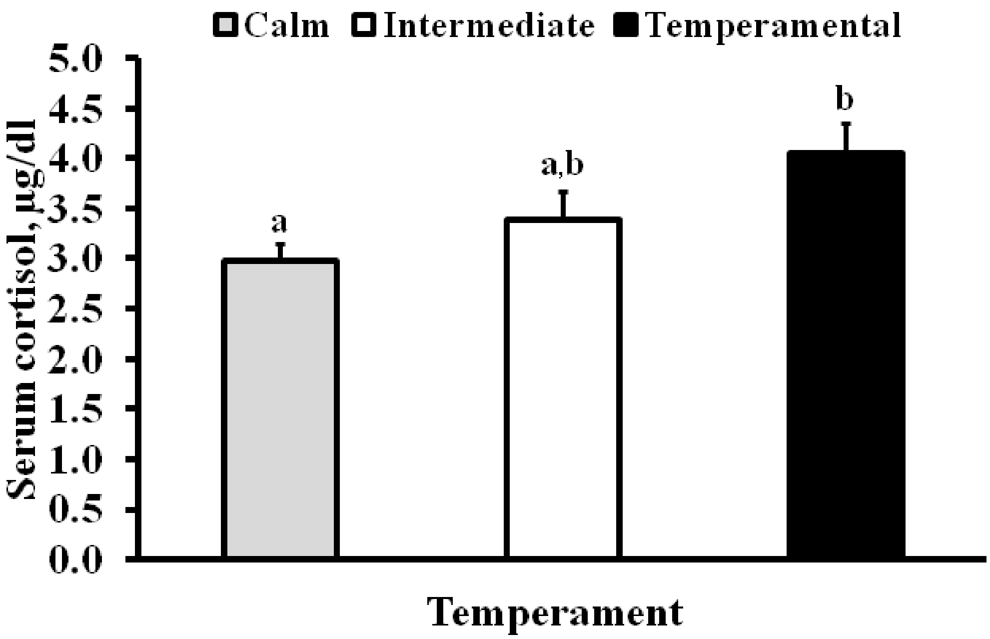

Figure 11 Serum cortisol concentrations in calm (gray bar), intermediate (white bar), and temperamental (black bar) Angus crossbred calves 69-71 days post-weaning.

Notes: Data presented as means ± SEM (µg/dl). [a,b]Bars with unlike letters differ (P = 0.016). Calm (n = 31), Intermediate (n = 32), and Temperamental (n = 28).

Temperament and Body Temperature

After management in the working facilities and restriction in the working chute, temperamental calves had greater rectal temperature ($P = 0.012$; Fig. 12) than calm and intermediate calves. In *Bos taurus* heifers and steers, Gruber et al. (2010) also reported greater ($P < 0.05$) rectal temperatures in nervous or temperamental calves than in their calm counterparts. Burdick et al. (2010) reported similar differences ($P < 0.05$) in rectal temperature between temperamental and calm Brahman bulls as a response to transportation. In our study, similarly to the cortisol concentrations, rectal temperature was positively associated with pen score (r = 0.20; *P* = 0.052) and exit velocity (r = 0.36; *P* = 0.0005). A similar association between rectal temperature and temperament score was also observed (*P* = 0.004; Table 3). In agreement with our data, Gruber et al. (2010) also reported a positive association (r = 0.33; *P* < 0.05) between rectal temperatures and pen scores in *Bos taurus* heifers and steers during management in a chute.

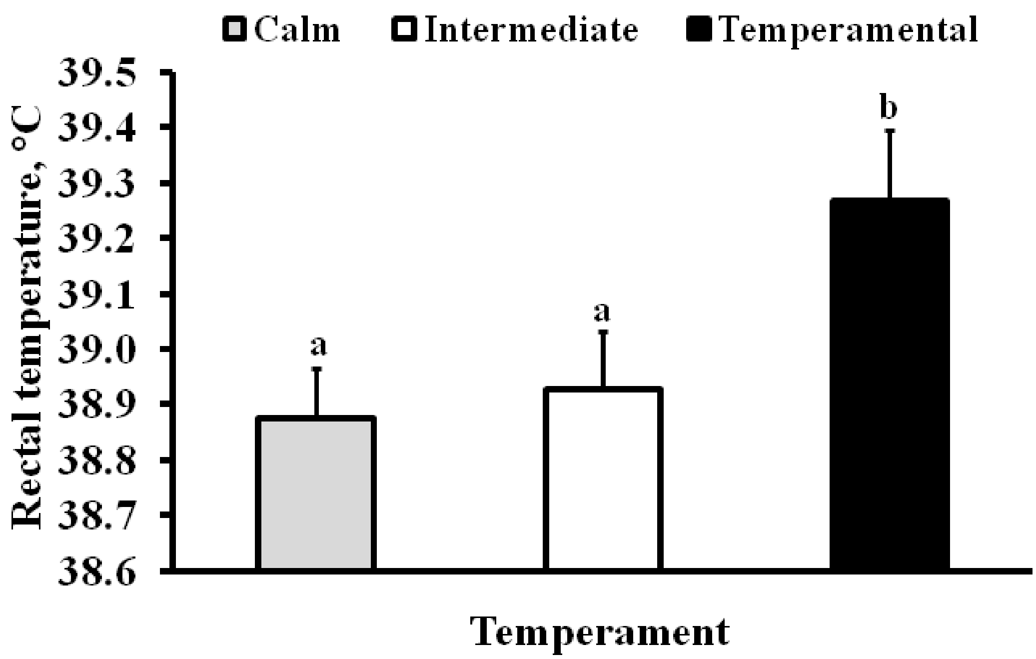

Figure 12 Rectal temperatures in calm (gray bar), intermediate (white bar), and temperamental (black bar) Angus crossbred calves 69-71 days post-weaning.

Notes: Data presented as means ± SEM (°C). [a,b]Bars with unlike letters differ (*P* = 0.012). Calm (n = 31), Intermediate (n = 32), and Temperamental (n = 28).

Normal management practices may represent a significant source of stress for cattle, especially in temperamental animals (Burdick et al., 2011b). Therefore, it may be reasonably assumed (as supported by the observed exit velocity values) that during normal management, temperamental animals will make physiological adjustments to redirect a significant portion of their blood flow from the visceral organs toward the skeletal muscles and central nervous system (Sapolsky, 1990). These changes in blood flow dynamics from the core body to the peripheral organs are essential for an increased rate in muscular activity (Sapolsky, 1990) which may result in energy loss as heat and a subsequent increase in core body temperature. In agreement with this assumption, Bass et al. (2010) found greater rectal temperature values ($P = 0.007$) in excitable *Bos taurus* beef heifers and steers that exit the chute running in comparison with calmer calves that exit walking or trotting.

In the present study and in agreement with previously published results, the rectal temperature, an established and extensively used index of the core body temperature (Brown-Brandl et al., 2003), was sensitive enough to capture differences in the thermal status associated with temperament classification in weaned Angus crossbred calves. Therefore, this variable may also help in the evaluation of other measurements of body temperature as alternative indicators of temperament in beef calves.

The significantly greater rectal temperature values observed in temperamental in comparison with intermediate and calm calves was not reflected in the skin temperature. Skin temperature values of 33.29 ± 0.49, 33.59 ± 0.51, and 33.61 ± 0.45°C ($P = 0.837$) were observed in calm, intermediate, and temperamental calves, respectively. Under normal conditions, core body temperature effect on superficial temperature may be greatly limited by the influence of external environmental factors (Spiers et al., 2004), resulting in low association between both temperatures. In fact, Umphrey et al. (2001)

reported that the rectal and skin temperatures correlation in dairy cows is approximately zero (-0.022 to -0.024).

Cattle can efficiently dissipate body heat at air temperatures lower than 25.6°C (Lefcourt and adams, 1996). After heat is transferred from the core body to the skin (predominantly through the circulatory system), a significant portion of this body heat dissipation is achieved by exchange between the skin and the external environment in favor of a temperature gradient (Finch, 1986). Therefore, external factors including air temperature, may limit the reliability of using skin or hair coat temperatures as indices of the thermal status in cattle. This is in agreement with Spiers et al. (2004) who reported that, in dairy cows, rectal temperature is a superior indicator of the thermal status of the animal than the skin temperature. In our study, the air temperature ranged from 0.18 to 10.46°C during the experimental days. Therefore, in these environmental conditions, such a large thermal gradient may have favor skin heat dissipation immediately after being transferred from the core body, avoiding the core thermal status to be reflected in the skin or hair coat temperature values.

In our study, the rectal temperature in temperamental calves was not related to the skin ($P = 0.554$) or hair coat temperatures ($P = 0.264$). Nor was rectal temperature associated with the skin ($P = 0.855$) or the hair coat ($P = 0.760$) temperatures in calm calves (Table 4). Therefore, even when calm calves had lower ($P = 0.052$; Fig. 13) hair coat temperature values than the intermediate and temperamental calves, those differences may not be attributed to their internal thermal status. Therefore, the skin and hair coat temperature values may not represent a reliable index of the internal thermal status of calm and temperamental calves under the environmental conditions existent during this study.

Table 4 Pearson correlation coefficients for rectal, hair coat, and skin temperatures in calm and temperamental calves; and for rectal and hair coat temperatures, and for Pulsatility Index and diameter (corrected for body weight) of the right jugular vein for heifers and steers in the weaned Angus crossbred calves used in this study.

	Calm (n = 31)	Temperamental (n = 28)
Rectal and hair coat temperatures	-0.06 0.760	0.22 0.264
Rectal and skin temperatures	0.03 0.855	0.12 0.554
	Heifers (n = 35)	**Steers (n = 44)**
Rectal and hair coat temperatures	0.18 0.303	0.30 0.045
Pulsatility Index and diameter[a]	-0.08 0.663	-0.29 0.054

Notes: [a]Diameter = cross sectional diameter of the right jugular vein corrected by body weight of the calf. Number of calves in each group is presented in parenthesis. Values are correlation coefficients over *P*-Values.

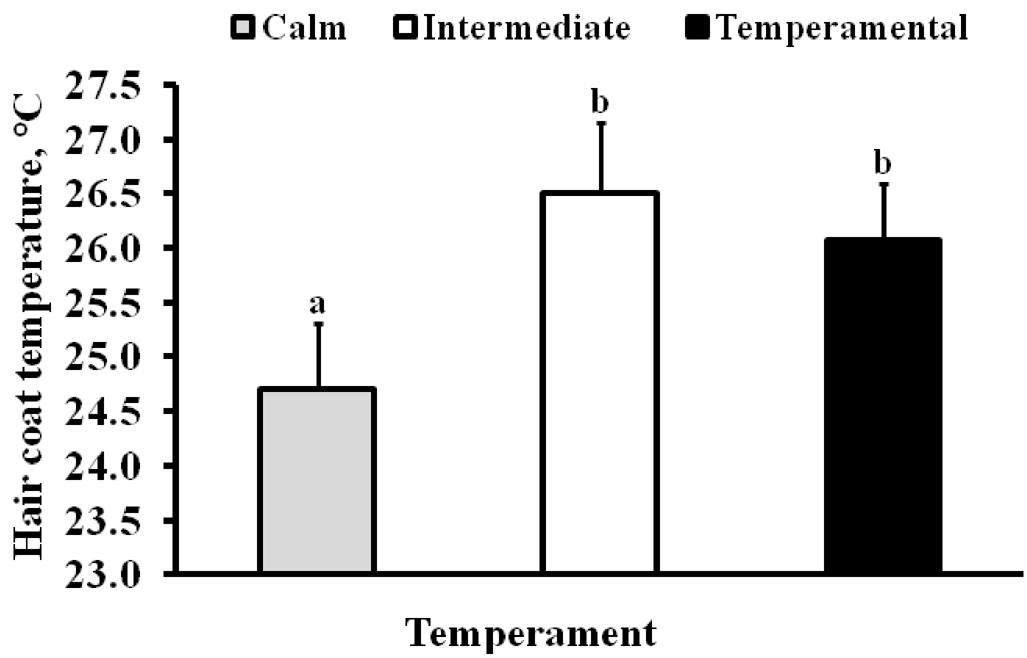

Figure 13 Hair temperatures over the central right jugular groove region in calm (gray bar), intermediate (white bar), and temperamental (black bar) Angus crossbred calves 69-71 days post-weaning.

Notes: Data presented as means ± SEM (°C). [a,b]Bars with unlike letters differ ($P = 0.052$). Calm (n = 31), Intermediate (n = 32), and Temperamental (n = 28).

Temperament, Luminal Diameter, and Pulsatility Index of the Right Jugular Vein

The Pulsatility Index is a vascular index able to provide semiquantitative evaluations of blood flow that are not subjected to the measurement restrictions (i.e., insonation angle) required for reliable volume of blood flow determinations (Herzog and Bollwein, 2007), and therefore, feasible for use in real-farm conditions. The Pulsatility Index provides information of vascular resistance distally to the examination point (Bollwein et al., 2004; Herzog and Bollwein, 2007), which is negatively associated with blood flow volume related variables (Panarace et al., 2006). Because for temperamental cattle, restriction in a chute may be highly stressful (Burdick et al., 2011b) resulting in a greater degree of physical activity during management (Bass et al., 2010) in comparison with calmer calves; in the present study, it was hypothesized that temperamental calves would exhibit a lower resistance to blood flow towards and away their central nervous system and muscles. Vascular resistance reduction is achieved by vessel luminal enlargement (MacAllister and Vallance, 1996; Paniagua et al., 2001). Therefore, this hypothesis implied an increase in vessel dimensions and blood flow from the brain to the vena cava through the jugular veins, and subsequently to the heart. Then, a decrease in Pulsatility Index was expected during vascular assessment of the jugular vein in temperamental calves in comparison with their calm counterparts.

However, the obtained diameter and Pulsatility Index values of the jugular vein seem not to be in agreement with our hypothesis. In the present study there were no temperament related differences (P = 0.204) in the diameter of the jugular vein (0.052 ± 0.002, 0.047 ± 0.001, and 0.052 ± 0.002 mm/kg for calm, intermediate, and temperamental, respectively). Temperamental calves tended (P = 0.095; Fig. 14) to have greater Pulsatility Index values than calm calves. Therefore, the study of the relationship between the measured body temperatures, the serum cortisol concentrations, and the Pulsatility Index and the diameter of the jugular vein may help to achieve a better understanding the obtained results.

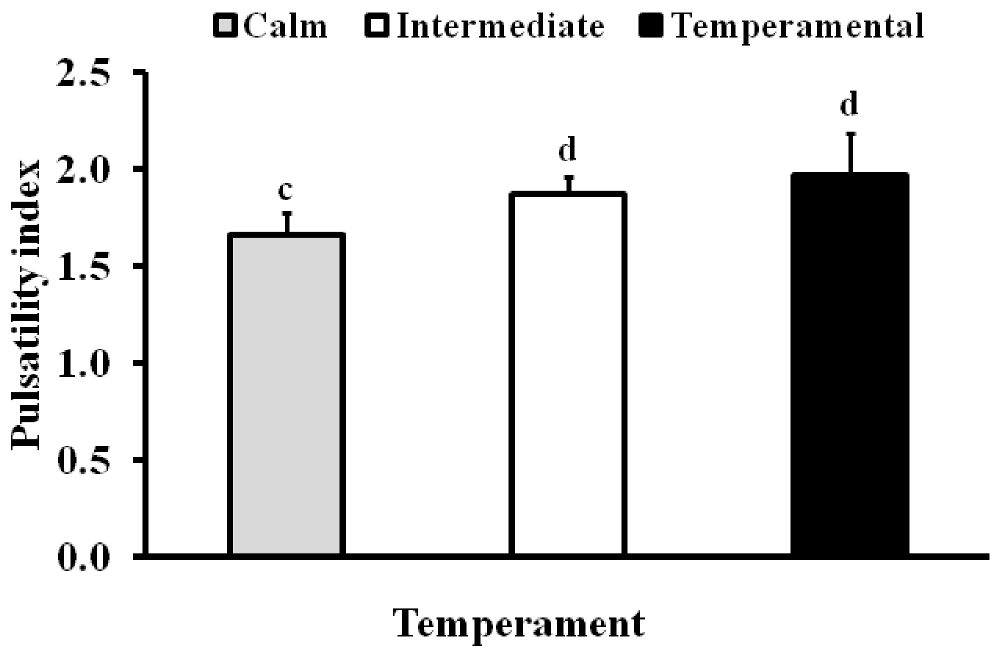

Figure 14 Pulsatility Index values on the right jugular vein in calm (gray bar), intermediate (white bar), and temperamental (black bar) Angus crossbred calves 69-71 days post-weaning.

Notes: Data presented as means ± SEM. [c,d]Bars with unlike letters tend to differ (P = 0.095). Calm (n = 31), Intermediate (n = 32), and temperamental (n = 28)

The diameter of the right jugular vein was not related to the rectal temperature (P = 0.650), the temperament score (P = 0.876), the serum cortisol concentrations (P = 0.548), or the skin temperature (P = 0.299; Table 3). However, there was a tendency towards a low positive correlation (P = 0.093) between the jugular vein diameter and the hair coat temperature (Table 3). The Pulsatility Index values of the right jugular vein were not associated to the rectal temperature (P = 0.719), the serum cortisol concentrations (P = 0.266) or the temperament score (P = 0.097) (Table 3). However, our Pulsatility Index values were negatively correlated to the hair coat (P = 0.0004) and skin temperatures (P = 0.017; Table 3). Therefore, the lack of differences in diameter and the observed apparent contradictory tendency towards greater Pulsatility Index values in temperamental calves in our study may not be attributed to the different temperament classifications. Thus, because of the proximity of the jugular vein to the skin, similar external uncontrolled variables to the factors affecting the hair coat and skin temperatures may also be influencing the Pulsatility Index and diameter of this vessel under the existing conditions in our study.

Sex and Serum Cortisol Concentrations

In the present study, no sex effect was observed in serum cortisol concentrations ($P = 0.104$; 3.88 ± 0.27 and 3.36 ± 0.21 μg/dl in heifers and steers, respectively). Our results are in agreement with Gruber et al. (2010) who reported that contemporaneous *Bos taurus* heifers and steers from the same herd had similar cortisol concentrations immediately after restriction in a working chute. Moreover, Hickey et al. (2003) found no differences in cortisol concentrations between beef *Bos taurus* bulls and heifers as a response to abrupt weaning. Therefore, this lack of gender related differences in this hormone has not only been observed between steers and heifers, but also between intact males and females.

Sex and Body Temperature

In the present study, heifers presented greater rectal temperatures than steers ($P = 0.002$; Fig. 15). In our calm and intermediate temperament groups, heifers tended to have greater pen score values ($P = 0.080$) and had greater temperament score values ($P = 0.039$) than bulls and steers, respectively (Table 2). This data is in agreement with Voisinet et al. (1997) who reported that heifers present greater temperament ratings than steers. Therefore, based on the greater rectal temperature values observed in temperamental cattle in comparison with their calm counterparts in our study and previously published (Burdick et al., 2010; Gruber et al., 2010), the rectal temperature differences among heifers and steers observed in our study may be a consequence of the aforementioned pen score and temperament score values.

Figure 15 Rectal temperatures in Angus crossbred heifers (black bar) and steers (white bar) 69-71 days post-weaning.

Notes: Data presented as means ± SEM (°C). [a,b]Bars with unlike letters differ ($P = 0.002$). Heifers (n = 35) and Steers (n = 44).

Also, in *Bos taurus* beef calves, it has been reported that heifers have greater body fat accumulation than steers (Murphey et al., 1985; Oury et al., 2007; Filipcik et al., 2009); especially greater subcutaneous fat tissue deposition (Marchello et al., 1970; Klastrup et al., 1984; Choat et al., 2006). In *Bos taurus* cattle, including newborn calves (Vermorel et al., 1989), heifers (Reid and Robb, 1971), steers (Mader et al., 1997), and cows (Thompson et al., 1983), subcutaneous adipose tissue has been identified as of significant importance in body temperature regulation because of its role in thermal insulation. Therefore, although not measured in this study, it can be reasonably assumed that greater subcutaneous adipose tissue deposition may interfere with heat exchange from the core body to the external environment resulting in storage of body heat with a subsequent increase in core body temperature. This assumption is reinforced by the fact that no differences ($P = 0.905$) were observed in skin temperature between sex groups (33.60 ± 0.39 and 33.66 ± 0.43°C for heifers and steers, respectively). In fact, even when a tendency ($P = 0.093$) toward greater hair coat temperature values was observed (Fig. 16) in the heifer group, it was not related to rectal temperature ($P = 0.303$; Table 4). Because the hair is directly exposed to the external environment, this tendency may be attributed to the influence of similar uncontrolled external factor including the air temperature.

Figure 16 Hair temperatures over the central right jugular groove region in Angus crossbred heifers (black bar) and steers (white bar) 69-71 days post-weaning.

Notes: Data presented as means ± SEM (°C). [c,d]Bars with unlike letters tend to differ (P = 0.093). Heifers (n = 35) and Steers (n = 44).

Sex, Luminal Diameter, and Pulsatility Index of the Right Jugular Vein

In the present study, there was a tendency (P = 0.067; Fig. 17) towards greater jugular vein diameter in steers than in heifers. However, no differences (P = 0.218) in Pulsatility Index of the right jugular vein were found between heifers and steers (1.66 ± 0.08 and 1.84 ± 0.11, respectively). When the diameter of the jugular vein values (without correction for BW) was used as the dependent variable in our model, BW had a significant effect (P = 0.017). In fact, a low, but significant positive association was observed between the uncorrected diameter of the jugular vein and the BW of the calf (r = 0.23; P = 0.028), which (as expected) suggest that the diameters of the jugular vein were directly associated with the BW of the respective calves. However, in our statistical analysis, the diameter of the jugular vein was already corrected by BW. Moreover, we did not observe differences in BW (*P* = 0.56) or even in age (*P* = 0.116) between heifers and steers. Therefore, this tendency towards greater jugular vein diameter in steers than in heifers cannot be directly attributed to any of the aforementioned factors and may also be the consequence of some external uncontrolled factor or even sexual dimorphism differences.

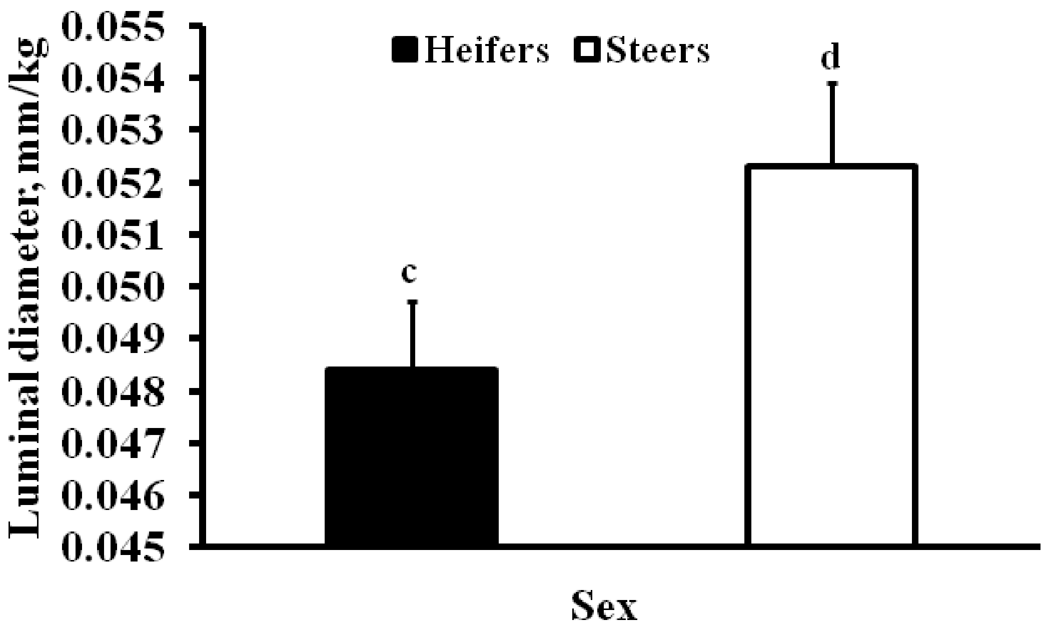

Figure 17 Jugular vein luminal diameter values corrected by BW in Angus crossbred heifers (black bar) and steers (white bar) 69-71 days post-weaning.

Notes: Data presented as means ± SEM (mm/kg). [c,d]Bars with unlike letters tend to differ (*P* = 0.067). Heifers (n = 35) and Steers (n = 44).

Conclusions

In the present study, the significance of the rectal temperature and cortisol concentrations as reliable physiological indices in temperament studies in beef calves able to differentiate between calm and temperamental animals was confirmed.

Interestingly, differences in rectal temperature, unrelated to temperament, were found between heifers and steers. Previously reported relationships between temperament and sex, as well as inter-sex variations in body adipose tissue accumulation (and its insulating properties) in cattle were suggested as possible explanations for this effect. Moreover, under the conditions existent in the present study, the evaluated vascular dynamic parameters in the jugular vein and superficial body temperatures did not result in reliable indicators of the temperament classification of the calf. Therefore, further studies on arterial perfusion and with alternative superficial body temperature recording methods in these cattle are recommended. Moreover, the skin and hair coat temperatures may also be evaluated under different environmental conditions to eliminate any potential external effect, especially any air temperature influence. Because of the direct influence of the cardiac output and of its anatomical location, the carotid arteries may represent a feasible option for future hemodynamic evaluations in beef cattle.

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CHAPTER V

EFFECTS OF RECOMBINANT BOVINE SOMATOTROPIN ON BLOOD FLOW TO THE MAMMARY GLAND AND THERMAL STATUS IN EARLY LACTATING HOLSTEIN COWS

Abstract

Color Doppler and B-mode ultrasound were used to evaluate the effects of recombinant bovine somatotropin administration on mammary gland blood flow volume (BFV), Resistance Index (RI) and vessel diameter in cycling, lactating Holstein cows (42.88 ± 3.53 m old; 2.25 ± 0.16 lactations; 32.25 ± 2.56 dim and 41.90 ± 2.35 kg of milk/d at the beginning of the study). Recombinant bovine somatotropin (rbST; n = 4; 500 mg, s.c.; Posilac, Monsanto Co., St. Louis, MO) was administered once at 40.25 ± 2.56 d postpartum, while Control cows (n = 4) received 1.75 mL of saline solution as a placebo. Ultrasound and temperature recordings were taken once daily at d -7, -5, -2, 0, 1, 2, 5, 7, 9, 12, and 14 (d 0 = immediately before treatment) and the periods before and after treatment were compared. Perfusion of the mammary gland was characterized by the BFV and RI of the left and right pudendoepigastric trunk arteries (PETA). Additionally, the diameter of both PETA, posterior view skin temperatures of the mammary gland via thermography, rectal, and air temperatures were recorded. No differences were observed between the right and left PETA or mammary gland sides ($P > 0.05$); therefore, both side values were combined for further analysis. In general, rbST administration resulted in an increase in BFV (P = 0.0002). In Control cows, a general decrease in BFV (P = 0.0002) was observed through the sampling period. The opposite trend was observed in RI ($P < 0.0001$). Cows treated with rbST presented greater (P = 0.003) PETA diameters than the Control cows. The variability of the superficial temperatures of the mammary gland was increased ($P < 0.05$) by rbST administration. However, rectal temperature and milk yield were not affected ($P > 0.05$) by the treatment. Air temperature was highly correlated (r = 0.46 to 0.87; P < 0.0001 to 0.02) to the recorded body temperatures. These preliminary findings suggest that color Doppler ultrasound may represent a feasible technique for the study of the vascular perfusion changes in the mammary gland associated with rbST administration. Further studies in later stages of lactation and under different environmental conditions are required to clarify the trends observed in rectal temperature and milk yield.

Keywords: Mammary gland blood flow, Holstein cows, recombinant bovine Somatotropin, Body temperature

Introduction

Bovine somatotropin (bST) "orchestrates" a wide range of metabolic changes in the dairy cow's physiology resulting in a more efficient use of nutrients for milk synthesis, enhancing milk yield and persistency (Bauman, 1992). In the mammary gland, it has been reported that two of these physiological effects resulting in higher milk yields after bST administration are: an increased blood flow volume (Boonsanit et al., 2010; Sitprija et al., 2010); and an increased rate of metabolic activity, both with subsequent heat production or release (West, 1994).

However, in dairy cows the positive response in milk yield associated with exogenous bST may be insignificant if this hormone is administered early in lactation before the production peak and a positive energy balance are achieved (Carriquiry et al., 2010; Etherton et al., 1998). Therefore, if the recombinant version of this hormone (rbST) is administered before the 9th or 10th week of lactation (57-70 d in milk; as recommended by the manufacturer), a significant increase in milk yield may not be observed. Moreover, cows can efficiently dissipate body heat in order to regulate their body temperature and maintain homeothermy at air temperatures lower than 25.6ºC (Lefcourt and Adams, 1996). Therefore, under such environmental conditions, an increase in body temperature associated with this hormone and representative of an existent higher metabolic rate (Johnson et al., 1991) or indicative of greater heat transfer from or towards the core body by means of the circulatory system, may not be observed.

Subsequently, during early lactation and at air temperatures lower than 25.6ºC, the direct assessment of the vascular status of the mammary gland may be essential for a better understanding of the physiological processes regulating the effects of this drug. Moreover, due to the aforementioned conditions, the thermal changes in the body and mammary gland need to be evaluated in order to determine if they may provide reliable information of the metabolic and circulatory changes induced by this hormone at this lactation stage. Therefore, the objectives of this study were to evaluate the performance of B-mode and color Doppler ultrasound as research tools for the assessment of the hemodynamics in the mammary gland, as well as to evaluate the vascular perfusion and thermal status of the mammary gland, the milk yield, and the overall thermal status of the animal as a response to exogenous administration of rbST during early lactation in Holstein cows.

Materials and Methods

Animal Handling

Eight lactating non-pregnant multiparous Holstein cows (42.88 ± 3.53 m old; 2.25 ± 0.16 lactations; and 41.90 ± 2.35 kg of milk/d) at the Mississippi Agricultural and Forestry Experiment Station, Joe Bearden Dairy Research Center, Mississippi State, MS were utilized in this experiment in compliance with the Institutional Animal Care and Use Committee of Mississippi State University (IACUC Approval Number 11-041). The study was conducted during the month of October 2011, with an average environmental temperature of 14.80 ± 0.39ºC during the sampling periods (Fig. 18). At the beginning of the study cows were 32.25 ± 2.56 d post-partum.

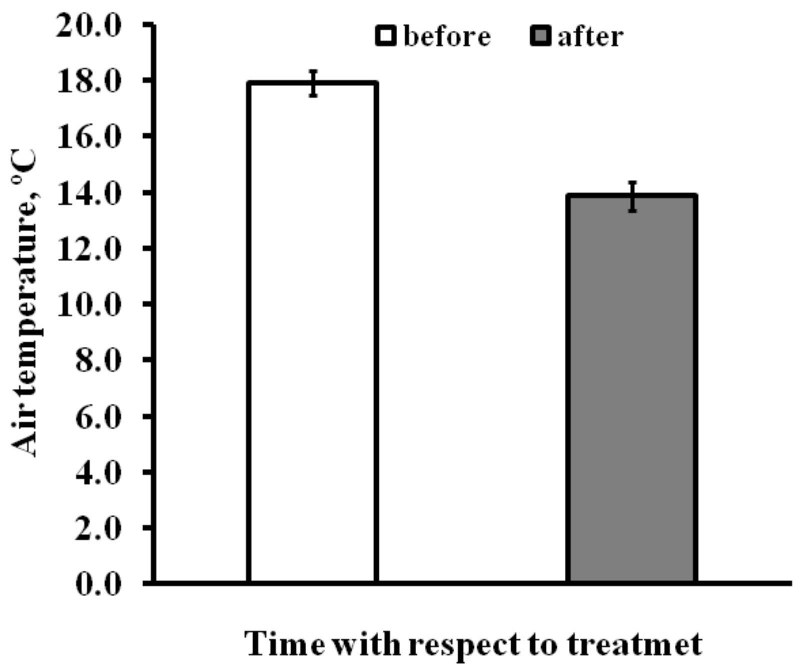

Figure 18 Average values of air temperature during October 2011 for the experimental periods of before (white bar) and after (gray bar) treatment.

Notes: All cows were treated at 40.25 ± 2.56 d post-partum; 500 mg of sometribove zinc and 1.75 mL of saline solution were administered subcutaneously to rbST and Control cows, respectively.

Treatments and Sampling

Cows were divided in two treatment groups. One of the groups (**rbST**; n = 4) received 500 mg of sometribove zinc (Posilac, Monsanto Co., St. Louis, MO) subcutaneously at the base of the tail at 40.25 ± 2.56 d post-partum. The remaining group (**Control**; n = 4) received the equivalent volume (1.75 mL) of saline solution as a placebo by the same route and on the same day. After the morning milking, cows were restrained in head-locks from 06:00–08:30 h everyday with *ad libitum* access to a total mixed ration as part of the normal management practices of the farm. Experimental samples (ultrasound and body temperatures) were recorded while the cows were restricted in head-locks during d -7, -5, -2, 0, 1, 2, 5, 7, 9, 12, and 14 (d 0 = treatment d) and the periods before and after treatment were compared. Every sampling day, cows were monitored in random order. Environmental temperatures were recorded every five minutes through all the sampling periods using a HOBO U22 Water Temp Pro V2 thermometer (Onset Computer Corporation, Cape Cod, MA) located in a cart used for transportation of the ultrasound machine at a height of 76.2 cm from the floor surface. The three air temperature recorded during the sampling period of each cow were averaged and used for further analysis.

Rectal Temperature and Infrared Thermography of the Mammary Gland

The rectal temperature of each cow was recorded using a digital thermometer (GLA M500, GLA Agricultural Electronics, San Luis Obispo, CA) with a stainless steel probe of 11.43 cm of length completely inserted in the rectum, immediately before all other variables were recorded. The probe was kept in the rectum until a stable measurement was obtained (60-90 s). Infrared thermograhy was employed to obtain images of a posterior view of the mammary gland in each cow immediately after the rectal temperature was recorded using a *FLIR SC660 Thermography unit (FLIR Systems, Sweden, AB*; (Fig. 19)). After holding the tail of the cow away, images were obtained at an angle of ~ 90°, an emissivity set at 0.98, and a distance of ~1 m from the posterior surface of the mammary gland. Thermal images were analyzed by means of the software ThermaCAM Research Pro 2.7 (FLIR Systems Sweden, AB). Posterior view mammary gland surface temperature (ºC) was quantified using the minimum, the maximum, the maximum - minimum difference, the average, and the standard deviation of the complete udder, the right quarter, the right teat, the left quarter, and the left teat, separately.

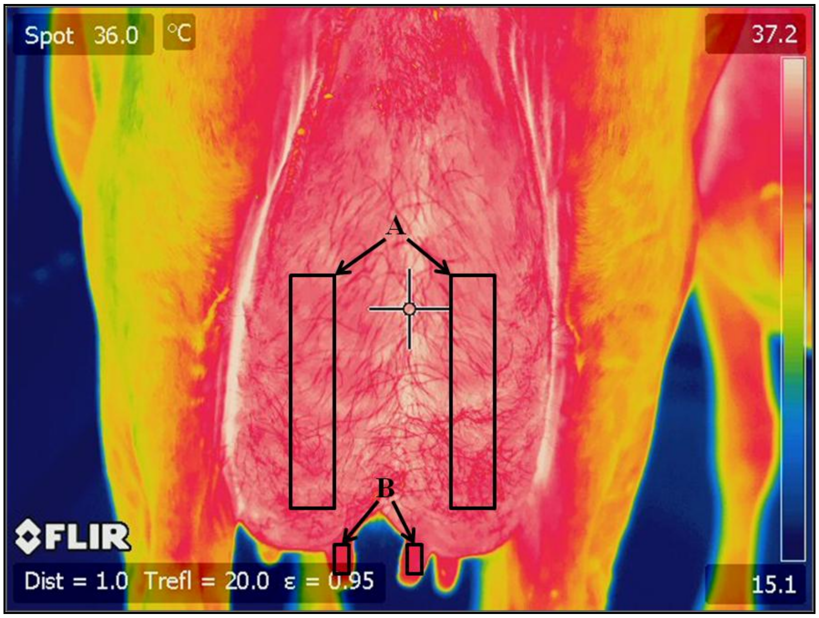

Figure 19 Infrared thermography image of the posterior view of the mammary gland in a cycling lactating Holstein cow.

Notes: A and B denotes the regions of temperature measurement in the left and right quarters and teats of the mammary gland, respectively. The four measurement areas were used for temperature determinations of the "complete mammary gland". In the present study images were obtained at an angle of ~ 90°, an emissivity set at 0.98, and a distance of ~1 m from the posterior surface of the udder. Temperature values were obtained by means of the software ThermaCAM Research Pro 2.7 (FLIR Systems Sweden, AB).

Doppler and B-mode Ultrasound

The vascular dynamic in the pudendoepigastric trunk arteries (PETA), the deepest point that can be reached by means of transrectal ultrasound and that branches in to the main source of arterial blood to the mammary gland (pudendal arteries; Klaus-Dieter and Habel, 2003) was characterized in the present study by means of the color Doppler ultrasound blood flow volume (BFV) and the Resistance Index (RI), and the B-mode ultrasound cross sectional luminal diameter.

Cows were transrectally ultrasound using a Sonosite, M-Turbo ver. 1.2.6. equipped with a L52X/10-5MHz transducer (color Doppler and B-mode) immediately after the rectal temperatures and the infrared thermography images of the mammary gland were obtained. Each ultrasound section lasted approximately 18 mins per cow and was performed by the same technician.

The right and left PETA were found following a technique previously described by Götze et al. (2010) and Potapow et al. (2010). Briefly, with the transducer in the rectum of the cow and facing dorsally, the abdominal aorta was easily identified and followed caudally until it subdivides in the external iliac arteries (right and left). By following the respective external iliac artery, in a cranial-ventral position with respect to the pelvic floor, the PETA is found immediately after the femoral artery branch.

An interrogation angle of approximately 60º between the direction of the blood flow and the ultrasound beam was used for all the Doppler measurements. In each side, a longitudinal view of the PETA (at the same measuring point) was obtained by means of color Doppler ultrasound. After at least three similar consecutive spectral waveforms were obtained, the image was frozen and the blood flow was characterized.   The blood flow volume (mL/min) was calculated by the machine in each selected waveform by means of the following equation:

$$BFV = TAMV\ (cm/s) * 60 * A\ (cm^2); \text{ (Götze et al. (2010),} \qquad \text{(Eq. 11)}$$

where: *TAMV* is time averaged maximum velocity of the waveform and *A* is the area of the blood vessel. This procedure was performed in triplicate in each side of each cow on each sampling day. Additionally, the same procedure was repeated in both PETA sides and the Resistance Index was calculated by the machine, also in triplicate, from the equation:

$$RI = (S-D)/S; \text{(Herzog and Bolwein, 2007)}, \qquad \text{(Eq. 12)}$$

where: *S* is the maximum systolic frequency shift, and *D* is the end diastolic frequency shift. Also, three frozen B-mode cross sectional images of each PETA side (at the same sampling point) were used for the determination of the luminal dimensions (i.e., diameter, area, and circumference) of these arteries by means of spherical calipers. Because arteries expand and contract during the cardiac cycle, triplicate luminal diameter values were collected. The sonographic measurements were performed always in the same order: first the BFV, second the RI, and third the cross sectional luminal diameter. In each measurement, the right side was always scanned first, followed by the left one. The average of these triplicate measurements (i.e., BFV, RI, and luminal diameter) was used for the statistical analysis.

Statistical Analysis

All statistical analyses were performed using SAS (SAS Inst., Inc., Cary, NC). Normality was tested using the Shapiro-Wilk statistic of the Univariate procedure (PROC UNIVARIATE of SAS). No differences were found between the right and left PETA sides for BFV ($P = 0.7634$), RI ($P = 0.6034$), or luminal diameter ($P = 0.6568$). Moreover, no differences were observed between the right and left sides of the mammary gland, including: minimum ($P = 0.9213$), maximum ($P = 0.4108$), difference ($P = 0.8759$), average ($P = 0.6645$), and standard deviations ($P = 0.2983$) of the mammary gland quarters; as well as, minimum ($P = 0.5598$), maximum ($P = 0.1597$), difference ($P = 0.1459$), average ($P = 0.2003$), and standard deviation ($P = 0.5960$) of the mammary gland teats. Therefore data from both anatomical sides were combined for the statistical analysis. The BFV, RI, luminal diameter, milk yield, rectal temperature, and mammary gland temperatures were analyzed by the General Linear Model procedure (PROC GLM of SAS). All models include: sampling period (before and after treatment), treatment group (rbST or Control), and the respective interactions as independent variables. Differences between means were assessed by the LSD test of SAS. The correlation procedure (PROC CORR of SAS) was used to generate Pearson correlations to determine the relationship between the BFV, RI, and luminal diameter of the PETA, body temperatures, air temperature, and milk yield. Significant differences were detected at a P-value ≤ 0.05, whereas a P-value between 0.06 and 0.10 was considered a tendency.

Results and Discussion

Pudendoepigastric Trunk Arteries

In the past, studies in blood flow to the mammary gland were performed at the external pudendal arteries, the main arterial blood supplies to both, the right and left sides of the udder (Budras and Habel, 2003). However, these studies required the employment of highly invasive techniques including dye infusion (Boonsanit et al., 2010) and flow probes implantation directly over the arteries (Davis et al., 1988; Gorewit et al., 1989; Delamaire and Guinard-Flament, 2006). Thus, the assessment of non-invasive techniques for blood flow evaluation in the vessels of large domestic species, including those vessels supplying the mammary gland, has received special attention during recent years. With the relatively recent accessibility of Doppler ultrasound to animal practitioners, transrectal assessment of blood flow to the mammary gland has been successfully evaluated in different research scenarios (Gotze et al., 2010; Potapow et al., 2010). The right and left PETAs, which are the closest arteries to the mammary gland that can be reached by means of transrectal ultrasound and branch to give origin to the external pudendal arteries and the caudal epigastric arteries, have been assessed in these studies. In the present study, the PETA presented overall average values of cross sectional diameter and blood flow volume of 20.0 ± 0.18 mm and 2902.72 ± 86.04 mL/min. Our values are inside the ranges of those previously published by others, including studies using different techniques. Gotze et al. (2010) reported an average ultrasound cross sectional diameter of the PETA of 16.2 mm in 40 lactating German Holstein cows. Using a dye dilution technique, Boonsanit et al. (2010) reported BFV values to the mammary gland of 2904-4129 and 2551-3158 mL/min in bST-treated and untreated early lactation crossbred Holstein cows, respectively. Davis et al. (1988), using an electronic flow probe around the left pudendal artery of mature Jersey cows, found BFV values of 3.3 and 4.4 L/min before and after growth hormone administration, respectively. Also, Delamaire and Guinard-Flament (2006) using flow meters in multiparous Holstein cows, reported mammary gland BFV of 1.23 ± 0.24 L/min. Moreover, Gorewit et al. (1989) using ultrasonic blood flow probes surrounding the right pudendal artery, reported BFV values of 1.23-2.23 L/min in non-pregnant multiparous lactating Holstein cows. Thus, the PETA values observed in the present study are in agreement with those previously published by others in the PETA and even with the BFV in the pudendal arteries.

Vascular Dynamics in the Pudendoepigastric Trunk Arteries and Recombinant Bovine Somatotropin

In the present study, treatment (rbST and Control) and sampling time (before and after treatment) interacted to significantly affect the BFV (Fig. 20; $P = 0.0002$) and the RI (Fig. 21; $P < 0.0001$), but not the luminal diameter ($P = 0.9470$) in the PETA. Overall decreases and increases during time in BFV values of the PETA were observed in Control and rbST-treated cows, respectively. The opposite trend was found in the RI values of the PETA. Overall increases and decreases in RI values during time were observed in Control and rbST-treated cows, respectively. However, our 11 BFV and RI of the PETA measurements were not associated in Control (r = -0.07; *P* = 0.6330) or in rbST-treated cows (r = -0.13; *P* = 0.4036). Independently to sampling time, the rbST-treated cows presented greater luminal diameters of the PETA than the Control cows (Fig. 22; *P* = 0.0027). Before treatment, the BFV and the luminal diameter of the PETA tended to be associated in rbST-treated cows (r = 0.45; *P* = 0.0807), but not in Control cows (r = 0.34; *P* = 0.1957). After treatment, BFV and luminal diameter of the PETA were correlated in rbST-treated cows (r = 0.37; *P* = 0.0500), and tended to be correlated in Control cows (r = 0.32; *P* = 0.0986).

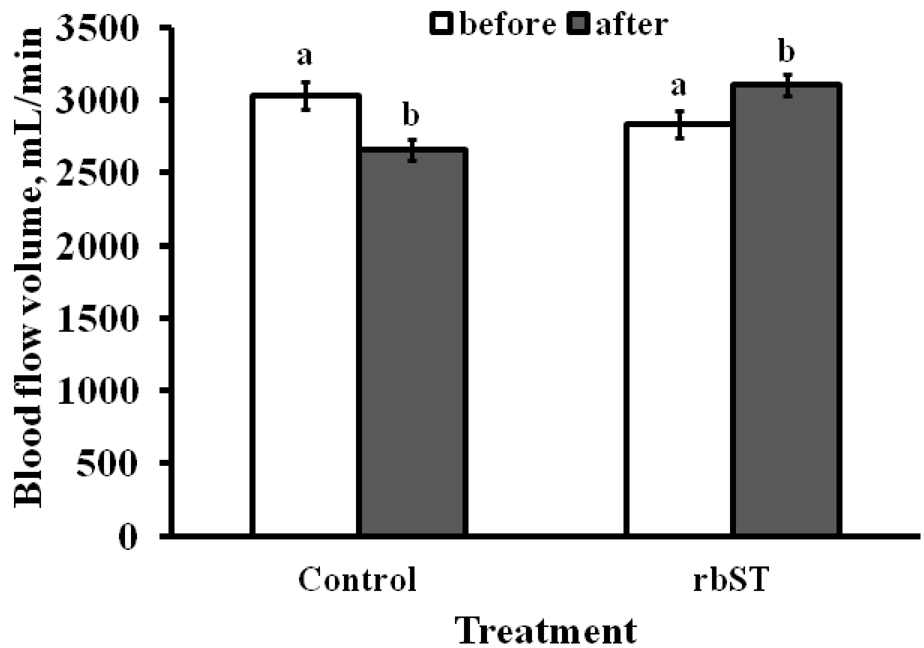

Figure 20 Blood flow volume in the pudendoepigastric trunk in rbST-treated and Control early lactating Holstein cows before (white bars) and after (gray bars) treatment.

Notes: Data presented as LSMeans ± SEM (mL/min). Values are the average of the right and left sides pudendoepigastric trunk. All cows were treated at 40.25 ± 2.56 d post-partum; 500 mg of sometribove zinc and 1.75 mL of saline solution were administered subcutaneously to rbST and Control cows, respectively. Different letters within treatment group indicate significant differences (treatment by time interaction; $P = 0.0002$).

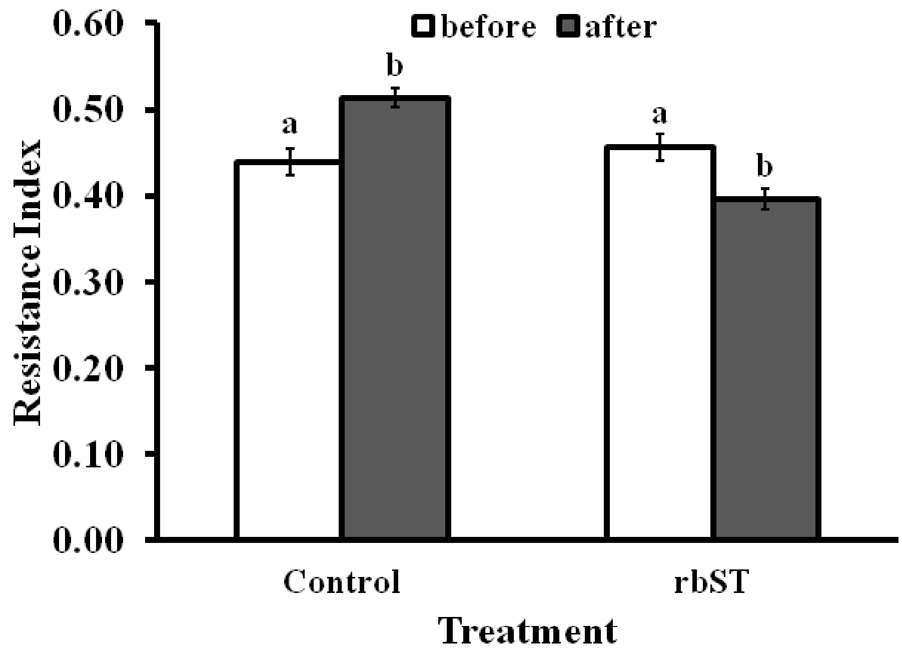

Figure 21 Resistance Index in the pudendoepigastric trunk in rbST-treated and Control early lactating Holstein cows before (white bars) and after (gray bars) treatment.

Notes: Data presented as LSMeans ± SEM. Values are the average of the right and left sides pudendoepigastric trunk. All cows were treated at 40.25 ± 2.56 d post-partum; 500 mg of sometribove zinc and 1.75 mL of saline solution were administered subcutaneously to rbST and Control cows, respectively. Different letters within treatments indicate significant differences (treatment by time interaction; $P < 0.0001$).

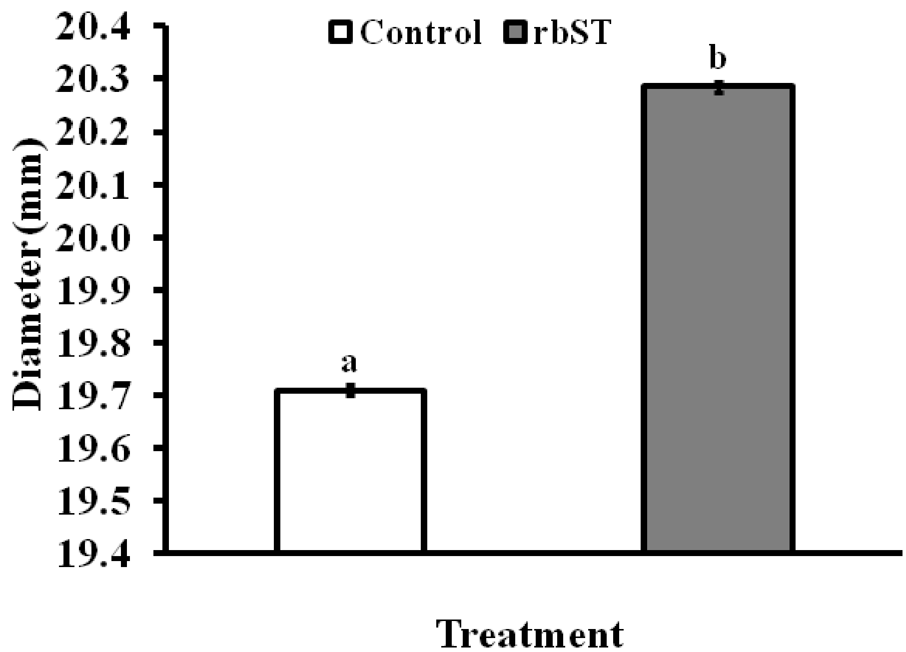

Figure 22 Luminal diameter of the pudendoepigastric trunk arteries in Control (white bar) and rbST-treated (gray bar) early lactating Holstein cows.

Notes: Data presented as Means ± SEM (mm). Values are the average of the right and left sides pudendoepigastric trunk. All cows were treated at 40.25 ± 2.56 d post-partum; 500 mg of sometribove zinc and 1.75 mL of saline solution were administered subcutaneously to rbST and Control cows, respectively. Different letters indicate significant differences (treatment; $P = 0.0027$).

The enhanced vascular perfusion through the mammary gland observed in our study is in agreement with the results previously published by others. Boonsanit et al. (2010) reported BFV values to the mammary gland of 2904 and 2551 mL/min in early lactating crossbred Holstein cows (75 d post-partum) under normal shade conditions with and without bST administration, respectively ($P = 0.02$). They also reported mammary gland BFV of 4129 and 3158 mL/min for bST-treated and untreated cows, respectively, under cooling with misters and fans conditions ($P = 0.02$; Boonsanit et al., 2010). Additionally, Davis et al. (1988), using an electronic flow probe around the left pudendal artery of mature Jersey cows, found BFV values of 3.3 and 4.4 L/min before and after growth hormone administration, respectively.

When exogenously administered to lactating dairy cows, bovine somatotropin "orchestrate" a wide variety of metabolic changes in the body tissues which subsequently result in a more efficient use of nutrients for milk synthesis, enhancing milk yield and persistency (Bauman, 1992). Between these physiological changes, this hormone is known to increase the volume of blood flowing to the mammary gland (Boonsanit et al., 2010).

A series of studies performed during the 80's and 90's using direct infusions to the pudendal arteries (the main arteries supplying the right and left sides of the mammary gland independently) in sheep and goats helped to achieve a better understanding of how exogenous bST is able to enhance the blood flow in the mammary gland. Mc Dowell et al. (1987) found no increases in mammary gland blood flow as a result of bST infusion to one pudic artery in lactating goats and ewes in comparison to the control side. However, Prosser et al. (1990 and 1994) reported that IGF-I infusion to one pudendal artery of lactating goats resulted in both, a milk yield and a blood flow volume increases in the infused side of the mammary gland in comparison with the control side.

Insulin-like growth factor I has been reported to play an essential role in the cardiovascular system (Vecchione et al., 2001) because of its vasodilatory effects (Perticone et al., 2008). For instance, in human kidneys, IGF-I stimulates a rapid increase in nitric oxide production (Tonshff et al., 1998). Nitric oxide has potent direct (Van Der Horst et al., 2003) and indirect (through prostaglandins; Salvemini et al., 1993) vasoldilatory properties. Thus, the vasodilatory effects attributed to IGF-I may be mediated through nitric oxide and vasodilatory prostaglandins (Tonshff et al., 1998). In fact, Vecchione et al. (2001) have reported that the vasodilation produced by IGF-I in rats is blunted by the inhibition of endothelial nitric oxide Synthase, one of the enzymes responsible for nitric oxide production. Thus, vascular tone is regulated by the effect of endothelial nitric oxide (Vecchione et al., 2001) which regulates the cyclooxygenase (COX) activity with a subsequent prostaglandin production (Salvemini, 1997), including PGE_2 which is known to be a potent vasodilator (Clyman et al., 1978).

Moreover, infusion of diethylamine NONOate (a nitric oxide donor) to one pudendal artery of lactating dairy goats induced a rapid and sustained increase in blood flow to the infused side of the mammary gland in comparison with the control side (Lacasse et al., 1996). However, infusion of N-nitro-arginine (an inhibitor of nitric oxide synthesis) decreased the blood flow in the infused side by 35%, but re-infusion of arginine (the nitric oxide precursor) to the already N-nitro-arginine treated side reestablished the blood flow values (Lacasse et al., 1996). Therefore, the increase in mammary blood flow observed after exogenous bST administration is not directly mediated by the growth hormone, but by the effect of the produced IGF-I on a subsequent up-regulation of the vasodilatory nitric oxide molecule.

Therefore, the observed increase in BFV and luminal diameter, as well as the decrease in RI of the PETA associated with rbST administration may be attributed to the IGF-I (mostly from liver origin) induced nitric oxide production. Moreover, the COX enzymes activation and the secondary messengers activated by nitric oxide may play an essential role in this effect mostly by the production of vasodilatory prostaglandins that may regulate the diameter in the blood vessels of the mammary gland.

Milk Yield

In the present study, even when Control cows presented greater daily milk yields than rbST-treated cows in both periods, before ($P = 0.0239$) and after ($P = 0.0462$) treatment; milk yield was not affected by placebo ($P = 0.3529$) or rbST ($P = 0.9284$) administration (Table 5). Etherton et al. (1998) and Carriquiry et al. (2008) reported that the positive response in milk yield associated with exogenous bST administration in dairy cows may be insignificant if this hormone is administered early in lactation before the production peak and a positive energy balance are achieved. In fact, our cows were treated only once at 40.25 ± 2.56 d post-partum. Therefore, the lack of significant effect on milk yield may be attributed to the fact that in the present study rbST was administered before the 9th or 10th week of lactation (57-70 d in milk; as recommended by the manufacturer). Thus, limiting the possibilities that the observed significant hemodynamic changes in BFV, RI, and luminal diameter of the PETA were translated in to an increased productive performance.

Table 5 Average daily milk yields in rbST-treated and Control early lactating Holstein cows before and after treatment.

	Before	After	*P*-Value
Control, kg/d	48.07 ± 1.43 (16)	46.42 ± 0.94 (28)	0.3529
rbST, kg/d	43.46 ± 1.31 (16)	43.61 ± 0.99 (28)	0.9284
***P*-Value**	0.0239	0.0462	

Note: All cows were treated at 40.25 ± 2.56 d post-partum; 500 mg of sometribove zinc and 1.75 mL of saline solution were administered subcutaneously to rbST and Control cows, respectively. *P*-Values at the bottom and at the right of the table correspond to comparisons between treatment groups (Control vs. rbST) and between time groups (before vs. after), respectively. Number of observations appears in parenthesis under its respective mean ± SEM.

Rectal and Mammary Gland Temperatures and Recombinant Bovine Somatotropin: Association with Air Temperature

Thermal changes in specific anatomical parts have the potential to affect the temperature of the whole body. Therefore, in the present study, the author hypothesized that any thermal change at the mammary gland, associated with a rbST-induced greater metabolic rate, would be translated to a body thermal change. In fact, in the present study, the temperatures of the: minimum and maximum complete mammary gland, the minimum, maximum, and average quarters, and the minimum, maximum, and average teats were associated with the rectal temperatures ($P < 0.0001$ to 0.02; Table 6). Also as expected, positive associations between the minimum and maximum complete mammary gland, the minimum, maximum, and average quarters, and the minimum, maximum, and average teats were observed in the present study (Table 6). Positive correlations (r = 0.25 to 0.61) were also observed between the rectal temperatures and the different temperatures of the mammary gland. At the same time, with the exception of the standard deviation and the maximum – minimum difference of the mammary gland quarter, the rectal temperatures were negatively associated (r = -0.38 to -0.33) with the differences (maximum – minimum) and the standard deviations of the mammary gland temperatures obtained from the thermography images; indicating that as the body temperature was increased, the range of variability in the temperature values of the cow was decreased, and vice versa.

However, no differences were observed in rectal temperature between the periods of before and after treatment in Control ($P = 0.1454$) or rbST-treated ($P = 0.4296$) cows (Table 7). Moreover, no differences were observed between Control and rbST-treated cows before ($P = 0.0730$) or after ($P = 0.2591$) treatment (Table 7). Cattle can efficiently regulate their body temperature and maintain homeothermy at air temperatures lower than 25.6ºC (Lefcourt and Adams, 1996). In our study, the air temperature values during the sampling periods of before and after treatment ranged from 14.69-21.03ºC and 5.82-19.57ºC, respectively (Fig. 18). In bovines, a significant portion of body heat dissipation is achieved by exchange between the skin and the external environment in favor of a temperature gradient (Finch, 1986). The relatively low air temperatures observed in our study may have propitiated a large thermal gradient for heat dissipation from the skin to the environment, which may have avoided any possible metabolic heat production difference between treatment groups at the mammary gland to be reflected in the rectal temperature values. However, also in the present study, greater variability in mammary gland temperatures was observed in rbST-treated than in Control cows ($P < 0.0001$ to 0.04; Table 8). Moreover, the luminal diameter of the PETA was associated with standard deviations of the mammary gland quarter temperatures (r = 0.34; $P = 0.0014$). Therefore, even when no differences in milk yield or rectal temperature were observed between treatment groups, it may be assumed that the hemodynamic effect associated with rbST administration may be the force driving the observed variability in the mammary gland temperatures probably due to the warmer temperature of the arterial blood flowing from the core body to the mammary glands of the cows.

In the present study the air temperature resulted in a highly important factor ($P <$ 0.0001 to 0.02) in the regulation of the different recorded body temperatures (i.e., rectal and thermography-derived temperatures; Table 6). As expected, the air temperature was positively correlated with the rectal and mammary gland temperatures (r = 0.46 to 0.87). Moreover, the differences (maximum – minimum) as well as the standard deviation values for the mammary gland temperatures obtained from the thermography images were negatively associated (r = -0.68 to -0.25) to the air temperatures; indicating that as the air temperature decreased, the range of variability in mammary gland temperature values of the cows was increased. This is evidenced by the mammary gland temperature values presented in Table 6. For all cows, including both treatment groups together, the presented mammary gland temperatures decreased (P = 0.04 to 0.0002) over time, while the mammary gland temperature differences increased (P = 0.01 to 0.0002) during the same period (Table 8).

Table 6 Overall Pearson correlation coefficients between the air, rectal, and mammary gland (thermography) temperatures for the complete dataset, including both treatments (rbST and Control) and time periods (before and after treatment).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1) Air temperature		0.46	0.73	0.74	-0.68	0.87	-0.50	0.69	0.83	-0.43	0.82	-0.39	0.51	0.84	-0.25
		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0002	<.0001	<.0001	0.0206
2) Rectal temperature			0.39	0.54	-0.34	0.57	-0.38	0.43	0.41	-0.33	0.43	-0.14	0.25	0.61	-0.04
			0.0002	<.0001	0.0012	<.0001	0.0003	<.0001	<.0001	0.0017	<.0001	0.189	0.021	<.0001	0.6836
3) Minimum complete MG				0.59	-0.99	0.72	-0.45	0.57	0.66	-0.38	0.66	-0.42	0.54	0.68	-0.33
				<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0003	<.0001	<.0001	<.0001	<.0001	0.0016
4) Maximum complete MG					-0.50	0.84	-0.56	0.71	0.76	-0.51	0.78	-0.33	0.36	0.88	-0.06
					<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0016	0.0007	<.0001	0.6062
5) Difference complete MG						-0.66	0.41	-0.52	-0.61	0.34	-0.60	0.40	-0.53	-0.61	0.35
						<.0001	<.0001	<.0001	<.0001	0.0015	<.0001	0.0001	<.0001	<.0001	0.0008
6) Average quarter							-0.58	0.73	0.82	-0.50	0.84	-0.56	0.64	0.94	-0.35
							<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0008
7) Standard deviation teat								-0.87	-0.49	0.88	-0.66	0.26	-0.19	-0.56	-0.003
								<.0001	<.0001	<.0001	<.0001	0.0136	0.074	<.0001	0.9772
8) Minimum teat									0.73	-0.92	0.79	-0.36	0.35	0.70	-0.12
									<.0001	<.0001	<.0001	0.0005	0.0009	<.0001	0.2741
9) Maximum teat										-0.39	0.96	-0.35	0.45	0.81	-0.18
										0.0002	<.0001	0.0009	<.0001	<.0001	0.086

Table 6 (continued)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
10) Difference teat											-0.50	0.28	-0.21	-0.47	0.05
											<.0001	0.0076	0.055	<.0001	0.6518
11) Average teat												-0.40	0.45	0.81	-0.19
												0.0001	<.0001	<.0001	0.0842
12) Standard dev. quarter													-0.83	-0.30	0.80
													<.0001	0.005	<.0001
13) Minimum quarter														0.44	-0.94
														<.0001	<.0001
14) Maximum quarter															-0.10
															0.3408
15) Difference quarter															

Note: Values are Pearson correlation coefficient over its respective *P*-Value from the complete dataset (both treatments and sampling periods). All cows were treated at 40.25 ± 2.56 d post-partum; 500 mg of sometribove zinc and 1.75 mL of saline solution were administered subcutaneously to rbST and Control cows, respectively. MG = mammary gland; Difference = maximum temperature – minimum temperature.

Table 7 Rectal temperatures in rbST-treated and Control early lactating Holstein cows before and after treatment.

	Before	After	*P*-Value
Control, ºC	38.37 ± 0.15 (16)	38.10 ± 0.11 (28)	0.1454
rbST, ºC	38.05 ± 0.10 (16)	37.94 ± 0.08 (28)	0.4296
***P*-Value**	0.0730	0.2591	

Notes: All cows were treated at 40.25 ± 2.56 d post-partum; 500 mg of sometribove zinc and 1.75 mL of saline solution were administered subcutaneously to rbST and Control cows, respectively. *P*-Values at the bottom and at the right of the table correspond to comparisons between treatment groups (Control vs. rbST) and between time groups (before vs. after), respectively. Number of observations appears in parenthesis under its respective mean ± SEM.

Table 8 Mammary gland posterior surface temperatures measured by infrared thermography in rbST-treated and Control early lactating Holstein cows before and after treatment.

	Before, ºC	After, ºC	*P*-Value
Complete mammary gland minimum	16.64 ± 0.53	10.62 ± 1.12	0.0002
Complete mammary gland maximum	38.43 ± 0.14	37.92 ± 0.13	0.0162
Complete mammary gland difference	21.79 ± 0.46	27.31 ± 1.05	0.0002
Quarter minimum	30.21 ± 0.49	28.06 ± 0.42	0.001
Quarter difference	6.13 ± 0.50	7.60 ± 0.37	0.0122
Quarter average	34.85 ± 0.17	33.81 ± 0.19	0.0003
Teat minimum	29.47 ± 0.54	27.54 ± 0.65	0.0423
Teat maximum	34.41 ± 0.18	33.35 ± 0.29	0.011
Teat average	33.05 ± 0.21	31.71 ± 0.35	0.0076
	rbST, ºC	**Control, ºC**	***P*-Value**
Teat difference	6.17 ± 0.54	4.83 ± 0.42	0.0351
Quarter minimum	27.93 ± 0.48	29.75 ± 0.44	0.0078
Quarter difference	8.04 ± 0.41	6.11 ± 0.41	0.0021
Quarter standard deviation	0.87 ± 0.04	0.60 ± 0.03	< 0.0001

Notes: All cows were treated at 40.25 ± 2.56 d post-partum; 500 mg of sometribove zinc and 1.75 mL of saline solution were administered subcutaneously to rbST and Control cows, respectively. All infrared thermography images were obtained from a posterior view of the mammary gland after holding the tail away. Difference = maximum temperature - minimum temperature.

Conclusions

In the present study, the feasibility of using B-mode and color Doppler ultrasound as research options to assess the hemodynamic status of the mammary gland of lactating dairy cows was corroborated. However, during relatively cool weather conditions (< 25.6ºC of air temperature), the increased thermal gradient from the cow skin towards the external environment may favor body heat dissipations to such a degree that any metabolic rbST-related thermal change in the mammary gland may not be translated to the rectal temperature values of the cow. Under such environmental conditions, the air temperature is a highly significant factor regulating the thermal status of the cows. Moreover, if administered early during lactation, rbST hemodynamic related changes in the mammary gland may not be translated in to an enhanced productive performance. Further studies under different environmental conditions and later in lactation are required to achieve a better understanding of these trends.

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CHAPTER VI

GENERAL CONCLUSIONS

There is a plethora of publications from studies performed using color Doppler ultrasound in humans during the 1970's, 80's, and 90's. However, it is not until 1998 that the first study about color Doppler ultrasound in large domestic species was published. Since this 1998 paper, a relatively small amount of studies in this area have been published. Such a considerably long period of time of applying this technology to human scenarios has driven the development of this ultrasound modality. Because the demand for a product is the major force driving the designs and features in the same, color Doppler ultrasound instruments have been developed towards its feasible use in human scenarios. However, most of the real farm conditions where studies with large domestic animals are performed may differ considerably from the conditions and limits presented in the human scenario. Therefore, this technology presents significant limitations when applied to large domestic animals, especially to excitable cattle.

However, because in theory, color Doppler ultrasound allows for the assessment of the vascular dynamic status in the blood vessels all around the body and because the circulatory system plays an essential role in the normal physiology of every organ in the body by supplying the cells with oxygen and nutrients, and by removing metabolic byproducts, this technology has significant importance in the area of physiology research. Moreover, the authors believe that as more animal practitioners get interested in the use of these instruments, the companies that produce the same will invest more resources in the development of technology that may counteract the already existent limitations in our research area. Subsequently, in the future, this technology may further allow for a better understanding of multiple physiological processes all around the body in these species.